

Comparative Sequence Analysis of Genes Encoding Outer Proteins of African Swine Fever Virus Isolates from Different Regions of Russian Federation and Armenia

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Abstract African swine fever (ASF) is an economically important disease of domestic pigs. ASF is endemic in most of sub-Saharan Africa, including Madagascar Island; the highest incidence of disease being recorded from the West and East Africa to the Southern Africa. Disease outbreaks have also occurred in Europe, South America and the Caribbean. In 2007 it was introduced into Georgia, and has since spread throughout the Caucasus and into southern Russia. There is no vaccine or treatment available to control ASF virus. Therefore timely ASFV detection and characterization are critical to understand and contain its spread. In this report, we describe the nucleotide structure analysis of genes E183L, KP177R and O61R encoding for proteins p54, p22 and p12, respectively, for different ASFV isolates collected within three years from South European regions of Russia and Armenia. The comparative analysis of these demonstrated variability in p12 sequences of the isolates from different geographic regions and hosts, whereas p54 and p22 sequences were conserved among the isolates. However, hydrophathy profile analyses did not reveal any structural variations for all three proteins. It suggests that p12 genes, but not p54 and p22 genes, is under strong selective pressure and can be a valuable genomic marker for studying of evolutionary pathways and genetic diversity of ASFV isolates.

Keywords African Swine Fever Virus, P54 Gene, P22 Gene, P12 Gene, Hydrophathy Profile, Phylogenetic Analysis, Comparative Sequence Analysis

1. Introduction

The causative agent of African swine fever (ASF) is a large deoxyribovirus that has been assigned to Asfarviridae family[1]. Dixon L.K. et al. noted, that although initially ASFV was classified as an *Iridovirus* based largely on virion morphology, increasing molecular biological data led to its reclassification as the sole member of a new DNA virus family, *Asfarviridae* (A s f a r - African swine fever and related viruses). African swine fever virus (ASFV) replicates in cytoplasm and has variable virulence to domestic pigs, with infections ranging from highly lethal to subclinical. ASFV genes encoding proteins which modulate host immune response, viral virulence to domestic swine, and the ability of ASFV to replicate and spread in its tick vector. ASFV is the only known DNA-arbovirus.

Domestic and wild pigs are susceptible. African bush pigs

and warthogs infected with ASFV are generally asymptomatic, with low level viremia. ASFV persistently infects ticks of the *genus Ornithodoros* from which ASFV can be isolated 5-10 years post infection[2]. ASFV infection of domestic swine results in several disease forms, ranging from highly lethal to subclinical depending on contributing viral and host factors. African swine fever is characterized by oedemas, ascites and haemorrhages, virus replication and spread within mononuclear-macrophage system, long-term carrier state, nonvirulent strains of virus induce latent infection.

Its virion diameter is ~200 nm, contains more than 50 proteins and consists of several concentric layers enclosing an electron-dense nucleoid, containing a double stranded DNA genome of approximately 190 kilobase pairs (kbp). The core is wrapped by an inner lipid envelope that lies beneath the icosahedral capsid[3, 4]. Extracellular particles possess an additional envelope derived from the plasma membrane[4].

Analyses of cell extracts revealed the presence of more than 100 viral proteins synthesized on different phases of ASFV life cycle[5-6]. ASFV p54 is an externally located

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viral structural protein of 25-27 kDa, encoded by the virus gene - the open reading frame (ORF) E183L[7-8].

ASFV protein p54 is involved in the adsorption of the virion on susceptible cells and the early steps of viral infection[9].

Published data have shown that ASFV p54, p30, p22 and p12 are essential virus proteins involved in early events of the replication[10, 11].

The protein encoded by the gene - ORF KP177R, is an early structural protein of apparent molecular weight 22 kDa, located externally in the viral particle. The protein contains a hydrophobic region at the N-terminus with the characteristics of a signal peptide and seems to appear transiently in the plasma membrane early after ASF virus infection[12].

The ASFV virus protein p12 (ORF O61R) is involved in virus attachment to the host cell and located within the outer envelope of the virions. This protein is not synthesized during early phases of infection and undergoes post translational modification[13]. The protein p12 has trans-membrane domain and cysteine-rich region which is responsible for the dimerization at the C-tail.

Multimers of the protein with an apparent molecular mass of 17 kDa in the absence of 2-mercaptoethanol were detected. Labelling experiments with [³⁵S] methionine and immunoprecipitation with specific antibodies directed against protein p12 indicated that the gene is expressed during the infection of swine macrophages with all of the viruses tested. The nucleotide sequence of a DNA fragment containing the gene encoding for protein p12 in several virus strains has shown that the 5' flanking region is conserved in all the virus isolates sequenced, whereas the intergenic region downstream of the gene varies among different isolates[14].

Currently, there is no vaccine available against ASF and the disease is controlled by animal quarantine and slaughter. It is believed that the lack of efficient ASFV vaccines might be due to unique molecular and biological properties of ASFV proteins responsible for virus-cell interactions: p54, p22 and p12.

It is believed that extensive studies of ASFV structure, genes, immune mechanisms which affect viral replication, virus-host interactions, and virulence will help to effectively control this dangerous agent.

This article is devoted to the investigation of the nucleotide structure of genes encoding outer proteins p54, p22 and p12 of African swine fever virus isolates from the different regions of Russian Federation and Armenia.

2. Material and Methods

2.1. Cells and Viruses

Primary porcine blood macrophage cell (PBMC) or leucocyte cultures were prepared from swine blood. Briefly, heparin-treated swine blood was incubated at 37°C for 1 h to allow sedimentation of the erythrocyte fraction. Mononuclear leukocytes were separated by flotation over a Ficoll-Paque (Pharmacia). The monocyte-macrophage cell

fraction was cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum (FBS, Sigma)[15].

The virulent ASFV isolates Krasnodar 2008 (domestic pigs), Elbrus 2008 (wild boar), Stavropol 2008 (domestic pigs), Orenburg 2009 (domestic pigs), Rostov 2009 (domestic pigs), Volgograd 2010 (wild boar) and Armenia 2007 (domestic pigs) were obtained in 2007-2011. These ASFV isolates were passaged no more than 2-5 times in leucocyte culture primary porcine blood macrophage cell cultures prior to DNA isolation, PCR-amplification and nucleotide sequencing.

2.2. Hemadsorption (HAD) Test

The hemadsorption test[16] is based on the ability of pig erythrocytes to adhere to the surface of pig monocyte or macrophage cells infected with ASFV. Three days old leucocyte culture in 96-well microplates was inoculated with 10-fold dilutions (200µl/well) of treated sterile sample of medium or virus (at least four wells per dilution).

After that 20 µl of freshly prepared 1% suspension of pig erythrocytes in the physiologic buffered solution (PBS) were added to each well. The inoculated cells were incubated at 37°, 5% CO₂ during 7 days and the plates were checked for the presence of HAD.

2.3. Constructing of p54, p22 and p12 Genes Containing Plasmids

The amplification of these genes was carried out with the following primers:

p54: 5'-CTTATAATATACTGCA GTATGTTGAGTC-3' and

5'-TTCTTGAGGATCCTTGAAA GTTGGTCC-3'[17];

p22: 5'-CAGAAA GGATCCAATATTATGTAGACC-3' and

5'-CGATGCA CAATATTATAAGCTTTAAACCG-3'[17];

p12: 5'-GCGGATCCTTGAATAAGCGTTAAC-3' and 5'-GCGGATCCGACATCATTATGGATGA-3'[14].

The DNA templates used for the amplification reactions were obtained either from peripheral blood of infected pigs, for the wild-type viruses, or from supernatants of ASFV infected cell cultures.

The DNA samples used in the PCR were heated at 95°C for 5 min before addition to the reaction mixture and PCR-amplification PCR fragments consisted of a single band that varied in size among the virus genes when analyzed by 1,5% agarose gel electrophoresis[14, 17]. PCR products were cloned into plasmids pTZ57R/T (Fermentas, Republic of Lithuania) and pAL-TA (ZAO Evrogen, Russia)[18]. DNA samples, containing full size copies of genes encoding p54, p22 and p12 (E183L, KP177R and O61R) were obtained by PCR amplification from purified DNA of different ASFV isolates using the specific primers. Recombinant clones were generated by inserting PCR-products of p54 genes and p22 genes into pTZ57R/T or PCR-products of p12 genes into pAL-TA. DNA samples of recombinant plasmids, containing p54 genes, p22 genes and p12 genes, were sequenced using the same primer pairs as for PCR.

The sequences were obtained for at least two independent clones of each virus isolate.

2.4. DNA Sequencing and Computer Analysis

Cloned p54, p22 and p12 genes were sequenced by the dideoxynucleotide chain terminator method, according to standard procedures[19].

Comparative analysis of DNA sequences of E183L, KP177R and O61R genes of different ASFV field isolates and strains was performed using the package Bio Edit 6.0.

Nucleotide sequences of genes encoded p54 of different ASFV strains and isolates: LIS/57 (Accession number EU874321.1), TENGANI/62 (EU874318.1), MOZ/1979 (EU874372.1), E70 (FJ174389.1), OURT 88/3 (AM712240.1, protein_id CAN10474.1), KEN/50 (EU874353.1), p22 – Tengani 62 (AY261364.1), E75 (FN557520.1, protein_id CBH29101.1), OURT 88/3 (AM712240.1, protein_id CAN10352.1), KENYA 1950 (AY261360.1) and p12 - LIS57 (M84178.1), TEN61 (M84177.1), E70(E75) (FN557520.1, protein_id CBH29198.1), OURT 88/3 (AM712240.1, protein_id CAN10447.1), KENYA 1950 (AY261360.1), KIR69 (M84183.1) deposited in GenBank were used. The sequences of Georgia 2007/1 strain complete genome (Accession number FR682468.1) were used for analyses and for comparison as reference sequences of genes p54 (protein_id CBW46791.1), p22 (protein_id CBW46645.1), p12 (protein_id CBW46764.1).

2.5. Hydropathy Profiles

Hydropathy profiles of ASFV proteins p54, p22 and p12 were obtained following the procedure of Hoop & Woods[20].

2.6. Phylogenetic Analysis

Bio Edit 6.0 software packages was used for the phylogenetic analysis. Phylogenetic trees were constructed using neighbour-joining algorithm[21]. A 603 bp, 534 bp, 138 bp fragments were used to construct phylogenetic trees for p54, p22 and p12 genes, respectively.

3. Results

3.1. Hemadsorption Test

ASFV protein CD2v is responsible for the attachment of erythrocytes to infected cells and this ASFV virus protein is the most variable protein[22, 23]; we examined all Russian isolates for the presence of the phenomenon hemadsorption during their reproduction.

HAD analyses demonstrated that all tested ASFV isolates from different regions of Russian Federation and Armenia (Krasnodar 2008, Elbrus 2008, Stavropol 2008, Orenburg 2009, Rostov 2009, Volgograd 2010 and Armenia 2007) produced phenomenon of hemadsorption (Figure 1).

Since the character of hemadsorption during the virus

reproduction with these isolates was identical, indicating a reasonable stability of the gene structure, we considered it is not necessary to analyze the gene encoding this protein.

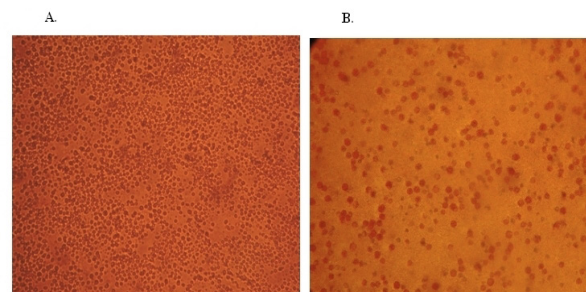


Figure 1. HAD test of Russian ASFV isolates from different regions in leukocyte cultures: A. The 3-days old leukocyte culture; B. The hemadsorption phenomenon produced by ASFV (Rostov 2009 isolate) in the leukocyte culture - 4 days post inoculation

3.2. Molecular Cloning and Sequencing of p54, p22 and p12 Genes

The results of sequencing and gene mapping with specific primers localization revealed that the ORF E183L (p54) located within nucleotide (nt) 162222-162776 of Georgia 2007/1 isolate genome. The rightwards transcribed ORFs coding p12 and p22 located within 128803-128988 nt and 3212-3781 nt, correspondingly (Fig. 2).

Therefore, the ORF E183L locates at the 5' end negative-chain DNA of the Georgia 2007/1 isolate genome (162222-162776 nt) and codes for a late induced structural glycoprotein p54 of 25 kDa (Fig. 2).

The ORF KP177R locates at the 5' end of the positive-chain DNA of Georgia 2007/1 isolate genome (3212-3781 nt) and codes for an early induced external viral structural protein p22 of 22 kDa (Fig. 2)

The ORF O61R locates at the positive-chain DNA central left part of Georgia 2007/1 isolate genome (128803-128988 nt) and codes for an early induced structural protein p12 of 12 kDa (Fig. 2).

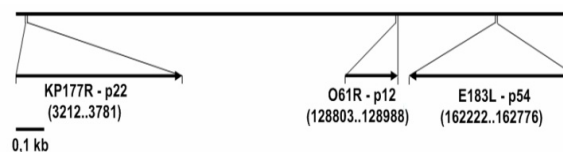


Figure 2. Positions of the E183L, KP177R and O61R ORFs on the genome map of African swine fever virus isolate Georgia 2007/1; size of complete genome ~189344 bp. The arrows indicate direction of the gene transcription and figures show positions of the genes

Nucleotide sequencing of the gene structure encoding of p54 was identical for six different viral isolates: Krasnodar 2008, Elbrus 2008, Stavropol 2008, Orenburg 2009, Rostov 2009 and Armenia 2007 (Figure 3, only Armenia 2007 and Stavropol 2008 sequences are shown).

Nucleotide sequence of same Georgia 2007/1 gene demonstrated a single nucleotide substitution from Russian isolates - within 438 nt (Figure 3).

African ASFV isolates had lower the p54 gene nucleotide sequence identity with that of European and American iso-

lates, indicating greater nucleotide heterogeneity among common origin for non-African isolates[24].
viruses from different geographic regions and indicating a

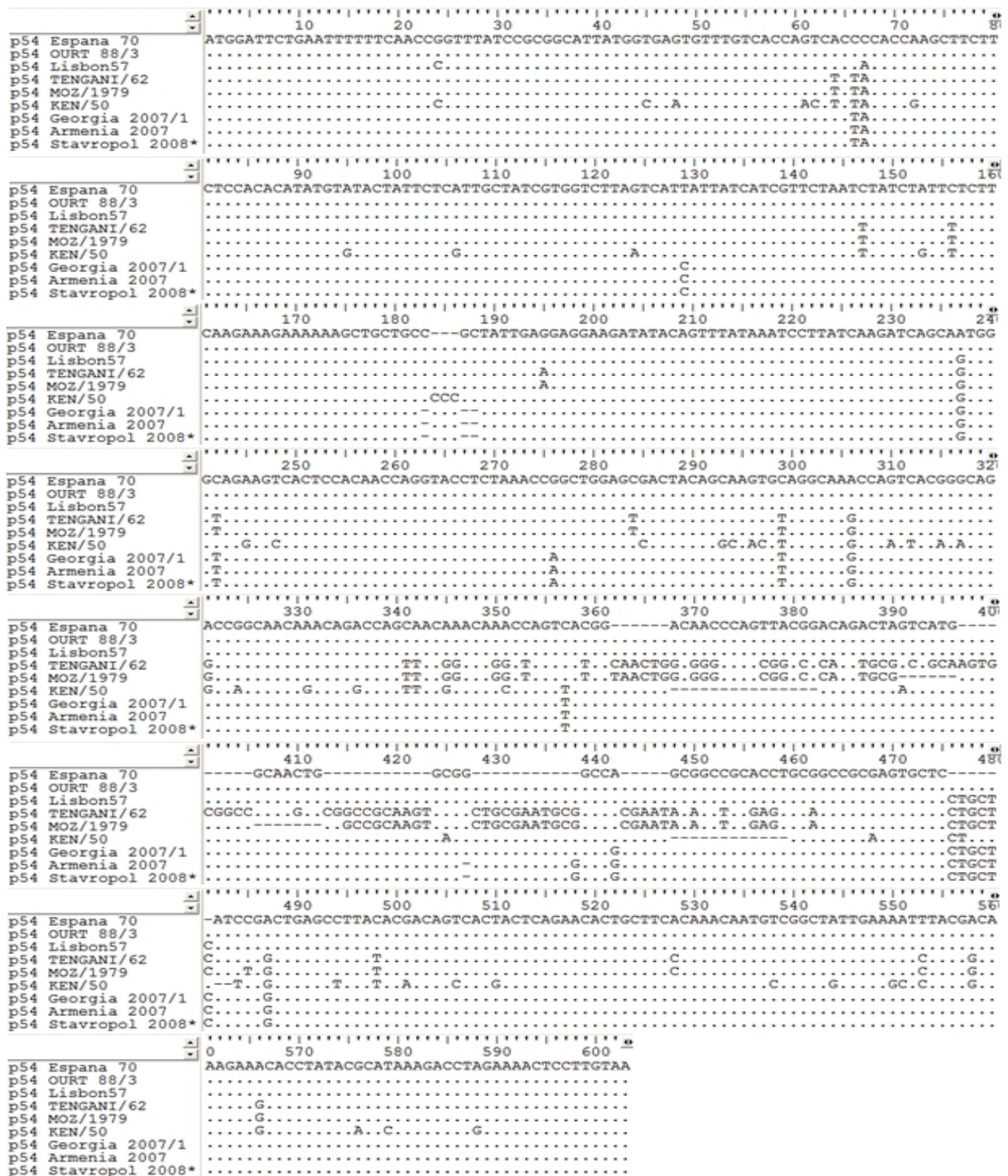


Figure 3. Multiple alignment of the p54 gene nucleotide sequences of ASFV isolates: · (point) - indicates identical nucleotide; - (dash) - indicates absence of nucleotide; *(asterisk) - indicates identical sequences of Krasnodar 2008, Elbrus 2008 and Orenburg 2009, Rostov 2009 E183L gene

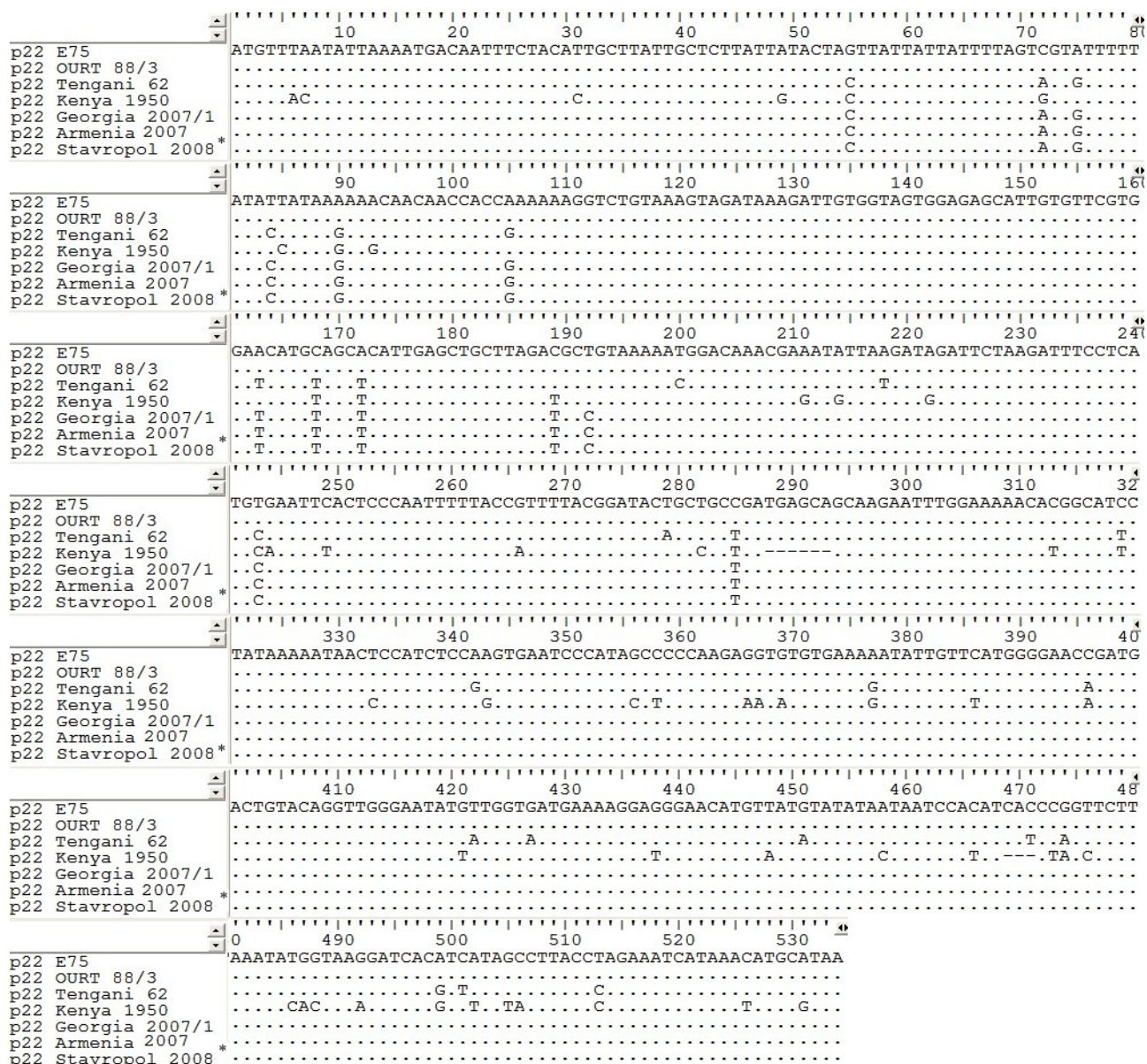


Figure 4. Multiple alignment of the p22 gene nucleotide sequences of ASFV isolates: · (point) - indicates identical nucleotide; - (dash) - indicates absence of nucleotide; * (asterisk) - indicates identical sequences of Krasnodar 2008, Elbrus 2008 and Orenburg 2009, Rostov 2009 KP 177R gene

Nucleotide sequence structure of the p22 gene was also identical for six different viral isolates from Rostov, Krasnodar, Elbrus, Stavropol, Orenburg, Armenia as well as Georgia. The p22 gene Kenya 1950 gene had maximal nucleotide substitutions of 9 strains compared (Figure 4, only Armenia 2007 and Stavropol 2008 sequences are shown). Thirteen nucleotide substitutions were recognized in ASFV Georgia 2007/1 and Russian isolates' the p22 gene sequences which were not found in E 75 strain genome. Also, 51 nucleotide substitutions were identified in ASFV Kenya 1950 genome, which were not found in Georgian, Armenian and Russian isolates genome sequences (Figure 4).

We analysed the variability of the gene, encoding protein p12 by comparing the sequences of 5 field virus isolates: Elbrus 2008, Stavropol 2008, Orenburg 2009, Volgograd 2010 and Armenia 2007.

The nucleotide sequence of the p12 gene of different ASFV isolates showed variability within regions from 195 to 248 and from 296 to 320 nucleotides (Figure 5).

We identified a 28nt-long insertion in p12 gene of Georgia 2007/1, Elbrus 2008, Stavropol 2008, Orenburg 2009, Volgograd 2010 and Armenia 2007 isolates (nt 218-245) which was absent in other European strain gene sequences with the exception of African - Kiravira 69, Tengani 61 and Kenya 1950 genes containing less than 28 nt insertions in the same region (Figure 5).

A few mutations in the sequence of p12 gene were observed between 170 to 265 nucleotides when different isolates were compared, whereas identical sequences were found when European group isolates were analysed (Lisbon 57, Espana 70 and OURT 88/3) (Figure 5).

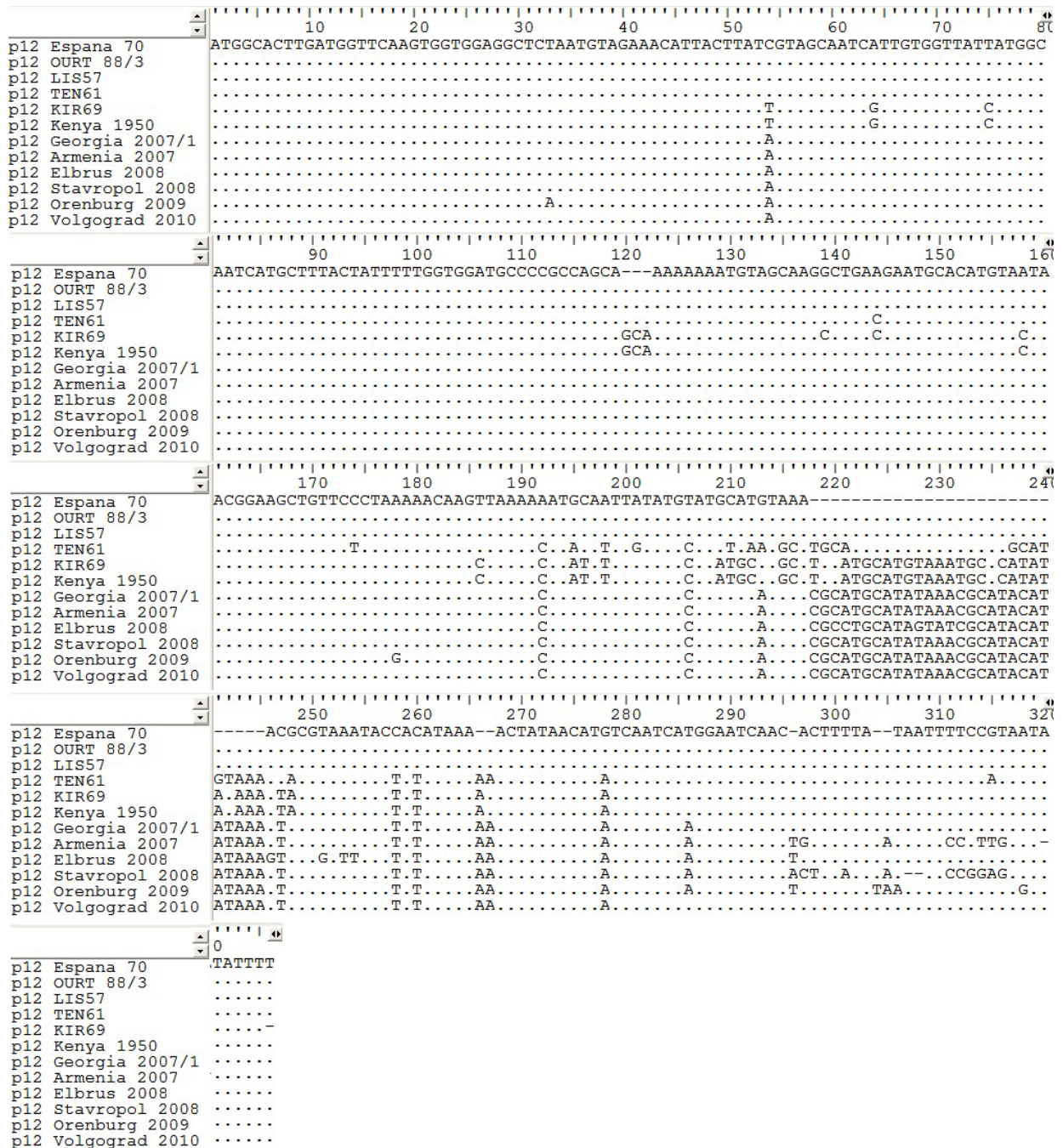


Figure 5. Multiple alignment of the PCR-product p12 gene nucleotide sequences of ASFV isolates: (point) - indicates identical nucleotide; (dash) - indicates absence of nucleotide

3.3. Hydropathy Analysis

Since we found single nucleotide substitutions in the p22 and p12 genes, and for the p12 gene nucleotide substitution occurred at the N-terminus with a net positive charge. It was necessary to check to see whether they affect the functional properties of encoded by these genes proteins. Hydropathy analysis makes possible recognition of protein function modifications when respective gene sequences vary. It was performed by using predicted amino acid sequences of ASFV p54, p22 and p12 proteins.

The hydrophilicity profile of p54 (Figure 6) revealed the

presence of a highly hydrophobic stretch, formed by 37 amino acid residues (a.a.) of p54 and a long hydrophilic C-terminus formed by 130 a.a. residues.

There was also a highly hydrophobic stretch, consisted of 26 amino acid residues and a long hydrophilic C-terminus formed by 140 a.a. residues in hydrophilicity profile of p22 (Figure 7).

Since nucleotide sequences of p54 genes were identical for six different viral strains - Krasnodar 2008, Elbrus 2008, Stavropol 2008, Orenburg 2009, Rostov 2009 and Armenia 2007 –we did not expect any differences in the functions of respective protein.

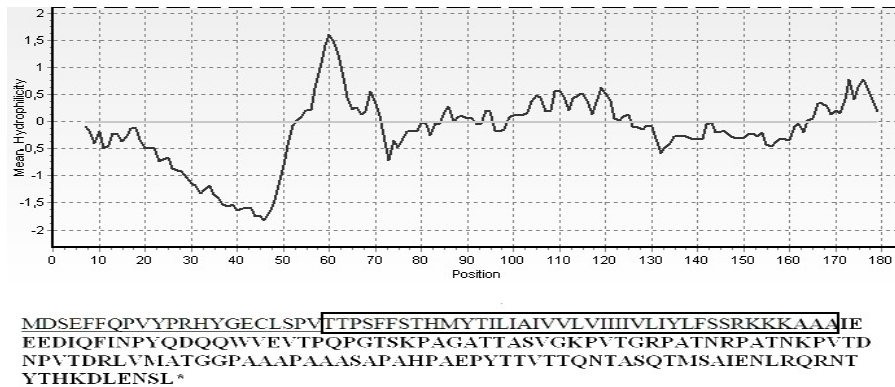


Figure 6. The Hoop & Woods scale mean hydrophilicity profile of Stavropol 2008 ASFV isolate p54 protein: the hydrophilic N-terminal domain formed by 20 residues is underlined; the boxed region corresponds to the hydrophobic stretch of 37 amino acid residues near the N-terminus of the polypeptide (residues 22 to 58); bold font corresponds to the hydrophilic C-terminus formed by 130 amino acid residues

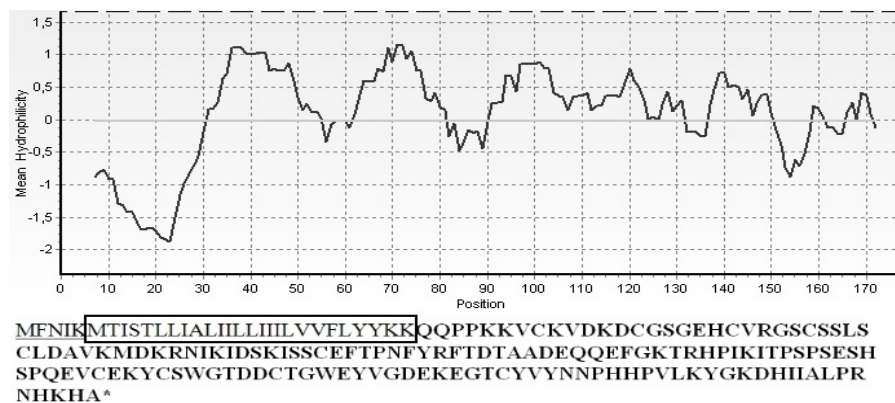


Figure 7. The Hoop & Woods scale mean hydrophilicity profile of Stavropol 2008 ASFV isolate p22 protein: five amino acids (positions 1 to 5) at the N-terminus with a net positive charge are underlined; the boxed region corresponds to a hydrophobic stretch of 26 amino acid (positions 6-31); bold font corresponds to the long hydrophilic C-terminus formed by 140 amino acid residues

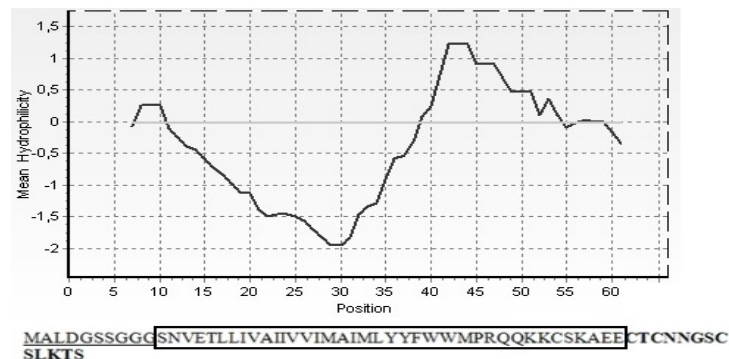


Figure 8. The Hoop & Woods scale mean hydrophilicity profile of Orenburg 2009 ASFV isolate p12 protein: ten amino acids (positions 1 to 10) at the N-terminus with a net positive charge are underlined; the boxed region corresponds to the hydrophobic stretch of 30 amino acid residues near the N-terminus of the polypeptide (residues 11 to 48); bold font corresponds to the short hydrophilic C-terminus formed by 13 amino acid residues

The deduced amino acid sequences of the p12 shows a stretch of 22 hydrophobic residues that functions as to anchor the protein in the external virus envelope. A hydrophilic C-terminus formed by 13 a.a. residues (Figure 8).

Despite few mutations present in the sequences of p12 gene of Russian and Armenian isolates within regions from 195 to 248 and from 296 to 320 nucleotides, we did not observe changes of the structure hydrophobic trans membrane segment, the hydrophilic C-terminus formed by 13 a.a. residues.

3.4. Phylogenetic Analysis of ASFV Isolates

The nucleotide sequences of orthologous genes from seven ASFV isolates (Armenia 2007, Krasnodar 2008, El-brus 2008, Stavropol 2008, Orenburg 2009, Rostov 2009 and Volgograd 2010) were analysed and phylogenetic trees were constructed for the genes encoding p54, p22 and p12.

The p54 gene-based neighbour-joining tree demonstrated that Russian isolates together with Georgia 2007/1 isolate form a separate clade within the European cluster. The other cluster comprises Southern African isolates - Tengani and Mozambique (Figure 9a).

The p22 gene phylogenetic analysis demonstrated that Russian isolates and Georgia 2007/1 isolate cluster together

and are closer related to European isolates than to Southern African isolates (Figure 9b).

All Russian and Armenian isolates had specific mutations in O61R gene. The phylogenetic analysis for p12 gene also demonstrated that Russian isolates and Armenia 2007 isolate formed a group separate from European and African isolates. Within this group Russian and Armenian isolates formed several subgroups with Stavropol and Armenia closest to each other and Elbrus, Orenburg and Georgia sharing higher homology with each other (Figure 9c). While Volgograd 2010 isolate formed another subgroup of its own.

Overall, Stavropolian and Armenian isolates share high homology between each other and with Georgia isolate suggesting that all Russian isolates may share a common origin with Georgia.

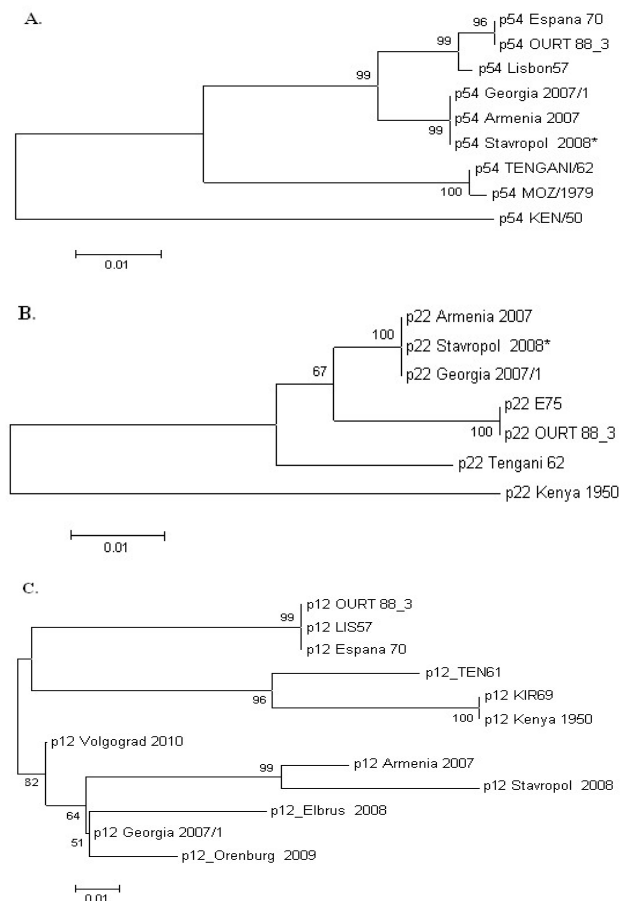


Figure 9. Neighbour-joining phylogenetic trees of E183L (p54) gene (a), KP177R (p22) gene (b) and intergenic region of p12 gene (c) sequences of some representative ASFV isolates tested

4. Discussion

The ASFV genome contains discrete variable regions and elements, which were evident through restriction mapping[25] and comparative sequences' analysis. ASFV isolates and cell culture-adapted viruses contain a conserved, centrally located 125-kbp genomic core in which major insertion-deletion events are rare, leaving larger-scale variability confined to the left 38- to 47-kbp and right 13- to

16-kbp terminal regions of the ASFV genome[26-28]. Rock D.L.[29] demonstrated that structure heterogeneity of p72 gene is no more than 10%, while for CD2v - 40%, p54 - 30%, p22 - 20% and Angulo A. et al.[14] described structure heterogeneity of p12 gene about 35%.

Previous results of Carrascosa A.L. et al. showed that the deduced amino acid sequence of p12 protein included a trans-membrane domain, cysteine rich C-terminal region and a stretch of 22 hydrophobic residues, which functions as to anchor the protein in the external virus envelope[4]. The absence of cleavable N-terminal signal sequences in the predicted amino acid sequence of p12 protein suggests that the polypeptide is inserted into the membrane through the mechanism, which has been proposed for other proteins of low molecular mass[30].

The hydropathy profile of p54 reveals the presence of a very hydrophobic stretch, formed by 21 amino acid residues, within the N-terminal region (residues 33 to 53) of the protein, which most likely represents a transmembrane domain[31].

The analyses of hydropathy profile allows to determine the functional properties of protein structure[32], in our case hydropathy profile of ASFV proteins p54, p22 and p12 corresponded to the results of a similar analysis for those proteins, but did not show significant differences of their structures.

We observed that the ASF virus, which has a high degree of variability, changes in the process of passaging *in vivo*. Prior to phenotypic changes, ASFV isolates accumulate silent genomic mutations specific for different isolates[33]. Therefore, we chose the outer ASFV proteins that are under selective pressure and involved in the processes of attachment and penetration into the cell.

Bastos A.D. et al.[34] analyzed strains of African swine fever virus from different geographic zones by partial p72 gene characterization and the phylogenetic analyses. Genotyping distributed all ASFV isolates to 10 clusters: genotype I included all European strains and some isolates from West Africa (from Angola to Senegal). Remaining 9 genotypes come from South, Central and East Africa and Madagascar.

The present genotyping strategy includes a two-step genetic characterisation approach whereby p72 gene sequencing is used to delineate genotypes, prior to intra-genotypic resolution of viral relationships by central variable region (CVR) characterisation of the 9RL ORF classify the ASFV isolates relative into the 22 currently known genotypes[35].

Malogolovkin A. et al.[36] compared sequences of genes encoding p72 and showed that gene B646L of all Russian isolates was highly conserved with no substitutions.

The serotype-based classification of ASFV strains previously developed by Balyshev V. et al.[37] distributed all ASFV strains in ten groups including eight serotype-based groups, one group of non-serotyped strains and one group consisting of heterogeneous isolates. This classification characterizes strains into different seroimmunotypes based on the results of *in vivo* the cross-challenge and *in vitro* the hemadsorption inhibition test[38]. We found out that the

immunodominant ASFV p54 is one of the virus proteins responsible for serotype specificity[24].

Phylogenetic analysis based on gene p54 conducted earlier in our laboratory allowed to divide all tested ASFV strains and isolates into 8 groups consistent with serotype-based classification[24]. All Russian, Georgian and Armenian isolates belonged to VIII seroimmunotype together with Rhodesia 1984 strain. Comparative sequence analysis of p54 genes of these isolates showed that they were identical, thus, we concluded that the isolates belonging to the same serotype have identical genes encoding p54.

Earlier data of Angulo A. et al.[14] demonstrated that the p12 5'-flanking region sequence was conserved among the isolates, whereas sequences downstream of this gene were highly variable in length and contained direct repeats in tandem. However, the main properties of the p12 protein were not altered in the original isolates and viruses adapted to grow in established cell lines. As noted by Angulo A., et al.[39], there is no correlation between the length of this sequence and any of the properties of these viruses, such as pathogenicity or capacity to grow in different cell types.

Rowlands R.J. et al.[39] obtained sequences of 4 regions of ASFV genome, including B646L (p72), E183L (p54), CP204L (p30) and B602L (CAP80). Phylogenetic analyses of this four genes showed that Georgia 2007/1 isolate belonged to genotype II, which circulated in Zambia, Mozambique and Madagascar. Our results are consistent with data of Rowlands R.J. et al.[40]: Krasnodar 2008, Elbrus 2008, Stavropol 2008, Orenburg 2009, Rostov 2009, Armenia 2007 isolates belonged to genotype II.

The phylogenetic analysis of 123 concatenated genes separated the viruses into two major clusters that correlate with their geographical distribution[41].

Gallardo C. et al. compared sequences of genes encoding p72, p54, p30 and central variable region (CVR) to increase resolution of additional loci and to study geographic distribution of ASFV isolates[42]. The CVR within the ORF B602L had been found to be the most useful locus for differentiation of closely related isolates and identification of p72-based virus subgroups.

Subsequent analysis of the genetic relatedness of Russian and Armenian ASFV isolates based on p12 gene intergenic region demonstrated some differences: Russian and Armenian isolates originate from Georgia, but contained 9 different nucleotide substitutions and 5 insertions.

5. Conclusions

Our assumption that the gene p12 (in particular, its intergenic region) analysis can provide reliable information about the phylogenetic relationship between different isolates was fully confirmed in this study phylogenetic and comparative sequence analyses. Between nucleotides 218 and 255 we found insertions typical for these isolates and Georgia 2007/1 strain. A characteristic feature of all investigated genomes of African isolates was the presence

of the shorter insertions in the same region. Unfortunately, we did not find more information in Gene Bank and had no data on the sequences of the p12 gene for the Mozambique 1979 and Rhodesia 1984 isolates, which would allow us to exactly determine the feasibility of using phylogenetic analysis for the specified region of ASF virus genome. Nevertheless, our findings suggest there is a need to analyze this region, because we found differences in the isolates with different passage history and of different origin.

The p12 based phylogenetic analysis allowed to define subgroups among Russian isolates while showed the same relatedness with European and African isolates as p22 and p54 based phylogenetic analyses.

The differences between p12 gene sequences may be reflective of different transmission pathways of the isolates. It is possible that the Volgograd 2010 isolated from a wild boar accumulated numerous genomic changes due to higher transmission rate in these hosts or that a diverse ASFV strain pool circulating in the wild boar population. While ASFV isolates from domestic pigs in Krasnodar, Stavropol and Orenburg regions are highly homologous between each other and their spread was likely mediated by humans.

Because our results have confirmed the stability of p54 genes in isolates belonging to one serotype, we can recommend analysis of this genomic region to identify changes in circulating virus' serotype, similar to that of long time epizootic in Spain and Portugal, when the virus I serotype was gradually supplanted by the IV serotype virus.

Overall, comparative analysis of the genes encoding p54, p22 and p12 proteins presented in this study indicates that p12 gene, but not p22 and p54 genes, is under strong selective pressure and can be a valuable genomic marker for studying of evolutionary pathways and genetic diversity of ASFV isolates.

6. Nucleotide Sequence Accession Numbers

The DNA sequence data in this report have been submitted to the GenBank data base under accession numbers p 54 genes: Krasnodar 2008 - JQ771683.1, Elbrus 2008 - JQ771682.1, Stavropol 2008 - JQ771686.1, Orenburg 2009 - JQ771684.1, Rostov 2009 - JQ771685.1 and Armenia 2007 - JQ771681.1, p 22 genes: Krasnodar 2008 - JQ771689.1, Elbrus 2008 - JQ771688.1, Stavropol 2008 - JQ771692.1, Orenburg 2009 - JQ771690.1, Rostov 2009 - JQ771691.1 and Armenia 2007 - JQ771687.1 and p 12 genes: Elbrus 2008 - JQ771677.1, Stavropol 2008 - JQ771679.1, Orenburg 2009 - JQ771678.1, Volgograd 2010 - JQ771680.1 and Armenia 2007 - JQ771676.1.

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