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Molecular Characterization of the First African Swine Fever Virus Genotype II Strains Identified from Mainland Italy, 2022

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Abstract: African swine fever (ASF) is responsible for important socio-economic effects in the global pig industry, especially for countries with large-scale piggery sectors. In January 2022, the African swine fever virus (ASFV) genotype II was identified in a wild boar population in mainland Italy (Piedmont region). This study describes the molecular characterization, by Sanger and next-generation sequencing (NGS), of the first index case 632/AL/2022 and of another isolate (2802/AL/2022) reported in the same month, in close proximity to the first, following multiple ASF outbreaks. Phylogenetic analysis based on the B646L gene and NGS clustered the isolates 632/AL/2022 and 2802/AL/2022 within the wide and most homogeneous p72 genotype II that includes viruses from European and Asian countries. The consensus sequence obtained from the ASFV 2802/AL/2022 isolate was 190,598 nucleotides in length and had a mean GC content of 38.38%. At the whole-genome level, ASF isolate 2802/AL/2022 showed a close genetic correlation with the other representative ASFV genotype II strains isolated between April 2007 and January 2022 from wild and domestic pigs in Eastern/Central European (EU) and Asian countries. CVR subtyping clustered the two Italian ASFV strains within the major CVR variant circulating since the first virus introduction in Georgia in 2007. Intergenic region I73R-I329L subtyping placed the Italian ASFV isolates within the variant identical to the strains frequently identified among wild boars and domestic pigs. Presently, given the high sequence similarity, it is impossible to trace the precise geographic origin of the virus at a country level. Moreover, the full-length sequences available in the NCBI are not completely representative of all affected territories.

Keywords: African swine fever virus (ASFV); genotype II; ASF outbreak; mainland Italy; wild boar population; next-generation sequencing (NGS); phylogenetic analysis

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1. Introduction

African swine fever (ASF), which is caused by a large enveloped linear double-stranded DNA virus [1], is responsible for important socio-economic consequences in the global swine industry, especially for countries with wide-scale pig production.

It was first identified in Kenya in 1921 [2], and prior to 2007 was mainly endemic in Africa. After a genotype I pandemic wave started in the Iberian Peninsula in 1960, the virus was eradicated worldwide, although it remained endemic in Sardinia Island [3]. In 2007, ASF was introduced to the Republic of Georgia through the port of Poti, likely via improperly disposed waste from international ships carrying contaminated pork or pork products, which were then used as swine feed [4]. Subsequently, the genotype II virus spread to Armenia and the Russian Federation. Afterward, the virus invaded the European Union in 2014 [5]. Later, in 2018, it was first confirmed in China [6] following several

outbreaks in Siberia in 2017 [7]. Since then, the disease has rapidly spread to 15 other Asian and Pacific countries [8]. In early 2020, it was also reported in two North-Eastern states of India [9]. The countries most recently affected include Papua New Guinea, Germany [10], the Dominican Republic [11] and Haiti [12].

ASF genotype I is still considered an endemic in Sardinia, although only sporadic indirect traces of the virus are currently detectable. In Italy, two ASF virus (ASFV) introductions in pigs have been reported in the past. The first was reported in 1967 in 28 Italian provinces. The second incursion was reported in Piedmont in March 1983 and represented the unique escape of ASFV from Sardinia; wild boar (*Sus scrofa*) meat imprudently imported from Sardinia has been incriminated as the cause of this outbreak. Strict quarantine and slaughter measures confined the spread of the disease, and the outbreaks were successfully eradicated. Recently, in 2022, ASFV was detected in a wild boar in mainland Italy (Piedmont region) [13].

ASFV is the only member of the family *Asfarviridae*, genus *Asfivirus*. The genome is approximately 170–190 kb in size and is divided into the left variable region (LVR, 38–48 kb), the central conserved region (CCR, approximately 125 kb) and the right variable region (RVR, 13–22 kb) [14,15]. There is a central variable region (CVR) of approximately 400 bp [16] within the CCR. The nature of CVR variation and the genetic mechanisms involved are unknown. The molecular basis of this variation includes alterations in the number and type of tandem repeated amino acid tetramers within a late viral gene, 9-RL. Analysis of the B602L amino acid sequences reveals a high degree of variability among different ASFV isolates [17–22].

Differences in the genome length are largely caused by the gain or loss of members of the five multigene families (MGF 100, 110, 300, 360 and 505/530) flanking the variable regions [23]. LVR and RVR can have a variable number of MGF genes. Variations within these regions are observed during the viral adaptation to monkey cell lines [24] and appear to be associated with reduced virulence [25]. Furthermore, minor length variations are associated with the number of tandem repeats located at loci within the coding regions and in intergenic regions (IGR) [26]. These variable regions are important for the evolutionary analysis of ASFV.

Molecular characterization of ASFV during outbreaks is significant for investigating the virus origin, quickly differentiating between closely related strains [27] and extening our knowledge of the molecular evolution of the virus and its epidemiology. The most common approach for the genotyping of ASFV during outbreaks is based on analyzing the C-terminal end of the B646L gene, which encodes the p72 capsid protein [28]. This approach has allowed for the identification of 22 distinct p72 genotypes (I-XXII) among virus strains from Eastern and Southern African countries [29]. ASFV genotype XXIII was identified in Ethiopia and was found to be derived from the same evolutionary branch as the IX and X genotypes prevalent in East African countries and the Democratic Republic of Congo [30]. The genotype XXIV has been identified in soft tick samples collected from Gorongosa National Forest Park, Mozambique [30]. The E183L gene, encoding the p54 protein and the CVR within the B602L gene, was also sequenced to distinguish between geographically and temporally constrained p72 genotypes [17–22].

Furthermore, to evaluate molecular differences between the ASFV strains, especially the evolutionary trend of strains from the same region, other approaches include the assessment of the: p30-encoding gene (CP204L) [31–33]; TRS within the IGR between I73R and I329L [32,33]; CD2v-encoding gene (EP402R) [33]; thymidine kinase (TK) gene [34]; J268L, Bt/Sj, KP86R [22] and O174L genes [35], and the C315R/C147L region [36]. Sequence analysis showed that the different isolates have partial differences in the length and sequences of the J268L, Bt/Sj and KP86R genes, which may be used to distinguish between evolutionarily similar isolates [22].

The recent innovations in whole-genome sequencing can facilitate comprehensive genotyping and provide data that are essential for elucidating the biology and genetic characteristics of ASFV. The use of next-generation sequencing (NGS) and bioinformatics

analysis for detecting and identifying ASFV from clinical samples isolated from outbreaks have been previously described [37–41].

In this study, we used Sanger sequencing and NGS to investigate the epidemiological links between the ASFV strains causing outbreaks amongst wild boars in mainland Italy in January 2022.

2. Materials and Methods

We analyzed bone marrow and spleen samples from the first index case (632/AL/2022) and one other virus (2802/AL/2022) collected from wild boars in the affected area during the outbreaks which occurred in Italy (Piedmont region) in the first two weeks of January 2022 [13]. The samples were tested at the National Reference Laboratory (CEREP) in a Biosafety Level 3 (BSL3) facility for diagnostic confirmation.

2.1. Samples Preparation

The ASFV was isolated from the positive samples as described in the *OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, Chapter 3.9.1, 2021. The strains were haemadsorbed in pig macrophage cultures. The ASFV purification was performed as previously described by Enjuanes et al. [42], with some modifications. Briefly, the virus suspension was treated at 37 °C for 90 min with an RNase Cocktail (5 U and 200 U of RNase A and RNase T1, respectively, Thermo Fisher Scientific: Baltic UAB, Vilnius, Lithuania) and 150 U Turbo DNase (Thermo Fisher Scientific: Baltic UAB, Vilnius, Lithuania). This was followed by treatment with trypsin (6 mg/mL) for 2.5 h at 37 °C and subsequent ultracentrifugation in a 5–20% sucrose gradient in phosphate buffer saline (PBS) at 4 °C for 90 min in a swing rotor (Sw 41) at 68,600× *g* (Beckman Coulter Inc., Brea, CA, USA). The original clinical materials (spleen, bone marrow, etc.) were subjected to three/four freeze–thaw cycles to homogenize the samples. After centrifugation at 6000× *g* for 10 min at 4 °C, the supernatants were filtered through 0.45 µm syringe filters. The filtered homogenates were treated at 37 °C for 90 min with the RNase Cocktail and Turbo DNase as previously described [41].

2.2. Partial Sequencing (Sanger)

The samples were genotyped by partial sequencing (Sanger) of a fragment of the B646L gene (p72), a fragment of the B602L gene (CVR) and tandem repeat sequences located between the I73R and I329L genes (ECORI), using previously described primers and thermal cycling conditions (refs) shown [33]. Viral DNA was extracted from the lysed samples and from the ASF isolates using the High Pure DNA nucleic acid kit (Roche Diagnostics GmBH: Mannheim, Germany) following the manufacturer's instructions. The selected positive samples were characterized by partial sequencing (Sanger) on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems: Waltham, MA, USA). Bidirectional sequencing was performed for each of the gene targets, and three independent reactions were performed for each sample. The sequences were then analyzed using the DNAStar package v.15 [43]. The nucleotide sequences were aligned using Clustal X with the ASF reference strains retrieved from PubMed at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/ accessed on 12 December 2022). The manual sequence editing was performed using BioEdit software (version 7.0.) [44], and phylogenetic analysis was performed using MEGA v. 7 [45] with the GTR model of sequence evolution and gamma distribution (GTR+G+I). The robustness of the clusters was tested by performing 10,000 bootstrap replicates: branches with bootstrap values below 70% are not shown [45].

2.3. Shotgun Metagenomic Analysis

The spleen sample of 2802/AL/2022 was selected for shotgun metagenomic analysis. The DNA samples were quantified using the Qubit® DNA HS Assay Kit (Thermo Fisher Scientific: Waltham, MA, USA) and then used for library preparation with Illumina® DNA Prep, (M) Tagmentation (96 samples) (Illumina Inc.: San Diego, CA, USA) according to the

manufacturer's protocol. Deep sequencing was performed on the NextSeq500 (Illumina Inc.: San Diego, CA, USA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2 (300 cycles) (Illumina Inc.: San Diego, CA, USA) and standard 150 bp paired-end reads. After quality checking and trimming of the raw reads data using FastQC v0.11.5 and Trimmomatic v0.36, respectively, host depletion was performed using Bowtie2 [46]. The reads were mapped using the BWA software package v.0.7.17 [47], with the ASFV Georgia 2007/1 sequence (accession number FR682468.2) as a reference. The iVar v1.3.1 tool was used to define a consensus sequence based on the mapping results [48].

Genome annotation was performed as previously described [37], using the GATU software [49] with the ASFV Georgia 2007/1 sequence (accession number FR682468.2) as a reference. The annotations were manually verified and curated using the Ugene software package [50].

A maximum likelihood (ML) phylogenetic tree was constructed using the Tamura–Nei parameter model in MEGA v.7 with 10,000 bootstrap replicates [45].

3. Results

Two representative isolates (632/AL/2022 and 2802/AL/2022) from the outbreak area in Northwest Italy were selected for molecular characterization.

The sample 632/AL/2022 was genotyped using partial sequencing (Sanger) of the B646L gene (p72), the B602L gene (CVR) and a TRS located between the I73R and I329L genes. The nucleotide sequences from the 478 bp region of the p72 genes were analyzed and compared with the previously defined reference sequences retrieved from GenBank. The phylogenetic analysis established that sample 632/AL/2022 was part of the largest and most homogeneous p72 genotype II cluster, which also includes strains from European and Asian countries (Figure 1). The sequence similarity ranged from 92.40 to 99.60% between the various subtypes. The phylogenetic tree is shown in Figure 1. It was not possible to carry out a complete genome sequence of the first index case (632/AL/2022) by NGS, as the starting material, represented by the bone marrow, was very poor.

The sample 2802/AL/2022 was selected for full–genome characterization by NGS. The sequence obtained showed a horizontal coverage of 100.00%, compared with that of the reference genome ASFV Georgia 2007 sequence (FR682468.2) and a mean vertical coverage of 885,*45x*. The consensus sequence of ASFV 2802/AL/2022 was 190,598 nucleotides in length and had a mean GC content of 38.38%. It contained 192 ORFs, encoding for structural–functional and uncharacterized proteins. The complete sequence was analyzed using BLASTn to identify the closest sequence match in the GenBank database. The whole-genome sequence of the 2802/AL/2022 ASFV positive sample showed a close genetic correlation with the other representative ASFV genotype II strains isolated between April 2007 and January 2022 from wild boars and domestic pigs in Eastern/Central European (EU) and Asian countries (Figure 2).

Additionally, in order to trace the outbreaks/cases, the genetic characterization of the two isolates was conducted using the EURL-genotyping standardized procedures.

A polymerase chain reaction (PCR) targeting the B602L variable region yielded a ~180 bp fragment from the two cases analyzed. Analysis of the amino acid tetramer repeat sequences within the CVR of the two ASFV isolates in this study revealed the presence of nine repeats identical to those identified among genotype II isolates from European and Asiatic countries (Figure 3). Comparison of the deduced amino acid sequences derived from the variable region of the B602L with 51 genotype II reference strains from European and Asian countries isolated between 2007 and 2022 identified 10 unique sequences of amino acid tetramers (BNDBNDBNAL) (Figure 3). Therefore, CVR subtyping clustered these ASFV isolates detected in Italian wild boar within the CVR variant majority circulating in the EU and Asian countries since their first introduction in Georgia in 2007.

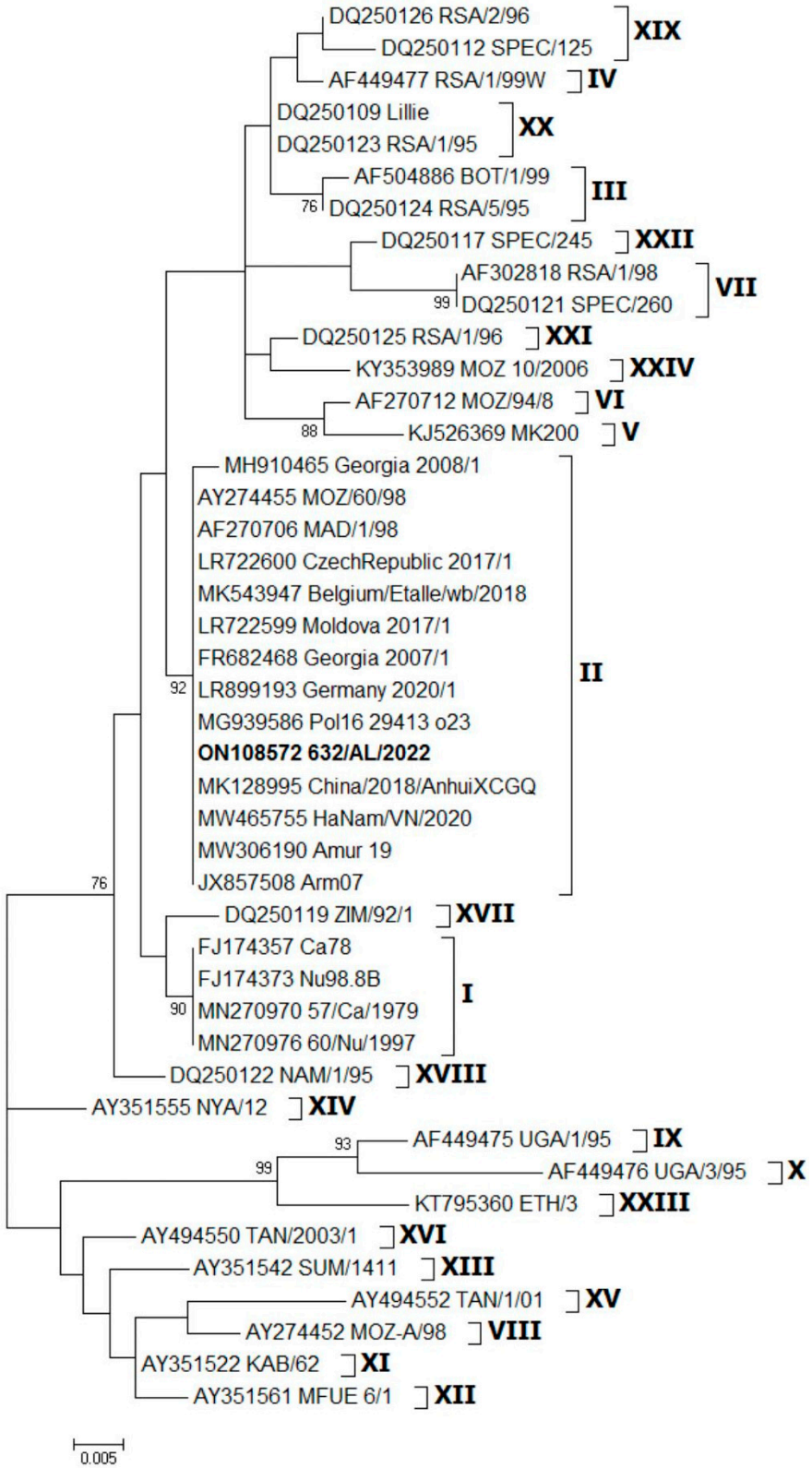

Figure 1. Phylogenetic tree reconstruction based on the C-terminal region of the B646L gene (p72). The sample ON108572 632/AL/2022 characterized in this study is shown in bold. Bootstrap values > 70 are indicated at their respective nodes. Bars indicate the number of nucleotide substitutions per site.

Figure 2. Maximum likelihood (ML) phylogenetic tree of 36 ASFV complete genome sequences. The sample ON108571 2802/AL/2022 characterized in this study is shown in bold. Bootstrap values > 70 are indicated at their respective nodes. Bars indicate the number of nucleotide substitutions per site.

GenBank/Strain	Amino acid sequence of the tetrameric repeats
FR682468 Georgia 2007/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
JX857523 Abk07	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLTDPER
MH910495 Georgia 2008/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
JX857522 Arm07	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLTDPER
KJ627203 Bel13/Grodno	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
NC044948 Odintsovo_02/14	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW306190 Amur 19/WB-6905	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW306191 Primorsky 19/WB-6723	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW306192 Ulyanovsk 19/WB-5699	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTKYTDLADPER
MT459800 Kabardino-Balkaria 19/WB-964	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK628478 LT14/1490	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
LS478113 Estonia 2014	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT647527 Est15/WB/Tartu1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYT~~~~~~~~~~~~~~~~CADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT647531 Est16/WB/Tartu17	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYT~~~~~~~~~~~~~~~~CADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT647554 Est16/WB/LANE22	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTYASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT647544 Est17/DP/Parnu1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTYASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
KJ627206 Pol14/Sz	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MH681419 POL/2015/Podlaskie	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MG939586 Pol16_29413_o23	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MG939589 Pol17_05838_C220	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT847620 Pol17_55892_C754	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT847621 Pol18_28298_O111	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT847623 Pol19_53050_C1959/19	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MN194591 Kyiv/2016/131	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
LR722599 Moldova 2017/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
LR722600 CzechRepublic 2017/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MN809122 RO/SM/2017	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MN715134 HU_2018	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
LR536725 Belgium 2018/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK543947 Belgium/Etalle/wb/2018	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
LR899193 Germany 2020/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK333180 Pig/HLJ/2018	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK333181 DB/LN/2018	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK128995 China/2018/AnhuiXCGQ	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT496893 GZ201801	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK645909 wbBS01	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MN172368 pig/China/CAS19-01/2019	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW361944 China/GD/2019	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MK940252 CN/2019/InnerMongolia-AES01	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW033528 wbShX01	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MN393477 Wuhan 2019-2	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW521382 HuB20	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW656282 Pig/Heilongjiang/HRB1/2020	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT851935 MNG/7/BU/2019	~~~~~~LHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLT
MN631140 Korea/Pig/Paju1/2019	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT445934 VNUA HY-ASF2	~~~~~~LHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLT
MT180393 NgheAn_2019	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW465755 VNUA-ASFV-05L1/HaNam/VN/2020	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MW396979 Timor-Leste/2019/1	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
MT851943 Indo/2020/Pig/West Java	~~~~~~LHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLT
MT642590 IND/AS/SD-02/2020	ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLADPER
ON108573 632_AL_2022	**ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLTDPER**
ON108571 2802_AL_2022	**ILSIADSCKTQTQKSKEAKTTIDSFLREHFVFDPNLHAQSAYTCADTNVDTCASMCADTNVDTCASMCADTNVDTCASTCTSTEYTDLTDPER**

Figure 3. Amino acid sequence of the tetrameric repeats that constitute the central variable region (CVR) of the B602L gene identified in Italy. The single letters refer to the code of each tetrameric repeat: B = CADT; N = NVDT/NVGT; D = CASM; A = CAST; L = CTST; H = NEDT; P = NADT; S = SAST; O = NASI; F = NAST; Q = NADI; V = NANT; M = NANI; T = NVNT; C = GAST; K = CANT [17,22,29].

To further define the most likely origin of the introduction of the ASFV genotype II in Italy, we compared TRS in the IGR between I73R and I329L with the representative ASFV genotype II strains. Intergenic region I73R-I329L subtyping clustered the Italian ASFV isolates with variants that have been frequently identified among wild boar and domestic pigs in EU countries since ASFV's introduction in 2014 and after 2018 in Asian countries (Figure 4).

GenBank/Strain	Partial nucleotide sequence alignment
FR682468 Georgia 2007/1	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MH910495 Georgia 2008/1	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
KJ620028 Arm07	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
KJ620035 Az08D	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
KP137629 Moscow_07/13	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
KP137633 Tula_08/13	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW306190 Amur 19/WB-6905	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW306191 Primorsky 19/WB-6723	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW306192 Ulyanovsk 19/WB-5699	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT459800 Kabardino-Balkaria 19/WB-964	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
NC044948 Odintsovo_02/14	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK628478 LT14/1490	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
LS478113 Estonia 2014	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MH681419 POL/2015/Podlaskie	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MG939586 Pol16_29413_o23	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT847620 Pol17_55892_C754	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MG939589 Pol17_05838_C220	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT847621 Pol18_28298_O111	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT847623 Pol19_53050_C1959/19	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT889536 Pol19_01529_C88/19	AAATAACAAGTATATAGGAATATATAGGAATATATAGGAATATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT951764 Pol20_12821-24_O1/20	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT951767 Pol20_29419-3_O6/20	AAATAACAAGTATATAGGAATATATAGGAATATATAGGAATATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
KJ620037 Ukr12/Zapo	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MN194591 Kyiv/2016/131	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
LR722599 Moldova 2017/1	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
LR722600 CzechRepublic 2017/1	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MN715134 HU_2018	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
LR536725 Belgium 2018/1	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK543947 Belgium/Etalle/wb/2018	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
LR899193 Germany 2020/1	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT496893 GZ201801	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK645909 wbBS01	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK333180 Pig/HLJ/2018	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK333181 DB/LN/2018	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK128995 China/2018/AnhuiXCGQ	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MN393477 Wuhan 2019-2	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MN172368 pig/China/CAS19-01/2019	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MK940252 CN/2019/InnerMongolia-AES01	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW033528 wbShX01	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW521382 HuB20	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW656282 Pig/Heilongjiang/HRB1/2020	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT852023 MNG/7/BU/2019	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MN603969 Korea/Pig/Paju1/2019	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MZ812370 VNUA Hanoi-ASF2-2019	AAATAACAAG----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT180393 NgheAn_2019	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW465755 VNUA-ASFV-05L1/HaNam/VN/2020	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MZ812411 VNUA BG-ASF3-2020	AAATAACAAGTATATAGGAATATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW396979 Timor-Leste/2019/1	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MW788581 MY/Beluran/VRI-1162-2021	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
MT851948 Indo/2019/Pig/North Sumatra	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
ON108574 632/AL/2022	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA
ON108571 2802/AL/2022	AAATAACAAGTATATAGGAA----------TATATAGGAATATATAGGAATATATAGAAATATATAGAAATAGCTAAGCTTA

Figure 4. Nucleotide sequence alignment of the partial intergenic region between I73R and I329L from African swine fever genotype II viruses. Viruses characterized in this study are indicated in bold.

The partial sequences and the complete genome sequence have been deposited in the NCBI (ON108572, ON108573, ON108574 and ON108571).

4. Discussion

To the best of our knowledge, Italy is the only country in Europe where the ASFV genotypes I and II coexist. In Sardinia, the ASFV genotype I was first identified in 1978 in the Cagliari province, presumably introduced from the Iberian Peninsula via food waste, which was subsequently fed to pigs [51]. The geographical location of the infection on an island has helped to limit the disease spread in the peninsula. In fact, in 40 years, only two introductions of the virus in mainland Italy have been reported. The first ASFV genotype II introduction in the mainland regions occurred in a wild boar, detected in Northwest

Italy in January 2022 [13]. This study represents a comprehensive attempt to resolve the intragenotypic relationships of the two genotype II isolates that are geographically and temporally linked to causing outbreaks in mainland Italy in January 2022 and to detect epidemiological links that may exist between outbreaks in previously affected countries. The molecular characterization of the different ASFV outbreaks is crucial for investigating the introduction of the virus and for quickly differentiating between closely related strains [27] to extend our knowledge of ASFV's viral evolution and epidemiology. Understanding the molecular evolution of ASFV isolates is necessary for applying effective prevention and control strategies.

The combined p72, ECORI and CVR approach and full–genome strategy have been used to achieve high levels of discrimination among closely related virus isolates. The two Italian strains, 632/AL/2022 and 2802/AL/2022, were defined as genotype II based on partial p72 gene and full–genome sequencing. The nucleotide sequences obtained from the two isolates revealed 100% similarity with the earlier characterized genotype II reference strains available in GenBank, and we have not identified any subgroups within genotype II (Figures 1 and 2). The B602L gene of ASFV is a hypervariable genetic marker that has shown useful for high-resolution discrimination of viruses that are identical according to their p72 and p54 genotypes. This region contains 12 base-pair repeats encoding four amino acids that vary in number and sequence when the genomes of different isolates are compared [26]. The CVR subtyping clustered the Italian wild boar ASFVs recently detected in Italy within the CVR variant I, with the majority circulating in Eastern European countries and Asian countries since the first introduction of the virus to Georgia in 2007. In addition, to further define the most likely origin of the strains, we compared the TRS in the IGR between I73R and I329L with 51 genotype II reference strains from Europe and Asian countries isolated between 2007 and 2022. The presence of TRS insertions in IGR between the I73R and I329L genes was first described in 1992 [52] and subsequently analyzed as an effective ASFV genome marker to discriminate closely circulating ASFVs from Eastern Europe [53], Russia [54] and Italy [33]. Recent ASF outbreaks in Italy were caused by ASFV strains of the IGR variant II, identical to the strains which have frequently occurred in wild boar and domestic pigs in EU countries since ASFV's introduction into EU in 2014 and Asian countries after 2018.

Overall, these results confirm the remarkable genetic stability of the ASFV genotype II. It is not unexpected that the sequences of 632/AL/2022 and 2802/AL/2022 have extremely high homology with sequences, which identify to genotype II strains over a wide geographic area. Particularly, the mean genetic diversity is very low among the ASFV genotype II strains collected in Italy and the other genotype II strains identified in different countries from 2007 to date. Generally, in countries where multiple mechanisms of ASF transmission (mixed sylvatic and domestic cycles) play crucial roles in disease epidemiology, higher levels of variation are observed among viruses. In Italy and other European countries, ASFV strains have not been circulating in a sylvatic cycle as they have to date within the wild boar population. Despite the limited data, the potential involvement of ticks in the transmission of ASFV can be excluded [55].

Presently, given the high sequence similarity, it is impossible to trace the exact geographic origin of the virus to Northwest Italy in January 2022 at a country level. Moreover, the full-length sequences available in the NCBI are not completely representative of all affected territories. Therefore, the exact route and time of ASFV's introduction remain unknown.

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