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Article

Genetic Diversity of Porcine Circovirus 2 Subtypes in Wild Boar and Domestic Pigs in Ukraine

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Abstract: Porcine circovirus type 2 (PCV2) is responsible for a number of porcine circovirus associated diseases (PCAD) that can severely impact domestic pig herds. For a non-enveloped virus with a small genome (1.7 kb ssDNA), PCV2 is remarkably diverse, with 8 subtypes (a-h). New subtypes of PCV2 can spread through migration of wild boars, which are thought to infect domestic pigs and spread further through the domestic pig trade. Despite a large swine population, the diversity of PCV2 subtypes in Ukraine has been undersampled, with few PCV2 genome sequences reported in the past decade. To gain a deeper understanding of PCV2 subtype diversity in Ukraine, samples of blood serum were collected from wild boars (n = 107) that were hunted in Ukraine during the November-December 2012 hunting season. We found 34/107 (31.8%) prevalence of PCV2 by diagnostic PCR. For domestic pigs, liver samples (n = 16) were collected from a commercial market near Kharkiv in 2019, of which 6/16 (37%) were positive for PCV2. We sequenced the genotyping locus ORF2, a gene encoding the PCV2 viral capsid (Cp), for 11 wild boar and 6 domestic pig samples in Ukraine using an Oxford Nanopore MinION device. Of 17 samples with resolved subtypes, PCV2 subtype b was most common in wild boar (10/11, 91%), while domestic pigs were infected with subtypes b and d. We also detected subtype b/d and b/a co-infections in wild boar and domestic pigs, respectively, and subtype f in a wild boar from Poltava for the first time in Ukraine. Building a maximum likelihood phylogeny, we identified a sublineage of PCV2 subtype b infections in both wild and domestic swine, suggesting a possible epizootic cluster and ecological interaction in northeastern Ukraine.

Keywords: porcine circovirus; PCV2; domestic pig; wild boar; subtype; phylogenetics; MinION; Ukraine

1. Introduction

1.1. Epidemiology of porcine circovirus 2-associated diseases in swine

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Between 2017 and 2021, the world produced 96-112 million tons of pork per year [1]. Swine raised in commercial facilities and backyard farms are at risk for epizootic infections. Some swine diseases, such as African swine fever (ASF) can only be controlled through biosecurity measures as there is no widely distributed vaccine for the causative virus [2]. The pork industry uses vaccines to avoid the increased cost from animal death and weight loss caused by other pathogens, particularly highly transmissible viruses such PRRSV (arterivirus), PEDV (coronavirus), classical swine fever (flavivirus), swine influenza A virus, and porcine circovirus type 2 (PCV2) [3]. However, vaccine formulations must often be updated to maintain efficacy against circulating genotypes of swine viruses.

Pigs of all breeds, as well as wild boars are susceptible to porcine circovirus (type) 2 infection, PCV2 can cause an array of diseases collectively known as porcine circovirus associated diseases (PCAD), with diverse clinical presentations [4,5]. In diseased pigs, particularly younger animals, PCAD signs often manifest as progressive weight loss (wasting), dyspnea, and jaundice and pathologically by lymphadenopathy, interstitial pneumonia, hepatitis, nephritis, spontaneous abortion, and/or death [5,6]. PCAD and related diseases include postweaning multisystemic wasting syndrome, porcine dermatitis and nephropathy syndrome, PCV2-associated lymphoid depletion, porcine respiratory disease complex, as well as enteritis, hepatitis, myocarditis, exudative dermatitis, CNS disease, abortions and reproductive failure, associated with PCV2. However, PCV2 can also be an asymptomatic infection. Alternately, when PCV2 co-infection with other pathogens or even different PCV2 variants may exacerbate PCAD [4,5,6].

Transmission of the PCV2 can occur horizontally (pig-to-pig contact) and vertically (sow to piglet). In domestic pigs, the source of infection are sick and latently infected animals of either sex, which can excrete the pathogen in feces, urine, saliva, milk, nasal or ocular secretions [7,8]. As a non-enveloped virus, PCV2 can survive for a long time in biological fluids and faeces and is easily transmitted orally or respiratory routes to other animals, and the amount of virus during transmission can vary depending on the age of the animals [8].

The pathogen is transmitted directly through contact with infected animals or through the use of virus-contaminated feed, sexual contact or artificial insemination [8,9, 10]. The attack rate of an infected pig is unknown, but two studies suggest transmission takes 6 weeks. In one study, PCV2 was transmitted 42 days after infection between infected and seronegative piglets in direct contact [11]. In another study, after intranasal infection of boars, PCV2 can be secreted in semen for 47 days, suggesting a sexual route of PCV2 transmission also exists [6].

It is thought that PCV2 can spread between countries by migration of wild boar, or through the trade in contaminated feed or subclinically infected pigs [8,12]. When a new subtype of PCV2 enters a country it can transfer between domestic pigs and wild boar populations, thus wild boars may act as both a reservoir and a vector for PCV2 [13].

Whole-virus inactivated and recombinant vaccines have reduced PCAD incidence, reduced weight gain from asymptomatic PCV2 infections in swine herds, and have reduced incidence of PCV2 infections [12]. However, vaccines have not prevented PCV2 infections, which has led to an increased selection pressure for escape variants in vaccinated pigs [9,12]. In one case, PCV2 in vaccinated pigs had the same non-synonymous nucleotide mutation rate as PCV2 in unvaccinated pigs [14]. The presence of PCV2 in vaccinated pigs, and possible emergence of escape variants, has spurred recent vaccine development and enhanced genomic surveillance in several countries including China, Italy, Korea, and the US [13,15,16,17].

1.2. PCV2 genome and subtypes

PCV2 is a non-enveloped virus with an icosahedral (T=1) capsid that contains a single stranded circular DNA genome of 1.7 kb [18]. The genome of PCV2 contains two major open reading frames (ORFs), and at least four other functional ORFs [18,19,20,21]. The two major genes are the replicase (ORF1) encoding Rep and Rep', and capsid (ORF2) encoding

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the capsid (Cp) protein. Subtypes of PCV2 are determined by ORF2, encoding Cp, with at least 8 subtypes (a-h) recognized [22].

Ukraine is a major pork producer, and harbors both large domestic swine (commercial and backyard) and wild boar populations. These swine populations have been subjected to PCV2 infection [23] and swine influenza [24] in wild boars, and porcine teschovirus 1 [25] and ASF in both wild and domestic animals [26,27]. The circulation of PCV2 subtypes in swine in Ukraine is poorly understood and suffers from the sporadic nature of surveillance. In 2015, Ukraine's major PCV2 subtype was b, with subtypes a, d, and g also in circulation [28,29,30]. Subtype d is currently a common subtype, may replicate better in vaccinated pigs, and was rare before 2014 [5,12,31]. With just 29 sequences reported in NCBI GenBank (to which we add 17 in this study), it is likely that PCV2 diversity in Ukraine has been underestimated.

As part of a One Health capacity-building project, focused on ASFV and swine pathogens co-circulating with ASFV in Ukraine, we developed protocols for virus and bacterial genome diagnostics and sequencing from samples obtained from domestic pigs, pork products, and hunted wild boar [23,25,26,32]. To this end, we built nanopore sequencing capacity at several research institutes in Ukraine, that included training of veterinary researchers in sequencing methods, bioinformatics, and data analysis.

To understand PCV2 circulation in Ukraine, we performed nanopore sequencing of PCV2 ORF1 (Cp) amplicons, generated from archived serum samples from wild boar collected in 2012, and liver samples from domestic pigs collected in 2019. We assembled one complete PCV2 genome from a wild boar. We conducted phylogenetics analysis of the Cp genes and found the most common PCV2 subtype in Ukraine was subtype b, and detected subtype a in wild boar and subtype d in domestic swine. Three domestic pigs and two wild boar also had evidence of co-infection with two PCV2 subtypes.

2. Materials and Methods

2.1. Sample collection

Blood sera were collected from hunted wild boar (n = 107) in the November-December 2012 hunting season in Ukraine. Samples were collected in 10 oblasts (provinces) across Ukraine (Poltava, Sumy, Zaporizhzhia, Chernihiv, Chernivetsk, Cherkasy, Kherson, Lviv, Volyn and Luhansk). Liver samples (n = 16) from clinically healthy pigs were collected from a commercial market in Kharkiv oblast in 2019. PCV2 vaccination status was not available. Sera and liver samples were stored at −30ºC, and total DNA was extracted from 200 µl of serum, or 10% liver suspension in PBS, using a commercial kit (QIAamp cador Pathogen Mini Kit, Qiagen, Valencia CA) according to the manufacturer's instruction. Extracted DNA was eluted in 100 µL elution buffer and stored at −30ºC at the NSC IECVM biosafetly level 2 laboratory in Kharkiv, Ukraine.

2.2. Diagnostic PCR to detect PCV2

All DNA samples were investigated for PCV2 viremia by conventional Taq PCR with Maxima Hot Start Green PCR Master Mix (ThermoFischer Scientific). Primers targeting ORF1 were: forward primer PCV-2F 5'-GAAGACGAGCGCAAGAAAATACG-3', and reverse primer PCV-2R 5'-CCAATCACGCTTCTGCATTTTCCC-3'. The diagnostic primers flank a 421 nt variable region of the tail Rep gene [23]. For each sample, 5 µL of the eluate was run in a 25 µL reaction, with an annealing step at 60 ºC, on a conventional PCR machine (Biometra TAdvanced Thermal Cycler, Analytik Jena GmbH).

2.3. Amplification and sequencing of ORF2 (Cp) and PCV2 genomes

Positive swine DNA samples were used to generate of ORF2 (Cp) gene DNA amplicons (798bp) by PCR for sequencing, using primers reported previously (Rudova et al. (2019). Briefly, PCR for Cp sequencing was performed using conventional PCR and Platinum SuperFi II Taq DNA Polymerase (ThermoFischer/Invitrogen), and primers targeting ORF2 (Cp): forward primer PCV-2seqF 5'-CCCATGCCCTGAATTTCCA-3', and reverse

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primer PCV-2seqR 5'-CCAATCACGCTTCTGCATTTTCCC-3'. For each positive DNA sample, 10 µL of the eluate was run in a 50 µL reaction, with an annealing step at 55ºC, on a Biometra TAdvanced Thermal Cycler PCR machine (Analytik Jena GmbH). To amplify nearly the complete genome of PCV2 (1768 bp), a 3-primer amplification scheme was employed using the same PCR machine with primers and conditions reported in the literature [33]. All PCR products were electrophoresed on a 1.5 % agarose gel stained with ethidium bromide and visualised using an ultraviolet transilluminator to assess DNA quality and concentration. PCR amplicons were purified using a SPRI beads (Agencourt AMPure XP beads at 1:1 sample:beads) prior to sequencing.

2.4. Nanopore sequencing and bioinformatics

All samples were sequenced in multiplex on a nanopore MinION Mk1B device in veterinary labs in Ukraine (NSC IECVM in Kharkiv; SSRILDVSE and NAAS IVM in Kyiv), using an end-ligation (SQK-LSK109) with native barcoding (EXP-NBD104) protocol according to the manufacturer's instructions (ONT: Oxford Nanopore Technologies, Oxford, U.K.). We basecalled and demultiplexed the raw sequence reads using Guppy version 3.6 (ONT). To generate consensus sequences from the raw reads, and discover mixed subtype co-infections, we took a competitive reference-based assembly approach. We filtered, binned, and competitively mapped Cp reads to a subset of PCV2 Cp sequences, representing two subtype a, one subtype b, one subtype d, one subtype g, and two subtype f ORF1 (Cp) sequences, with minimap2 v2.22 [34] to generate consensus genomes. For additional detailed description of bioinformatics methods for consensus genome assembly, see Supplementary Methods.

2.5. Sequence curation and availability

We found references for manual curation by blasting each consensus against the PCV2 database on Genbank (taxid: 85708). The top hit for each consensus was then used to detect and remove indels in the consensus. We also checked and corrected reading frames using transeq from the emboss package v6.6 [35]. All sequences were deposited in NCBI GenBank under BioProject (*pending*) and Accessions (*pending*).

2.6. Phylogenetics analysis

We built a database for phylogenetic tree construction with consensus genomes from our samples, and with PCV2 Cp sequences on GenBank. Our database contained 5862 Cp sequences downloaded from Genbank (Supplemental File 1). Cp sequences in our database were aligned with Mafft V7.407 [36] and manually inspected to remove sequences with early stop codons or incomplete reading frames with Genious v2020.2.1 [37]. We removed recombinant sequences from the inspected Cp sequences with RDP4 v4.101 using settings similar described previously [18]. We then removed all gaps and stop codons in our remaining, aligned Cp sequences using Geneious v2020.2.1. Our final database contained 429 Cp sequences (Supplemental file:**.**

We inferred a maximum likelihood (ML) tree using IQtree, our consensuses, and a sub-sample of our Cp sequence database. Sub-sampling was done with cd-hit using parameter -c 0.985 to reduce our database to a manageable size. After sub-sampling, our Cp sequences left in our database were at least 1.5% different. We added Cp sequences from Ukraine that were removed by sub-sampling back into our sub-sampled database. We built a consensus tree with IQtree v1.6.12 [38] using parameters -st CODON -m MFP+MERGE -bb 1000, the sub-sampled Cp database, and the Cp genes from our consensuses. The tree inference was statistically tested (100 bootstraps). The consensus tree was edited in R studio using the treeio, ape, ggplot2, and ggtree [15,38].

2.7. Mantel test

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Studies of the correlation of geographical and genetic distances of Ukrainian isolates were conducted by Mantel test [39]. We analyzed Mantel test output using R software (https://www.r-project.org) as described [40].

3. Results

3.1. Detection of PCV2 in wild boar and domestic pigs in Ukraine

To analyze PCV2 infection in wild boar, blood sera (n = 107) were opportunistically collected from disease surveillance of hunted wild boar over the November-December 2012 winter hunting season in Ukraine. Samples were collected across mixed forest/agricultural regions of Ukraine, from Poltava, Sumy, Zaporizhzhia, Chernihiv, Chernivetsk, Cherkasy, Kherson, Lviv, Volyn and Luhansk oblasts (**Table 1**). Blood samples were screened by a diagnostic PCR assay against ORF1 to determine PCV2 viremia [23]. Thirty-four (31.8%) samples were positive for PCV2, with samples of good DNA quality from 6 different regions across Ukraine (Zaporizhia, Chernivtsi Poltava, Volyn, Chernihiv, and Luhansk) selected for sequencing (**Figure 1**).

To genotype PCV2 infection in domestic swine, liver samples (n = 16) were collected in a commercial market near Kharkiv in 2019 (**Figure 1**). Although PCV2 infection and PCV2 vaccination status was not known, pork products sold at markets in the Kharkiv region appeared to be derived from generally healthy animals. Nevertheless, in this small sampling, 6 of 16 liver samples (37%) were positive for PCV2 by PCR and Cp gene sequenced.

Table 1. Porcine circovirus 2 detection in hunted wild boar in Ukraine by PCR (2012).

Region in Ukraine (*Oblast*)	Blood Sera Collected	Positive for PCV2 (PCR)	PCV2 Prevalence (%)	Number selected for sequencing
Poltava	15	3	20%	2
Sumy	8	5	63%	-
Zaporizhia,	13	12	92%	1
Chernihiv	17	5	29%	4
Chernivtsi	5	2	40%	2
Cherkasy	10	1	10%	-
Kherson	1	0	0%	-
Lviv	13	3	23%	-
Volyn	13	1	8%	1
Luhansk	12	2	17%	2
Total:	107	34	31.8%	12

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Figure 1. Sampling for PCV2 in Ukraine in wild boar and domestic pigs. Map of Ukraine showing the oblasts (provinces) where wild boar blood serum samples were collected opportunistically over the November-December 2012 winter hunting season (red asterisks). Domestic pig liver samples were collected from a commercial market near Karhkiv in 2019, site of the NSC IECVM veterinary lab (white asterisk). Other PCV2 sequences downloaded from GenBank were from the named oblasts on the map.

3.2. Subtyping of PCV2 by sequencing

To analyze subtype(s) of PCV2 in wild boar and domestic pig samples, we amplified and sequenced a 798 bp amplicon of ORF2 (Cp) using a nanopore (MinION) platform. We generated consensus sequences from nanopore sequence reads by reference-based assembly, and tested for co-infections (mixed subtype infections), by quantitative assembly to subtypes a, b, d, g, and f. All samples, except one (Luhansk-1), had a read depth of 14,941-635,035 reads and a mean Q-score of 12.9-14.6 for reads matching the major PCV2 subtype identified (**Table 2**).

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Table 2. PCV2 ORF2 capsid gene (Cp) amplicon sequencing results.

Sample*	Host Type	Sequence Yield (Mbp)	Number of Reads	Mean quality (Q-score)	Major PCV2 Subtype
Chernihiv 1	Wild Boar	1349.3	170304	13	b
Chernihiv 2	Wild Boar	558.0	70235	13.2	b
Chernihiv 3	Wild Boar	568.1	71070	13.2	b
Chernihiv 4	Wild Boar	383.1	47930	13.1	b
Chernivtsi 1	Wild Boar	1149.3	144338	13.1	b
Chernivtsi 2	Wild Boar	1163.6	146489	13.2	b
Luhansk 1	Wild Boar	NA	NA	NA	NA
Luhansk 2	Wild Boar	365.1	44527	13.2	b
Poltava 1	Wild Boar	696.9	87848	13	b
Poltava 2	Wild Boar	605.2	76231	12.9	f
Volyn	Wild Boar	320.0	39156	13.1	b
Zaporizhzhia	Wild Boar	121.1	14941	13.1	b
Kharkiv 1	Domestic pig	3599.6	453425	14.5	b
Kharkiv 2	Domestic pig	1240.9	156283	14.6	d
Kharkiv 3	Domestic pig	1828.5	229888	14.6	b
Kharkiv 4	Domestic pig	560.0	70490	14.6	d
Kharkiv 5	Domestic pig	5036.4	635035	14.5	b
Kharkiv 6	Domestic pig	213.9	26903	14.6	d

3.3. PCV2 full-length genome from Ukraine.

We attempted to generate full length genomes of 10 total DNA samples using a 3-primer PCR protocol reported previously [33]. Although all samples yielded long reads, we successfully assembled one complete genome from a wild boar (3194 reads with mean Q = 12.5, read depth of 32X for reads >1700 nt length). This was a subtype **b** genome annotated as PCV2/Chernihiv-1/2012 (1767 nt length; Accession: pending). This genome matched its closest relative in NCBI GenBank for 1764/1767 nt with 3 single nucleotide variations (SNV). The amino acid sequences of the Rep, Cp, and ORF3 proteins of this virus are an exact match to its closest PCV2 subtype **b** relatives sequenced in the same year 2012 in China (JX406426.1 and HQ395035.1).

3.4. Co-infections with two PCV2 subtypes

We tested for co-infections in our samples with a co-infection pipeline that quantitatively mapped reads to different PCV2 subtypes (see Supplementary Methods). We found five incidences of co-infection by more than one PCV2 subtype (**Table 3**). Two of 12 wild boar samples that were positive for PCV2, both collected in Chernivtsi in 2012, had >3% of their reads from a co-infection with another (minor) PCV2 subtype, **f**; while 3 of 6 infected domestic pig samples collected in 2019 had 5-28% of their reads from a co-infection with subtype **d**. Thirteen of 18 isolates resolved (60%) showed evidence of only one (major) subtype of PCV2. according to our sequencing analysis (**Table 2**).

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Table 3. Co-infections of two PCV2 subtypes in swine in Ukraine. Percent and number of reads binned for each minor variant in a co-infection listed.

Sample	% Minor sub-type	No. Reads	Major sub-type	Minor Sub-type
Chernivtsi 1 (wild boar)	3.47	5191	b	a
Chernivtsi 2 (wild boar)	3.93	6000	b	a
Karhkiv 4 (domestic pig)	28.41	28356	d	b
Karhkiv 5 (domestic pig)	5.81	39560	b	d
Karhkiv 6 (domestic pig)	25.82	9398	d	b

3.5. Phylogenetic analysis of PCV2 in Ukraine

We analyzed the relationship among PCV2 isolates and subtypes identified in Ukraine by phylogenetic tree construction. PCV2 ORF2 (Cp) amplicon sequences were aligned using MAFFT V7.407 with a set of **N** contemporary PCV2 reference sequences including all previous good quality sequences from Ukraine, subsampled from NCBI GenBank (Supplemental File 1). The alignment was used for inference of a maximum likelihood (ML) tree of PCV2 Cp sequences using IQtree v1.6.12. We found that the PCV2 sequences from Ukraine grouped into clades containing reference genomes representing subtypes a, b, d, f, or g. The range of bootstrap values distinguishing subtypes, and branched clusters (sublineages) within subtypes, on the phylogenetic tree are consistent with the mutation rate of PCV2 (1.2×10^{-3} substitutions/nt/year) that is relatively high for a virus with a ssDNA genome [41]. However, bootstrap support analysis allowed inference of potential PCV2 transmission chains or clusters within subtypes **b, d**, and **a** by analysis of the phylogenetic tree (**Figure 2**).

Most of the major PCV2 strains from wild boar across Ukraine (10 of 11 resolved, BB = 93 of 100 bootstraps) fell into the subtype **b** clade along with a reference genome from Ukraine (KP420197) isolated from a wild boar in Zaporizhzhia in 2010 (**Figure 2**). In addition, two of the minor co-infecting sequences in wild boar grouped with subtype **b**. The remaining wild boar PCV2 isolates included minor subtype **a** detected as two co-infections of subtype **b** (Chernivitsi-1 and -2). These two minor variants fell into a subtype **a** (reference sequence HM038034) that contained PCV2 strains of subtype **a** from wild boar in Ukraine in 2012 (KP420202, KP420203, KP420186, KP420194, and KP420199). A divergent PCV2 subtype **f** sequence was also found in wild boar (Polatava-2, BB = 93) in a clade with a 2013 subtype **f** reference strain (LC004750). Subtype **f** is found in Asia and elsewhere, but not previously in Ukraine.

In domestic swine samples collected from Kharkiv in 2019, 3 of 6 were PCV2 subtype **b,** and 3 of 6 were inferred as subtype **d** (BB = 60) with a reference PCV2 subtype **d** genome from China in 2012 (KC515014), and not with subtype **g** (BB = 100). The Ukraine domestic pig subtype **d** sequences fell into in a sublineage (BB = 91) with a Ukraine isolate from wild boar in Poltava in 2012 (KP420187). One domestic pig infected by PCV2 subtype **b** also had a minor subtype **d** co-infection (Kharkiv-5).

3.5. Co-circulation of PCV2 subtypes

These results suggest there was co-circulation of at least three PCV2 subtypes (**b, a,** and **f**) in wild boar in 2012, and two subtypes (**b** and **d**) in domestic pigs in 2019, in Ukraine (**Table 2**). Co-infections between subtypes **b** and **a** in wild boar, and **b** and **d** in domestic pigs, were also observed (**Table 3**), and are not unprecedented considering the finding of animals in Ukraine infected solely with these subtypes. Analysis of introduction and epizootic spread of PCV2 subtypes among wild boar, and infection of domestic swine, is considered in the Discussion.

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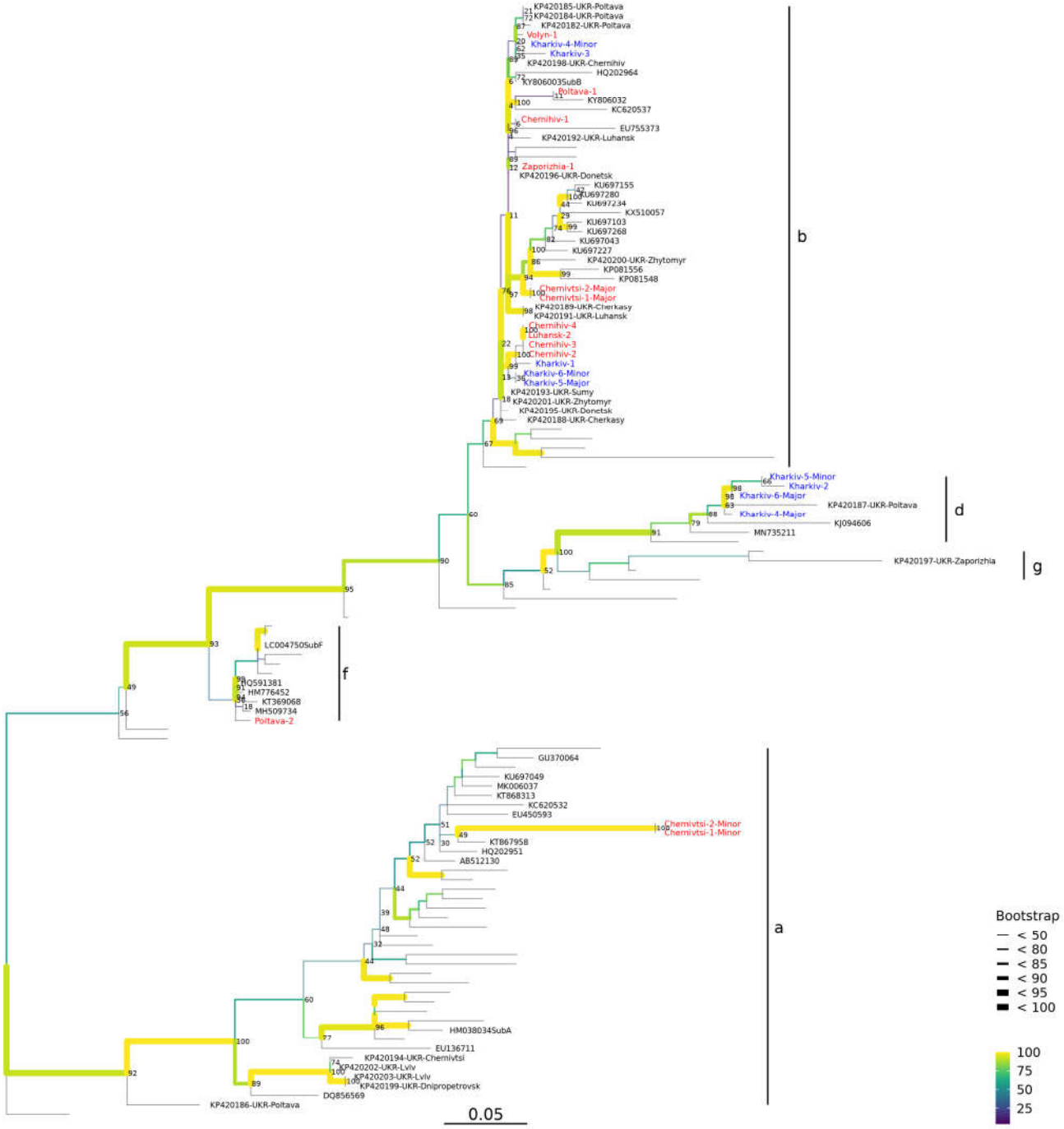

Figure 2. Phylogeny of porcine circovirus 2 in Ukraine. A maximum likelihood tree was inferred for PCV2 ORF2 (Cp) genes from our study (red, wild boar; blue, domestic pig), and sequences downloaded from GenBank (Accessions). PCV2 co-infections sequenced in this study are labeled (–major and –minor). The subtype-defining reference sequences are the major PCV2 lineage assignment with indicated GenBank Accessions (designated sub or described in the text). Bootstrap values are indicated by thickness of the tree branches and color key.

3.6. Genetic structure of PCV2 subtype b

We analyzed the genetic structure of the PCV2 subtype **b** population circulating among wild boar populations in Ukraine in 2012, by estimating the correlation of geographical and genetic distance using the Mantel test. We excluded repetitive sequences Chernivtsi-1 and Chernivtsi-2, and the subtype **f** Poltava-2, and the domestic swine samples. The remaining subtype **b** wild boar sequences (**Table 2**) formed eight conditional genetic units associated with their pairwise geographic distances in Ukraine. We found

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no statistically significant relationship between geographical distance and genetic structure of wild boar subtype **b** sequences (Mantel test p = 0.763; correlation p = 0.3742; **Figure 3**). Thus, we did not detect a geographical structure to the population of wild boars carrying this subtype of PCV2 in the limited data set available.

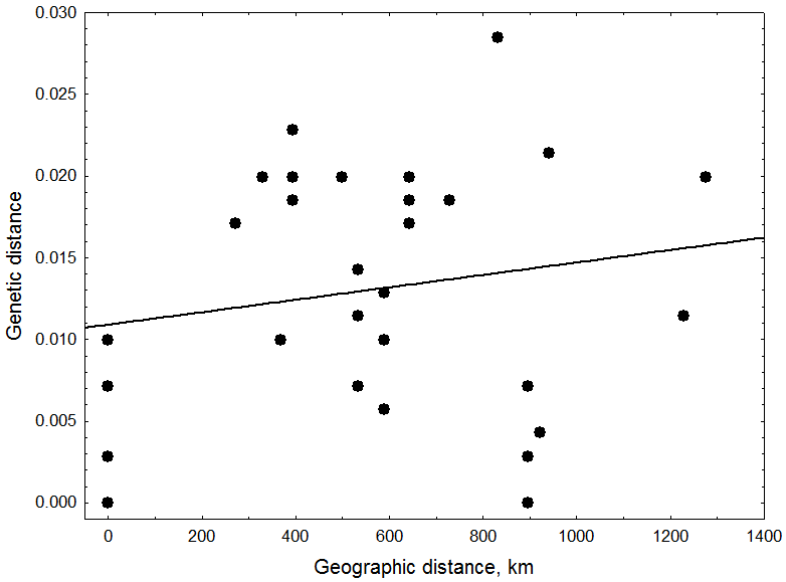

Figure 3. Correlation of geographical and genetic distances for PCV2 subtype b. Eight subtype b wild boar ORF2 (Cp) sequences genetic distances (*y-axis*) were correlated with distance between the geographical centers of their respective locations (*oblasts*) in Ukraine (*x-axis*), and analyzed by Mantel test, with significance tested by linear regression (p-values, *not significant*).

4. Discussion

Despite a small (1.7 kb) ssDNA genome with limited coding capacity (a replicase, a capsid protein, and accessory proteins), porcine circoviruses are widely spread pathogens that cause a diverse spectrum of diseases (PCAD) in swine [3,4,5]. We conducted a genomics inquiry to identify subtypes of PCV2 that have spread through the large wild boar and domestic pig populations of Ukraine. PCV2 subtypes **a, b, d,** and **g** have been detected previously, if sporadically, in Ukraine mainly in wild boar [30], with additional sequences deposited in GenBank [from an unpublished study by Podgorska, K., Sytiuk, M., Stepniewska, K. and Kus, K., "*Porcine circovirus type 2 infections in wild boars in Ukraine*", 2015]. We applied a PCR diagnostics assay that we had developed previously [23], to examine archived blood samples from wild boar opportunistically hunted across Ukraine in 2012, and found a PCV2 prevalence of 31.8% (N = 34 of 107 samples). We also collected and identified PCV2 in a sampling of domestic pork products (liver) from a market in Kharkiv in 2019 (37% prevalence).

4.1. PCV2 subtypes detected in Ukraine

To understand PCV2 diversity and spread, we sequenced ORF2 (capsid, Cp) genes in 18 samples, and recovered 17 sequences for PCV2 subtyping and phylogenetics analysis. We found that both domestic pigs and wild boars were infected with PCV2 subtypes **b** and **d**, with subtype **b** being the most common subtype with 10/12 wild boar and 5/6 domestic pig samples (83% subtype **b**). We found evidence of PCV2 co-infections of subtype (major/minor) **b/d, d/b,** and **b/a**, where there is a dominant (major) subtype and co-infection with a different second (minor) subtype, in 5 of 18 cases (28%). This finding similar to reports of PCV2 co-infections in previous studies, and **b/d** co-infections were the most common co-infection seen in the US in 2015 [31]. We also found one wild boar sample infected with PCV2 subtype **f,** a subtype not previously detected in Ukraine. Although limited by a relatively small number of sporadic samples in our own and previous studies,

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cumulatively, our phylogenetics analysis suggests that the large swine population in Ukraine might host a diversity of PCV2 subtypes, riddled with wild boar to domestic pig transmission events (**Figure 2**).

4.2. Transmission and Epidemiology

Recent studies have investigated the prevalence and subtype diversity of PCV2 in wild boar and domestic swine, in the forested countries of Central and Eastern Europe. PCR studies indicate that the prevalence of PCV2 typically 20-40% in domestic pigs on farms in regions of PCV2 prevalence even with vaccination, for example in Germany [42,43]. Serological studies also indicate high seroprevalence, for example 48% in a study of Iberian wild pigs [44]. Apparently, PCV2 maintains a high enzootic (endemic) prevalence in swine herds in countries in Europe with similar agricultural practices, a significant wild boar population, and mixed forest/farmland ecologies, including Poland, Hungary, Germany, Romania, and other countries including Ukraine. In wild boars, PCV2 DNA was detected in 20.5% of pathological material samples in Hungary [45], 18.1% in Germany [46], 13.5% in Romania [47], and up to 75% in Poland [48], illustrating a wide variation that may be due to outbreak dynamics or host susceptibility.

PCV2 genotypes in wild boar have been detected in domestic pigs, suggesting the possible spread of PCV2 from a wild boar reservoir to domestic swine and contributing to the diverse spectrum of diseases in domestic pigs [8,13]. There is genetic evidence for sharing of PCV2 strains among wild boar and domestic pigs in Europe, a pattern we found in phylogenetics analysis of PCV2 subtypes in Ukraine (**Figure 2**). Recent studies showed PCV2 sequences from wild boar and domestic pigs were closely related in Slovenia [49], Germany [46], and Hungary and Romania [45,47]. In Poland, nine PCV2 subtype **b** sequences from wild boar showed very high identity with sequences from domestic pigs, a pattern also noted among subtype **a** [48,50].

The genetic similarity of PCV2 sequences indicates a shared ecological interaction between wild boar and domestic pigs, with sufficient close contact for the transmission of PCV2 and other pathogens such as ASF [8,45,51]. Thus, introduction new antigenically distinct strains of PCV2 may lead to outbreaks (epizootics) in swine, and symptomatic cases of PCAD.

However, with its high global prevalence, closely similar genetic isolates of PCV2 subtype **b** have also been found far afield, for example a wild boar isolate from Germany was closely similar to a domestic pig isolate from China [46]. In Hungary, some strains were closely related to the isolates in Canada, while others were similar to the isolates from Germany. The full genome we sequenced, PCV2/Chernihiv-1/2012 from a wild boar, is very similar (3 nt differences) to contemporary strains from China. This confounding data suggests that PCV2 also may benefit from anthropogenic modes of transmission, possibly involving transboundary trade in live pigs and/or pork products.

4.3. A wild boar transmission chain

We detected a potential transmission chain between wild boars in the non-neighboring *oblasts* (provinces) of Luhansk and Chernihiv. At least two other potential transmission chains between non-neighboring *oblasts* have been found in Ukraine [30], suggesting that circulation of PCV2 is undersampled in Ukraine. It is possible that a combination of wild boar migrations and human activity, such as live pig trade and mixing of herds from different farms, may contribute to PCV2 spread [5,8]. In addition, PCV2 co-infections (mixed infections with 2 or more subtypes), and possibility of genomic recombination events, may contribute to the epidemic and genetic diversity of PCV2 genotypes [52].

A small cluster of PCV2 subtype **b** sequences from Luhansk (Luhansk-2) and Chernihiv (Chernihiv-2, -3 and -4) had a branch length of zero in the ML phylogenetic tree (**Figure 2**). This suggests the existence of an epizootic transmission chain between Luhansk and Chernihiv, or along the border region in northeastern Ukraine (BB = 100). We also found a cluster of PCV2 subtype **b** in domestic pig liver samples from a

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commercial market in Kharkiv *oblast* in proximal to this branch (BB = 99). Although separated by 7 years, with PCV2 from wild boar hunted in 2012 and domestic pig samples from 2019, this is remarkable and suggests there is an endemic sublineage of PCV2 subtype **b** that spans the northeastern region of Ukraine. This region, which includes northern parts of Kyiv, Chernihiv, Sumy, Kharkiv and Luhansk *oblasts* in Ukraine, and spans the border with the Russian *oblasts* of Kursk, Belgorod, and Voronezh, harbors a considerable wild boar population that also has exhibited evidence of cross-border transmission of African swine fever [26,53], and epizootics of swine influenza [24] and porcine teschovirus-1 [25].

Other potential PCV2 transmission chains in our data set can be inferred among wild boar within Chernivsti in 2012 (Chernivitsi-1 and -2). These two hunted wild boar samples were collected in close proximity in November-December 2012 share both PCV2 of the dominant major subtype **b** (BB = 100), and subtype **a** minor variant (BB = 100, approximately 3% of reads in each sample), although further details are not available. They exist within a poorly sampled sublineage or subclade of PCV2 subtype **b** with representatives from *Zhytomyr from wild boar 2012*, Cherkasy and Luhansk, and numerous isolates from elsewhere in Eurasia (BB = 76). Other members of this subtype **b** sublineage may include isolates from Poltava, Chernihiv, Luhansk and Volyn *oblasts*, and one of our PCV2 sequences from domestic pig liver in Kharkiv (Kharkiv-3).

4.4. PCV2 subtypes in Ukraine reflect global diversity

By phylogenetic analysis, we found examples of PCV2 sequences that clustered with sequences outside of Ukraine, for subtypes **b, d, f** and **a** (**Figure 2**). These results suggest that PCV2 subtypes in wild boar and domestic swine in Ukraine are potentially result of multiple introductions, or exchanges, across borders. Thus, PCV2 should be analyzed in a regional context (a Central/East European group), keeping in mind possible long distance transfer of virus across Eurasia. Whether genomic surveillance for PCV2 might provide data for mapping swine disease ecology is unclear, but this is a pressing question for understanding not only PCAD, but also spread of other swine diseases, such as ASF, PEDV, and swine influenza, that infect both wild boar and domestic swine [1,2,3].

PCV2 subtype **b** is found in domestic pigs in Europe, Asia and North America, so it is evident that this is a widely transmitted subtype of the virus, along with subtype **d** [12]. We discovered 12 new subtype **b** infections in Ukraine; four fall into a cluster, but the others do not (**Figure 2**). With the paucity of PCV2 subtype **b** samples from Ukraine, it is not possible to build a robust time-demarked phylogeny, thus it is difficult to infer whether there was one or only a few ancestral introductions of this PCV2 subtype **b** subclade into Ukraine, or multiple occurrences.

The complete genome we assembled of the Chernihiv-1 isolate (PCV2/Chernihiv-1/2012) within this subclade had 3 SNV in comparison to its closest match from China (PCV2/China FX1102/2012, Accession JX406426.1), but no amino acid variations in the major proteins involved in PCV2 replication. The PCV2 subtype **b** is conceivably also a strain designation, as this subtype exhibits higher transmissibility than other subtypes, and is antigenically distinct, requiring a specific vaccine formulation [12].

We also identified PCV2 subtype **d** in the domestic pig samples and one wild boar sequence (NCM) sampled before 2012. Subtype **d** infections were identified in domestic pigs in the US and Korea, and wild boar in Korea and Italy, among other places, only after 2013 [12,28,31]. We report the first discovery of a PCV2 subtype **f** in Ukraine, that infected a wild boar in Poltava *oblast*. The prevalence of this subtype is uknown, but it reflects the diversity of PCV2 in swine in Ukraine, a necessary step to develop and deploy effective vaccines against PCAD.

4.5. Limitations

This study is limited by the sample size and lack of longitudinal sequence data on PCV2 circulating in Ukraine. There may be hidden genetic diversity, particularly in

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subtype **b**, that has not been sampled in either wild boar or domestic swine. Alternately, the PCV2 subtype **b** circulating among wild boars in Ukraine may actually be relatively homogeneous. We did not observe a stark geographical structure in our data. There was an absence of correlation between genetic and geographical distances using the Mantel test (**Figure 3**), although this result is tempered by a limited sample size. Consistent with this finding, many PCAD cases in domestic pigs are thought to be of anthropogenic origin in Ukraine, where agricultural practices play a key role in the spread of this disease. It cannot be assumed they are all subtype **b**, as we also found evidence of subtypes **a** and **f** in wild boar in Ukraine, and co-infections in domestic pigs with subtypes **b** and **d**. Thus a broader biosurveillance and sequencing effort is needed to understand potential wild boar to domestic swine transmission of PCV2, and other swine diseases.

4.6. Application of MinION capacity building for One Health

To understand PCV2 diversity and spread, we conducted ORF2 (Cp) and full genome PCR amplification and sequencing. Sequencing ORF2 (Cp) alone is a reliable phylogenetic marker for PCV2 subtype assignment [54]. We accomplished this by deploying a cost-effective, accurate long-read nanopore sequencer (Oxford Nanopore Technologies MinION) in veterinary diagnostic and research labs in Ukraine. This work was part of an effort to build capacity in One Health through genomics, with parallel training of junior scientists in sequencing library preparation and bioinformatics of raw data processing, genome assembly, and phylogenetics. Apart from genotyping analysis of PCV2, as part of this Ukraine-US effort, we sequenced other viral and bacterial pathogens of swine including African swine fever virus, porcine teschovirus-1, swine influenza, porcine epidemic diarrhea virus (PEDV), and *Salmonella enterica* [26,32; and personal communication). The emergence of novel genotypes, such as PCV3 in Poland, highlights potential of this rapid sequencing-based diagnostic approach for veterinary disease [55]. Expanding on this effort, some Ukrainian scientists in the program have leveraged skills in nanopore sequencing and phylogenetics to study other animal and human pathogens in Ukraine, including avian influenza, avian paramyxoviruses, *Brucella abortis* (isolated from sheep), and SARS-CoV-2 [56,57,58]. These efforts highlight the applicability of nanopore methods for multi-pathogen biosurveillance, and contribution to understanding host-pathogen ecology.

5. Conclusions

Porcine circovirus 2 is the etiological agent of a spectrum of viremic and inflammatory diseases in swine (PCAD) in part attributable to genetic variation of the pathogen. In this study we found that PCV2 subtypes **b, d, a** and **f** were present in Ukraine between 2012 and 2019. In domestic pig liver samples, we found PCV2 subtypes **b, d,** and **b/d** co-infections. In wild boar, we found subtypes **b, b/a** co-infections, and subtype **f** for the first time in Ukraine. Using phylogenetics, we noted an epizootic cluster of PCV2 subtype **b** in both wild and domestic swine, suggesting a possible ecological interaction in Chernihiv, Kharkiv, and Luhansk oblasts in Ukraine. Our study illustrates the utility and the need for expanded genomic epidemiology to map the reservoirs and transmission routes of PCV2 in Ukraine, to understand the ecological context of this swine disease.

Supplementary Materials: Supplementary Methods; Supplementary File S1.

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