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BRIEF REPORT

The unique genetic variation within the O174L gene of Polish strains of African swine fever virus facilitates tracking virus origin

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More than ten years after the emergence of African swine fever (ASF) in Georgia, the disease continues to pose a serious threat not only to European countries, which are leading pork producers, but also to worldwide trade of swine. The front of the ASF zone is constantly expanding westward, and recently, the first cases were reported in Belgium, suggesting a crucial role of human activity in ASF spread, considering the exceptional resistance of the causal agent to environmental conditions [1–4]. Moreover, the disease was confirmed in numerous pig herds in China, raising worldwide concern about an approaching ASF pandemic [5]. In Poland, the disease continues to spread into new areas. At the end of 2017, ASF cases were confirmed in wild boars in the area surrounding Warsaw, indicating an unexpected incursion of the disease into a previously ASF-free area of the country. From 2014 to the end of 2018, a total of 3347 cases in wild boars and 213 outbreaks in pig herds were reported in Poland [6].

Previous reports on the genetic diversity of African swine fever virus (ASFV) have reported a low level of genetic variability among European isolates [7–12]. Recent investigations of ASFV genotypes have been based on sequencing of the B646L and E183L genes and analysis of the intergenic region (IGR) between I73R and I329L genes [7, 8]. Another study of the molecular evolution of the EP402R and MGF505-2R genes of 67 Polish strains revealed minor genetic diversity within these genes, indicating slow but consistent molecular evolution in these regions [9].

Analysis of the whole genome sequences of seven Polish ASFV isolates revealed 14-nucleotide sequence variation within the O174L gene, which encodes the DNA polymerase beta-like protein [13]. Analysis of 46 ASFV isolates collected from ASF cases in wild boars and outbreaks in pigs between 2014 and 2018 in Poland confirmed the presence of a specific mutation – a 14-nucleotide insertion that might be useful to distinguish closely related ASFV isolates belonging to the highly pathogenic genotype II. This might aid in achieving a better understanding of ASFV transmission routes and sources of infection in Eastern and Central European countries. In this brief report, we present the identification of a new ASFV genetic marker within the nucleotide sequences of some ASFV Polish isolates. A spatiotemporal analysis in combination with phylogenetic data partially allowed the spread of ASFV in Poland to be traced.

Swine pulmonary alveolar macrophages (PAMs) were used to obtain and propagate 14 ASFV isolates for 3 passages. Total DNA was extracted from infected PAMs as described elsewhere [9], and viral DNA was amplified using an UPL real-time PCR method as described by Fernández-Pinero *et al.* [14]. Next, the whole genome was sequenced using a Miseq sequencer (Ilumina, San Diego, USA). The complete genome sequences of seven Polish ASFV isolates were submitted to the GenBank database under the accession numbers MG939583-MG939589. A genetic variation was observed within the O174L genes (encoding the beta protein of DNA polymerase) of three sequences collected in 2017 originating from two wild boars (cases #201 in Łosice and #754 in Piaseczno) as well as from domestic pigs (outbreak#81 in Radzyń Podlaski) from different locations. Subsequently, 46 ASFV isolates from the ASF National Reference Laboratory (NRL), collected between 2014 and 2018 (Table 1), were selected for further genomic analysis. In order to confirm the 14-nucleotide-insertion within the O174L gene, a pair of primers amplifying the 673-bp region of interest was designed. The primer binding sites were as follows: forward primer, 5'- TGGCTCAGACGATATTTC AACTC-3', nt 128,160-128,182; reverse primer, 5'-GCC TCCACCACTTTGAACCAT-3', nt 128,813-128,832. The optimal annealing temperature was 46°C. PCR was performed, and the products were subjected to sequencing by

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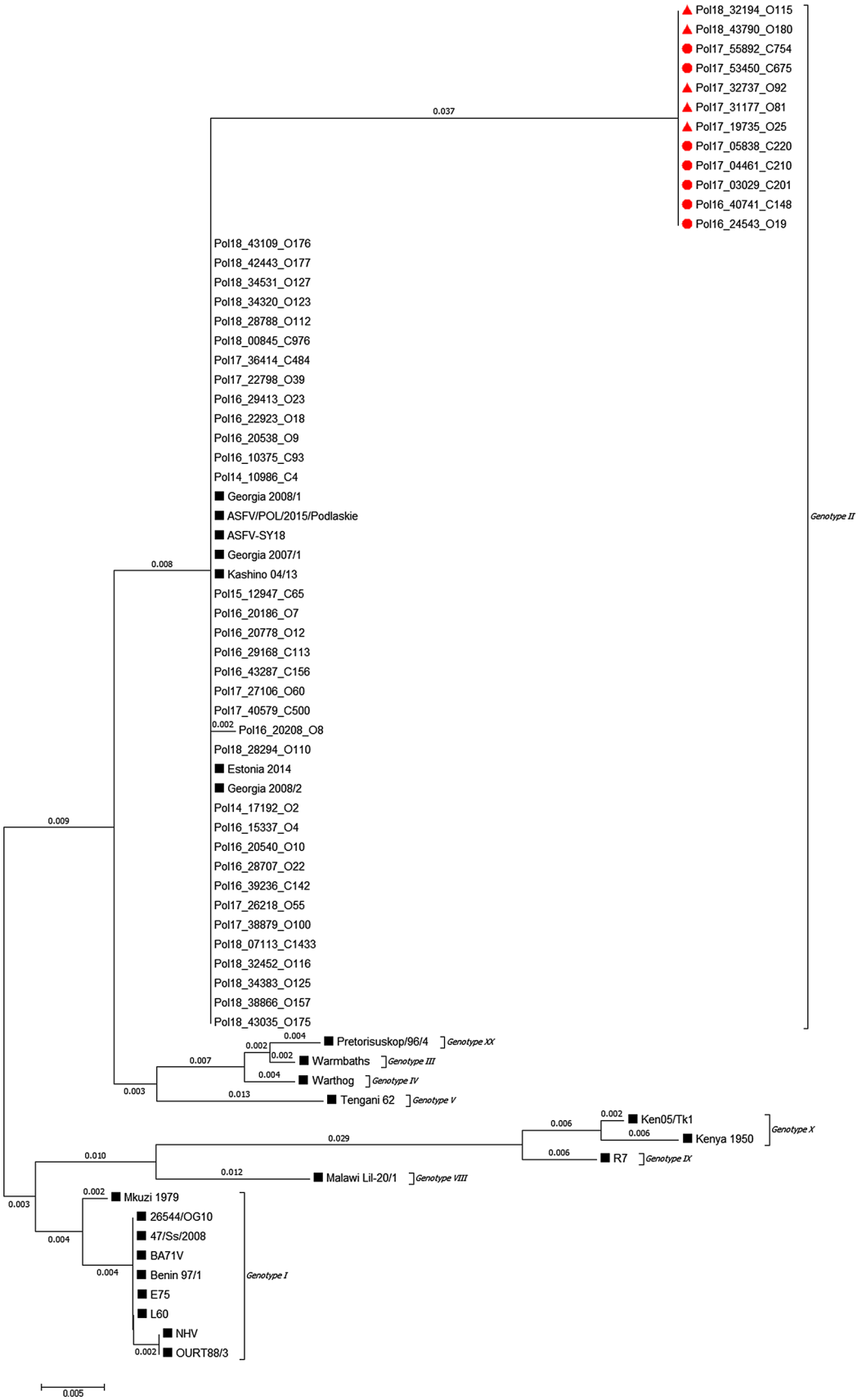
Table 1 African swine fever isolates originating from Poland from 2014 to 2018, selected for a study of genetic variation within the O174L gene. Isolates containing a mutation resulting in the insertion of 14 nucleotides within the O174L gene are indicated by gray shading

Isolate name	Accession no.	Year	Case no.	Outbreak no.	Voivodeship	District	County
Pol14_10986_C4	MH764342	2014	4	n/a	Podlaskie	Sokółka	Krynki
Pol14_17192_O2	MH764339	2014	n/a	2	Podlaskie	Białystok	Gródek
Pol15_12947_C65	MH764343	2015	65	n/a	Podlaskie	Białystok	Gródek
Pol16_10375_C93	MH764346	2016	93	n/a	Podlaskie	Hajnówka	Narewka
Pol16_15337_O4	MH764344	2016	n/a	4	Podlaskie	Hajnówka	Hajnówka
Pol16_20186_O7	MH764332	2016	n/a	7	Podlaskie	Białystok	Choroszcz
Pol16_20208_O8	MH764348	2016	n/a	8	Podlaskie	Zambrów	Zambrów
Pol16_20538_O9	MH764337	2016	n/a	9	Podlaskie	Wysokie Mazowieckie	Wysokie Mazowieckie
Pol16_20540_O10	MH764330	2016	n/a	10	Podlaskie	Wysokie Mazowieckie	Sokoły
Pol16_20778_O12	MH764322	2016	n/a	12	Podlaskie	Bielsk Podlaski	Bielsk Podlaski
Pol16_22923_O18	MH764321	2016	n/a	18	Podlaskie	Mońki	Goniądz
Pol16_24543_O19	MH764316	2016	n/a	19	Lubelskie	Biała Podlaska	Leśna Podlaska
Pol16_28707_O22	MH764340	2016	n/a	22	Podlaskie	Siemiatycze	Nurzec Stacja
Pol16_29168_C113	MH764323	2016	113	n/a	Podlaskie	Mońki	Trzcianne
Pol16_29413_O23	MH764336	2016	n/a	23	Podlaskie	Siemiatycze	Nurzec Stacja
Pol16_39236_C142	MH764350	2016	142	n/a	Podlaskie	Siemiatycze	Nurzec Stacja
Pol16_40741_C148	MH764305	2016	148	n/a	Lubelskie	Biała Podlaska	Zalesie
Pol16_43287_C156	MH764347	2016	156	n/a	Podlaskie	Hajnówka	Czeremcha
Pol17_03029_C201	MH764313	2017	201	n/a	Mazowieckie	Łosice	Sarnaki
Pol17_04461_C210	MH764310	2017	210	n/a	Mazowieckie	Łosice	Sarnaki
Pol17_05838_C220	MH764307	2017	220	n/a	Podlaskie	Siemiatycze	Mielnik
Pol17_19735_O25	MH764309	2017	n/a	25	Podlaskie	Mońki	Trzcianne
Pol17_22798_O39	MH764334	2017	n/a	39	Podlaskie	Grajewo	Radziłów
Pol17_26218_O55	MH764345	2017	n/a	55	Lubelskie	Radzyń Podlaski	Kąkolewnica
Pol17_27106_O60	MH764325	2017	n/a	60	Lubelskie	Włodawa	Włodawa
Pol17_31177_O81	MH764314	2017	n/a	81	Lubelskie	Radzyń	Kąkolewnica
Pol17_32737_O92	MH764306	2017	n/a	92	Mazowieckie	Siedlce	Korczew
Pol17_36414_C484	MH764328	2017	484	n/a	Podlaskie	Sejny	Krasnopol
Pol17_38879_O100	MH764331	2017	n/a	100	Podlaskie	Augustów	Lipsk
Pol17_40579_C500	MH764349	2017	500	n/a	Podlaskie	Augustów	Bargłów Kościelny
Pol17_53450_C675	MH764308	2017	675	n/a	Mazowieckie	Warszawa	Warszawa
Pol17_55892_C754	MH764315	2017	754	n/a	Mazowieckie	Piaseczno	Góra Kalwaria
Pol18_00845_C976	MH764329	2018	976	n/a	Warmińsko-Mazurskie	Braniewo	Płoskinia
Pol18_07113_C1433	MH764335	2018	1433	n/a	Podlaskie	Suwałki	Szypliszki
Pol18_28294_O110	MH764333	2018	n/a	110	Lubelskie	Chełm	Wierzbica
Pol18_28788_O112	MH764319	2018	n/a	112	Podlaskie	Sejny	Giby
Pol18_34320_O123	MH764324	2018	n/a	123	Lubelskie	Chełm	Dorohusk
Pol18_32194_O115	MH764312	2018	n/a	115	Lubelskie	Parczew	Jabłoń
Pol18_32452_O116	MH764338	2018	n/a	116	Lubelskie	Włodawa	Hańsk
Pol18_34383_O125	MH764318	2018	n/a	125	Podlaskie	Sokółka	Dąbrowa Białostocka
Pol18_34531_O127	MH764326	2018	n/a	127	Lubelskie	Biała Podlaska	Sławatycze
Pol18_38866_O157	MH764341	2018	n/a	157	Mazowieckie	Siedlce	Wiśniewo
Pol18_42443_O177	MH764320	2018	n/a	177	Warmińsko-Mazurskie	Bartoszyce	Sępopol
Pol18_43035_O175	MH764317	2018	n/a	175	Lubelskie	Lubartów	Ostrów Lubelski
Pol18_43109_O176	MH764327	2018	n/a	176	Warmińsko-Mazurskie	Kętrzyn	Srokowo
Pol18_43790_O180	MH764311	2018	n/a	180	Mazowieckie	Mińsk Mazowiecki	Siennica


Pol18_32194_O115
Pol18_43790_O180
Pol17_55892_C754
Pol17_53450_C675
Pol17_32737_O92
Pol17_31177_O81
Pol17_19735_O25
Pol17_05838_C220
Pol17_04461_C210
Pol17_03029_C201
Pol16_40741_C148
Pol16_24543_O19
0.037
Pol18_43109_O176
Pol18_42443_O177
Pol18_34531_O127
Pol18_34320_O123
Pol18_28788_O112
Pol18_00845_C976
Pol17_36414_C484
Pol17_22798_O39
Pol16_29413_O23
Pol16_22923_O18
Pol16_20538_O9
Pol16_10375_C93
Pol14_10986_C4
Georgia 2008/1
ASFV/POL/2015/Podlaskie
ASFV-SY18
Georgia 2007/1
Kashino 04/13
Pol15_12947_C65
Pol16_20186_O7
Pol16_20778_O12
Pol16_29168_C113
Pol16_43287_C156
Pol17_27106_O60
Pol17_40579_C500
Pol16_20208_O8
Pol18_28294_O110
Estonia 2014
Georgia 2008/2
Pol14_17192_O2
Pol16_15337_O4
Pol16_20540_O10
Pol16_28707_O22
Pol16_39236_C142
Pol17_26218_O55
Pol17_38879_O100
Pol18_07113_C1433
Pol18_32452_O116
Pol18_34383_O125
Pol18_38866_O157
Pol18_43035_O175
Genotype II
0.008
0.002
0.009
0.004
Pretorisuskop/96/4
Genotype XX
0.002
0.002
Warmbaths
Genotype III
0.007
0.004
Warthog
Genotype IV
0.003
0.013
Tengani 62
Genotype V
0.002
0.006
Ken05/Tk1
0.006
Kenya 1950
Genotype X
0.029
0.006
R7
Genotype IX
0.010
0.012
Malawi Lil-20/1
Genotype VIII
0.003
0.002
Mkuzi 1979
26544/OG10
47/Ss/2008
BA71V
0.004
0.004
Benin 97/1
E75
L60
NHV
0.002
OURT88/3
Genotype I
0.005

◂**Fig. 1** Minimum-evolution (ME) phylogenetic tree of African swine fever virus isolates from Eastern European strains based on the sequences of the O174L gene, including 46 sequences from Poland (Table 1) and 24 sequences of various genotypes. Black squares (■) indicate sequences available in the GenBank database and reference DNA from the NRL collection, which was also sequenced in this study. Red triangles and dots (▲, outbreaks, ●, cases) indicate samples containing the additional 14-nt insertion within the O174L gene. The scale bar indicates nucleotide substitutions per site.

the Sanger method. The resulting sequences were aligned to those of closely related European genotype II isolates and those of other ASFV genotypes, with sequences available in the GenBank database. The following sequences were used for comparison (numbers in brackets indicate the: p72 genotype and the GenBank accession number): 47/Ss/2008 (I, KX354450.1), 26544/OG10 (I, KM102979.1), ASFV-SY18 (II, MH766894.1), ASFV/POL/2015/Podlaskie (II, MH681419.1), BA71V (I, NC_001659.2), Benin 97/1 (I, AM712239.1), E75 (I, FN557520.1), Estonia 2014 (II, LS478113.1), Georgia 2007/1 (II, FR682468.1), Georgia 2008/1 (II, MH910495.1), Georgia 2008/2 (II, MH910496.1), Kashino 04/13 (II, KJ747406.1), Ken05/Tk1 (X, KM111294.1), Kenya 1950 (X, AY261360.1), L60 (I, KM262844.1), Malawi Lil-20/1 (VIII, AY261361.1), Mkuzi 1979 (I, AY261362.1), NHV (I, KM262845.1), OURT88/3 (I, AM712240), Pretorisuskop/96/4 (XX, AY261363.1), R7 (IX, MH025917.1), Tengani 62 (V, AY261364.1), Warmbaths (III, AY261365.1), and Warthog (IV, AY261366.1). Additionally, two related reference DNA specimens from the NRL collection, Ukr12/Zapo and Lt14/1490, were also sequenced and included in the analysis. Nucleotide and amino acid sequence alignments were performed with a similarity cost matrix, using the Geneious R9 global alignment algorithm, and a phylogram was constructed using the minimum-evolution (ME) algorithm in MEGA 6 software (Fig. 1) [15]. All O174L gene sequences of Polish ASF viruses generated in this study were submitted to the GenBank database under the accession numbers MH764305-MH764350.

An alignment of O174L nucleotide sequences of ASFV revealed the presence of an unique variation in 12 isolates originating from Poland. An insertion of 14 nucleotides (TCACTACTGAAAAA) was found, corresponding to a tandem repeat of nt 128282-128283 with reference to the Georgia 2007/1 strain. This insertion resulted in a shift to a different reading frame containing a premature stop codon. The wild-type and mutated proteins thus shared only 93% amino acid sequence identity. The disrupted O174L gene encodes a reparative DNA polymerase belonging to the family X DNA polymerases [16]. The ASFV polymerase X is able to efficiently repair single-nucleotide DNA breaks by base-excision repair (BER) during viral infection [17]. Previous studies have shown that deletion of the ASFV polymerase X gene results in accumulation of DNA damage with an increase of mutation frequency in swine macrophages [17]. Therefore, this particular gene is essential for maintaining viral genetic information. However, the effect of the observed insertion on the function of the polymerase remains unknown and needs to be investigated.

The 14-nucleotide-long insertion was found in 12 of the sequences listed in Table 1 and was found in isolates from wild boars (n = 6; designated as "C" [case] in the isolates names) as well as isolates from pigs (n = 6; designated as "O" [outbreak]). A spatiotemporal analysis using ArcGIS software showed that most of the ASFV isolates possessing the insertion were from the region near the Biała Podlaska district, where the mutation in O174L was first identified (outbreak #19, August 2016, 15 km from the Belarusian border) (Fig. 2). Seven other isolates collected between 2016 and 2018 originated from within approximately 50 km of outbreak #19. The remaining four isolates were from more distant locations: outbreak #25 (June 2016, Mońki district) 140 km from the Biała Podlaska district, and three isolates within the so-called "Warsaw cluster", located between 100 and 130 km from outbreak #19. It is noteworthy that all three samples obtained from this particular cluster contained the mutation. Taking into consideration that wild boars have a rather sedentary life style and the fact that the emergence of ASFV within the "Warsaw cluster" was sudden, unexpected, and distant from the sites of previous disease reports, we presume that the virus was introduced into this particular area by humans [18]. Furthermore, the emergence of ASF in the Mońki district might also have resulted from human activity; however, only one out of four isolates tested (outbreak #18, case #113, outbreak #25 and outbreak #39) from this region contained the mutation, suggesting that ASFV was introduced into this particular district on at least two separate occasions. In 2018, 11 isolates originating from pig outbreaks were analyzed, of which only two contained the specific mutation. One of them was collected in the Parczew district, but five other isolates collected from 75 km away did not contain the mutation, again showing that multiple introductions had occurred in this region.

Analysis of whole genome sequences of nine Polish ASFV isolates with confirmatory sequencing by the Sanger method revealed a new genetic marker within the O174L gene that might be useful for distinguishing closely related ASFV isolates in Poland. The data suggest that the simultaneous emergence of ASFV on domestic pig farms in various locations in Poland was probably due to multiple independent, unrelated disease outbreaks. However, an involvement of human activity in the spread of ASF in Poland, particularly into previously ASF-free and geographically distant areas could not be excluded, since the migratory activity of wild boars is very limited [18]. The genetic variability within the O174L gene provides an opportunity for deeper insight into

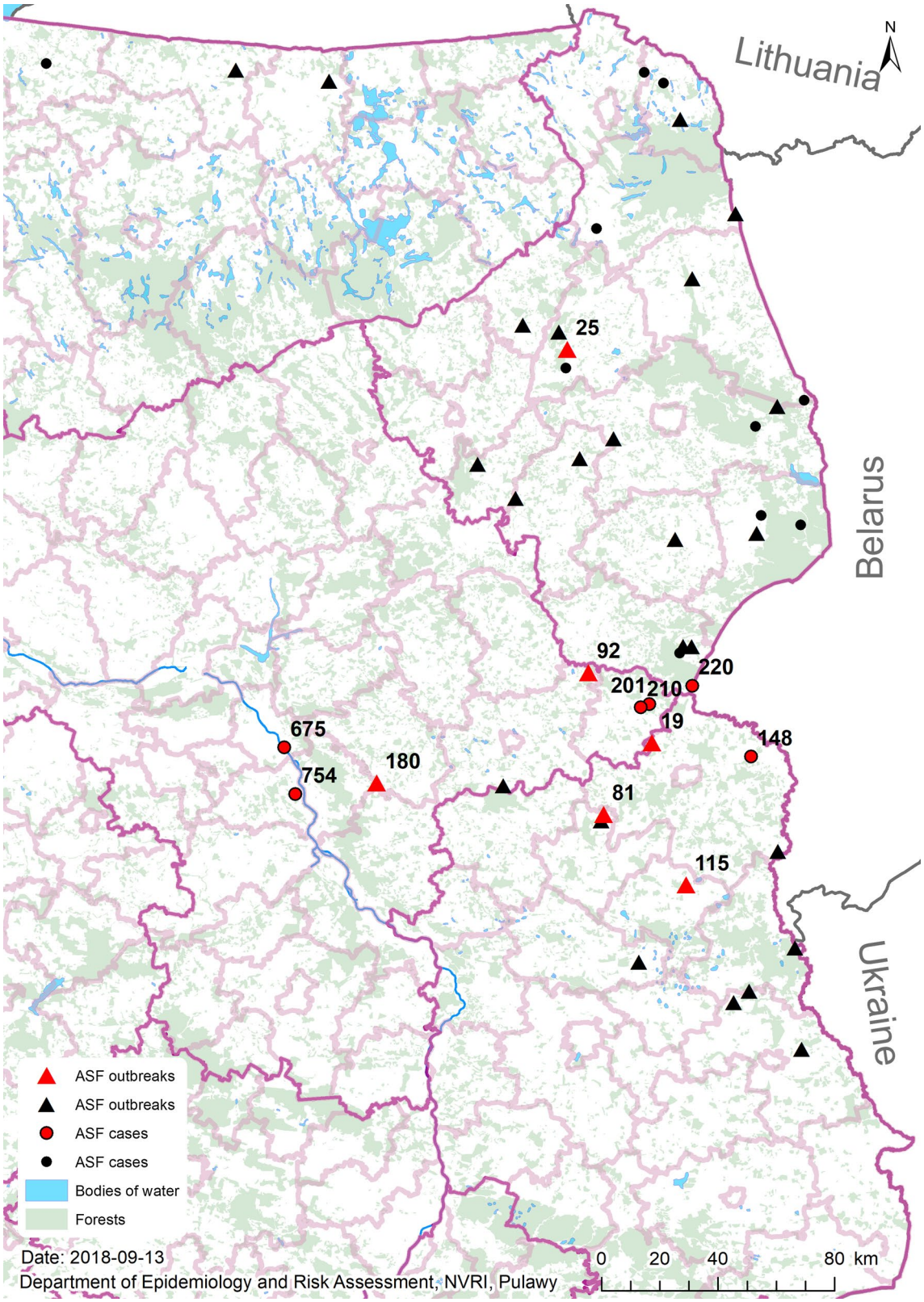


Fig. 2 Geographical origin of the ASFV isolates investigated. Numbers correspond to the isolates containing the specific mutation, which are indicated in red (▲, outbreaks, ●, cases). Isolates without the mutation are shown in black and are not numbered.

the spread of ASFV in Poland and provides evidence of a relationship between geographically separated outbreaks, as was the case with the emergence of ASFV in the region surrounding Warsaw, where the virus seems to be to have been imported from the region of Biała Podlaska. The predicted amino acid protein sequence differs moderately from the original ASFV Georgia 2007/1 strain and shows only 93% sequence identity, whereas the encoded DNA polymerase is highly conserved among various ASFV genotypes (Fig. 1). The duplication of the 14-nt long sequence seems to have occurred suddenly, since no intermediate sequences between original and mutated sequences were detected, indicating that this tandem repeat has a higher evolution rate that the whole ASFV genome, a phenomenon that has been observed with other DNA viruses [19]. Genetic data like those presented in this report are essential for the study of ASFV evolution. However, an investigation of the effect of the variation described here on biology of the virus is still required.

Data availability The datasets generated and analyzed in the current study are available in the GenBank repository under accession numbers MH764305-MH764350.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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