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Detection, Genotyping and Phylogenetic Characterisation of Porcine Circovirus 2 and Porcine Circovirus 3 in Wild Boar (*Sus scrofa*) from West Bačka District, Vojvodina, Serbia

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Simple Summary

Wild boar live alongside farmed pigs in many parts of Europe and can carry the same viruses. Two of these are porcine circovirus 2 and porcine circovirus 3. The first causes disease in farmed pigs and is costly to the pig industry, while the role of the second in disease is less clear, so it is useful to know how often both occur in wild animals. We examined tissue from 90 wild boar shot by hunters in West Bačka District, in the north of Serbia, during the 2021 and 2022 seasons using material that had already been collected for routine disease monitoring. The first virus was found in 17 animals and the second in 12, and no animal carried both. We determined the genetic sequence of several of these viruses and compared them with sequences recorded elsewhere. They closely resembled viruses reported from farmed pigs in Serbia and in other European countries, showing that wild boar and farmed pigs carry very similar viruses. Our results cannot show in which direction the viruses moved between the two groups, but they support continued monitoring of wild boar alongside farmed pigs.

Abstract

Porcine circovirus 2 (PCV2) and porcine circovirus 3 (PCV3) are widespread in domestic pigs and common in wild boar, but Serbian wild boar data come predominantly from the east. Tissue pools from 90 wild boar hunted in West Bačka District, Vojvodina, in 2021 and 2022, collected within national swine fever surveillance, were screened by real-time PCR. PCV2 was detected in 17 animals (18.9%; 95% CI 11.4–28.5) and PCV3 in 12 (13.3%; 95% CI 7.1–22.1), with no co-infection. Four partial PCV2 capsid sequences were obtained, three PCV2b and one PCV2d, all within the diversity already reported from Serbian wild boar and European domestic pigs. Five complete PCV3 genomes differed by no more than 1.46% and met the distance criteria for PCV3-a; at the complete-genome level, the closest reference sequences came mainly from domestic pigs in Serbia and Hungary. Neither of the two PCV3 recombination signals met the acceptance criteria, so no event could be attributed to these sequences; breakpoint analysis of the combined reference-and-study alignment detected topological incongruence at the Cap coding-region boundary, an exploratory finding that does not demonstrate recombination in these genomes. Both viruses circulate

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in this population, carrying diversity that these data do not separate from that of European domestic pigs.

Keywords: porcine circovirus 2; porcine circovirus 3; wild boar; *Sus scrofa*; genotyping; phylogenetic analysis; recombination; Serbia

1. Introduction

Porcine circovirus 2 (PCV2) and porcine circovirus 3 (PCV3) are small, non-enveloped viruses with circular single-stranded DNA genomes, classified in the species *Circovirus porcine2* and *Circovirus porcine3* (genus *Circovirus*, family *Circoviridae*). Both encode a replication-associated protein (Rep) and a capsid protein (Cap), and the capsid gene, which concentrates antigenic and evolutionary variability, serves as the reference region for genetic classification [1,2]. PCV2 is the essential infectious component of porcine circovirus-associated disease and among the most costly viral agents of intensive pig production; vaccination since 2006 has suppressed clinical disease but not viral circulation [3]. Eight genotypes, PCV2a to PCV2h, are recognised under the criteria of Franzo and Segalés, namely a maximum intra-genotype *p*-distance of 13% across the capsid gene (ORF2), bootstrap support above 70% and at least fifteen sequences [1]; a proposed ninth, PCV2i, has not been generally adopted [4]. PCV2a was displaced by PCV2b in the early 2000s, and PCV2d is now the most frequently reported genotype in European and Asian pigs [3,5].

PCV3 was identified in 2016 in sows with porcine dermatitis and nephropathy syndrome and reproductive failure in which PCV2 could not be demonstrated [6], and it has since been reported worldwide from both diseased and clinically normal pigs. Because detection alone has repeatedly been mistaken for causation, a case definition requiring multisystemic lymphoplasmacytic to lymphohistiocytic perivascular inflammation together with viral nucleic acid within the lesions has been proposed [7]; most infections appear to be subclinical. The genetic classification of PCV3 is more contested than that of PCV2. Unified criteria proposed in 2020, namely a maximum within-genotype distance of 3% for complete genomes and 6% for ORF2, bootstrap support above 90%, congruence between the two datasets and at least five sequences per genotype, recognised a single genotype, PCV3-a, with a minor clade left unnamed [2]. Study-specific labels have since proliferated [8], and a recent reassessment of all available ORF2 data has questioned whether PCV3 supports formal genotypes at all [9].

Wild boar populations have expanded across much of Europe, increasingly overlapping with outdoor and low-biosecurity pig holdings. PCV3 was first demonstrated in wild boar in 2018 in an Italian regional park, where roughly 30% of animals carried viral DNA without clinical signs [10], and comparable or higher frequencies have since been reported from Germany [11], several Italian regions [12–14] and Portugal [15]. In northern Italy, PCV3 was more frequent and more variable in wild boar and rural holdings than in intensive units [16], and Portuguese sequences provide evidence of viral exchange between wild boar and domestic pigs [17]. Serbia had just under three million pigs in 2020, some 1.26 million of them in Vojvodina Province, and more than half are kept in holdings with low or absent biosecurity; such holdings are particularly numerous in western Vojvodina, where they coexist with intensive production [18]. Serbia also has the highest pig density in the Western Balkans [19], and wild boar abundance is rising, in part because realised culling falls short of planned culling [20]. Contact between the two hosts is documented: domestic pig–wild boar contact was recorded on every free-range Serbian farm examined [21], and wild boar with more than 10% domestic ancestry are concentrated in Serbia and neigh-

bouring countries [22,23]. In Serbia, PCV2b predominated in domestic pigs and wild boar sampled before 2012 [24], and PCV3 was first reported from pig farms in 2019 [25]. In wild boar from South Banat District, at the eastern edge of Vojvodina, PCV2 was detected in 40.3% of 124 animals in 2018–2019, with no PCV3 [26]; in the 2021/2022 season, the same hunting grounds yielded PCV2 in 17.5% and PCV3 in 15.5% of 103 animals, the first report of PCV3 in Serbian wild boar [27]. Higher figures have since been reported from eastern Serbia and South Banat [28] and from western Serbia and the Republic of Srpska [29]. West Bačka District, which borders Hungary and Croatia and combines intensive pig production with an extensive network of hunting grounds, has not been examined. PCV3 characterisation in Serbian wild boar has so far rested on partial capsid sequences, and neither virus has been screened for recombination in Serbian suids.

This study screened wild boar hunted in West Bačka District during the 2021 and 2022 seasons, sampled within the national African and classical swine fever monitoring programme, for PCV2 and PCV3 by real-time PCR. PCV2 capsid-gene sequences and complete PCV3 genomes obtained from positive animals were genotyped, placed in a phylogenetic context and examined for recombination.

2. Materials and Methods

2.1. Study Area and Sample Collection

Sampling was carried out in West Bačka District, in north-western Vojvodina, an area of intensive arable farming and pig production bounded by the Danube to the west. The area corresponds to the diagnostic catchment of the Veterinary Specialist Institute "Sombor" rather than to a sampling frame designed for this study. The 90 animals came from ten hunting grounds and eleven localities (Figure 1, Table 1; cross-tabulated in Table S5); because a locality may share its name with the hunting ground in which it lies, hunting grounds are identified as such throughout. Bački Monoštor (26 animals) and Lalinske livade (23) dominated at locality level, and no other locality contributed more than eleven animals. Eight localities, accounting for 64 animals, belong to Danube floodplain hunting grounds within or bordering the Gornje Podunavlje reserve; the other three (Lalinske livade, Lalić and Odžaci) lie inland.

Table 1. Wild boar sampled in West Bačka District during the 2021 and 2022 hunting seasons, by locality, with the number of animals positive for PCV2 and for PCV3 by real-time PCR.

Locality	Animals Tested	PCV2 Positive	PCV3 Positive
Bački Monoštor	26	5	3
Lalinske livade	23	4	2
Apatinski rit	11	3	1
Sonta	10	0	3
Bezdan	6	2	2
Kozara	4	2	0
Kurjačica	3	0	0
Kolut	2	1	1
Kameni Pesak	2	0	0
Lalić	2	0	0
Odžaci	1	0	0
Total	**90**	**17 (18.9%)**	**12 (13.3%)**

No animal was positive for both viruses, so the totals correspond to 29 positive animals.

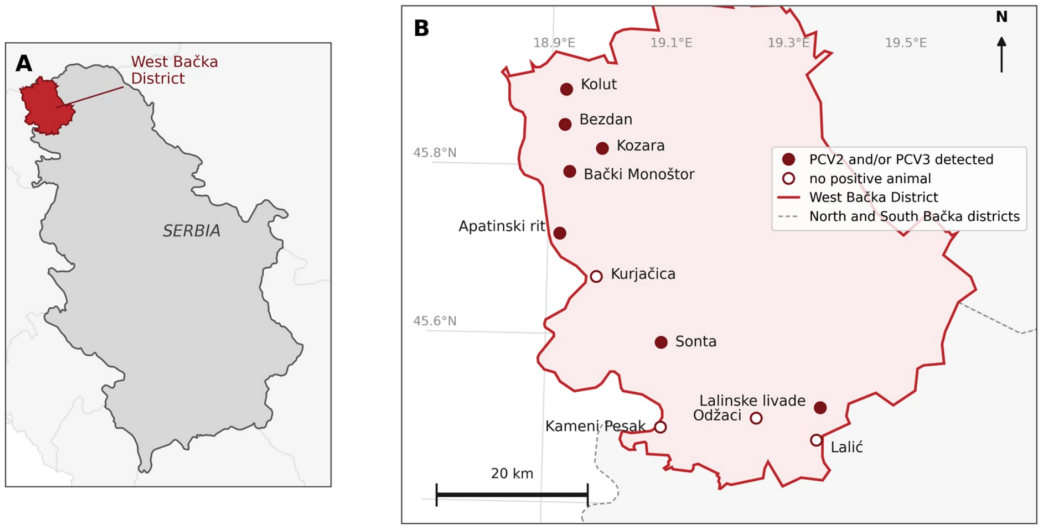


Figure 1. Study area. (**A**) Location of West Bačka District (red) within Serbia. (**B**) The eleven localities at which wild boar were sampled during the 2021 and 2022 hunting seasons, shown against the boundary of West Bačka District (solid red); the adjoining North and South Bačka districts are outlined in grey for context. Filled symbols mark localities from which at least one animal was positive for PCV2 or PCV3; open symbols mark localities from which no positive animal was obtained. Projection UTM zone 34N (EPSG:32634); graticule in decimal degrees. District boundaries © OpenStreetMap contributors (ODbL); national outline from Natural Earth.

Lymph node, tonsil and spleen were collected at post-mortem examination; none of the carcasses showed macroscopic lesions suggestive of acute systemic disease. For each animal, approximately 20 mg of pooled tissue, made up of the three organs in equal proportions, was homogenised in 600 µL of phosphate-buffered saline in tubes pre-filled with glass beads (TissueLyser, Qiagen, Hilden, Germany; 30 Hz, 3 min) and clarified by centrifugation at 1500× *g* for 2 min, so that one animal corresponded to one test unit. Samples were handled in a dedicated PCR cabinet.

2.2. Nucleic Acid Extraction

Total nucleic acid was extracted from clarified homogenates with the MagFast DNA/RNA extraction kit (Innovative Diagnostics, Montpellier, France) on an IDEAL32 automated platform (ID VET, Grabels, France) according to the manufacturer's instructions. Extracts were dispensed into labelled microtitre plates in a separate cabinet and held at −80 °C until testing.

2.3. Detection of PCV2 and PCV3 by Real-Time PCR

Both viruses were detected by probe-based real-time PCR on an AriaMx instrument (Agilent Technologies, Santa Clara, CA, USA). PCV2 was detected with the genesig Standard Kit for Porcine circovirus 2 (Primerdesign Ltd., Chandler's Ford, UK), which targets the 3′ non-coding region with a FAM-labelled probe (primer and probe sequences are not disclosed), according to the manufacturer's instructions, with 5 µL of template in 20 µL reactions: 95 °C for 2 min, followed by 50 cycles of 95 °C for 10 s and 60 °C for 60 s. PCV3 was detected with the capsid-targeted primers and probe of Yuan et al. [30], in duplex with an endogenous β-actin assay [31], using Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) in 20 µL reactions containing 0.4 µM of each primer, 0.2 µM of probe and 3 µL of template DNA. Cycling followed the master mix manufacturer's protocol: 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Relative to the original protocol [30], the master mix, oligonucleotide concentrations and cycling conditions were therefore changed.

Amplification of β-actin was taken only as evidence that the extract contained amplifiable nucleic acid. Interference between the PCV3 and β-actin targets was not formally evaluated, but the PCV3 positive control amplified as expected in the duplex format. Positive controls were the PCV2 kit control template, DNA of PCV2 strain 1010-Stoon and DNA of a characterised PCV3 field strain (Laboratory of Virology, Faculty of Veterinary Medicine, University of Belgrade), and nuclease-free water served as the no-template control in each run. Samples were considered positive when a characteristic amplification curve was obtained with a cycle threshold (Ct) below 35 for both viruses. Primer and probe sequences, the basis of the cut-off, and the interpretation of the internal control are given in Table S1.

2.4. Amplification and Sequencing

A uniform cut-off was applied for sequencing: only samples with a Ct of 25 or below, i.e., those with the lowest Ct values and sufficient template DNA, were amplified by conventional PCR, namely four of the 17 PCV2-positive and five of the 12 PCV3-positive animals (Table S11). Reaction conditions followed the source publications, with two overlapping PCV2 fragments of 353 and 293 bp spanning the variable region of the capsid gene [3] and three overlapping PCV3 amplicons of 553, 1030 and 895 bp covering the complete genome [32,33]. Each run included DNA of PCV2 strain 1010-Stoon or of the PCV3 field strain as a positive control and a no-template control.

All nine samples yielded a usable sequence. Products were sequenced in both directions by the Sanger method (Macrogen Europe, Amsterdam, The Netherlands), and consensus sequences generated in MEGA12 [34] were assembled into a 503 nt ORF2 fragment for PCV2 and complete genomes for PCV3, compared with GenBank entries by BLAST (http://blast.ncbi.nlm.nih.gov, accessed on 12 January 2026), and deposited under accession numbers PX564516–PX564519 and PX564520–PX564524, respectively.

2.5. Reference Sequences

Reference sequences were retrieved from GenBank in January 2026 to represent the recognised genotypes and geographic range of each virus (Tables S2 and S3). Sequences from Serbia, neighbouring countries and the rest of Europe were included as far as available, whereas sequences from China, which make up most of those available for both viruses, were reduced to a subset, and identical sequences were partly removed, mainly among those from China, to keep the trees readable. The PCV2 dataset comprised 89 ORF2 fragment sequences, 85 of them from GenBank, including PCV1 (FJ475129.2) as an outgroup. The PCV3 dataset comprised 48 complete genomes, 43 of them from GenBank, from which a 645-nt ORF2 window was analysed separately. The four PCV3 sequences previously reported from Serbian wild boar (OP784790.1–OP784793.1) [27] cover only part of the capsid gene; they were compared with the sequences reported here over the 543 capsid-gene positions shared by all nine sequences, using *p*-distances with partial deletion at 95% site coverage (Table S10).

2.6. Alignment and Phylogenetic Analysis

Sequences were aligned with MUSCLE in MEGA12. Because PCV3 genomes in GenBank begin at different positions, all genomes were first brought to the same orientation and rotated manually in MEGA12 to a common start 216 nt upstream of the putative Rep initiation codon so that Rep occupies alignment positions 217–1104. The complete genomes, 2000 nt long for the sequences reported here, were then aligned and trimmed to the 1996 columns covered by all sequences, and the alignment was checked visually. Only three of these columns had less than 95% site coverage and were excluded by partial deletion, leaving 1993 positions; no position was excluded from the 645-position ORF2 window (alignment columns 1336–1980), which was analysed as a separate dataset. All PCV3

positions given in this paper, including breakpoints, refer to columns of this alignment. Substitution models were selected in MEGA12 by the Bayesian information criterion: Hasegawa–Kishino–Yano for the PCV2 fragment and PCV3 ORF2, and Tamura three-parameter for PCV3 genomes, each with gamma-distributed rates (five categories). These models, with the gamma shape parameter estimated from each dataset (given in the figure legends), were used to infer maximum likelihood trees by nearest-neighbour interchange, with 1000 bootstrap replicates; genetic distances were calculated as uncorrected p-distances. For PCV2, the 95% coverage criterion would have retained only 200 of 509 positions, so all sites were used for the tree, with pairwise deletion for distances. The PCV2 tree was rooted with PCV1. No outgroup was included for PCV3, so the PCV3 trees are unrooted and are drawn with the root placed at the midpoint of the longest path between two sequences; this affects only the display, not the topology or genotype assignment.

2.7. Genotype Assignment

PCV2 genotypes were assigned by phylogenetic clustering and p-distance according to Franzo and Segalés [1]. Genotypes of the reference sequences were taken from the original publications, checked against the GenBank records where available, and confirmed by their clustering in the trees reconstructed here. Because the 13% threshold is defined on complete ORF2, distances over the 503 nt fragment are treated as supporting evidence rather than as a formal application of the criterion; with pairwise deletion, the number of positions compared also varies between pairs, so the nearest sequence by distance need not be the sister sequence in the tree. PCV3 genotypes were assigned according to Franzo et al. [2]. Because only one sequence of the divergent clade was available, support for the bipartition delimiting PCV3-a could not be assessed, and assignment rested on the distance criteria and on congruence between the genome and ORF2 trees. MG372488.1, the sole representative of the tentative PCV3b clade of that proposal, was retained as a divergent reference but excluded from within-genotype distances; the clade was never formally recognised and has since been set aside by the same authors, its divergence being consistent with a sequencing artefact [9]. Genotype designations are therefore used as a framework internal to this dataset, in keeping with recent reservations about standardised PCV3 genotyping [9].

2.8. Recombination Analysis

Recombination was screened in RDP5 [35], with PCV3 genomes treated as circular and PCV2 fragments as linear, using nine algorithms (RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq, LARD and PhylPro) with Bonferroni correction and 1000 permutations. Only signals detected by at least three methods at $p < 0.001$ were considered further, and candidate events were examined through breakpoint distributions, algorithm plots, region-specific phylogenies and the alignment itself.

Breakpoints were also assessed with the genetic algorithm for recombination detection (GARD) on the Datamonkey platform [36]. Models with successive numbers of breakpoints were compared by the small-sample-corrected Akaike information criterion (c-AIC), and the best partitioned model was compared with a single-topology model allowing branch lengths to vary, to distinguish topological incongruence from rate variation. RDP5 and GARD were applied to the PCV3 genome alignment (1996 positions) and to the PCV2 fragments (88 sequences, 509 positions, outgroup excluded). For PCV3, GARD was repeated after exclusion of the divergent sequences MG372488.1 and KY924473.1–KY924475.1, and on the reference genomes alone (43 sequences, 39 without the divergent sequences). Breakpoints are given as alignment columns: for GARD, the last column of the upstream partition, and for RDP5, the columns delimiting the putative recombinant segment.

2.9. Statistical Analysis

Detection rates were expressed as proportions of the animals examined, with 95% confidence intervals calculated by the exact (Clopper–Pearson) binomial method. No further statistical analysis was performed, as the study was descriptive and metadata permitting comparison between subgroups were not available for all animals.

3. Results

3.1. Detection of PCV2 and PCV3

Circoviral DNA was detected in 29 of the 90 wild boar (32.2%; 95% CI 22.8–42.9): PCV2 in 17 (18.9%; 95% CI 11.4–28.5) and PCV3 in 12 (13.3%; 95% CI 7.1–22.1), with no co-infection. Ct values ranged from 19.2 to 33.6 for PCV2 and from 20.0 to 32.5 for PCV3 (Table S11). Positive animals came from seven of the eleven localities (Table 1). Six of these belong to Danube floodplain hunting grounds and account for 23 of the 29 positive animals; the seventh, Lalinske livade, is the most heavily sampled inland locality. Because sampling followed the diagnostic catchment and locality contributions were uneven, the two groups of localities are not compared.

3.2. Partial PCV2 Capsid Gene Sequences and Genotype Assignment

Four PCV2-positive samples yielded sequences covering 503 nt of the variable region of ORF2 (PX564516–PX564519; Table 2). In the maximum likelihood tree constructed from 89 sequences, the reference strains resolved into groups corresponding to genotypes PCV2a through PCV2h (Figure 2). None of the sequences from this study formed a separate lineage. PX564516, PX564517 and PX564518 grouped with PCV2b reference sequences, whereas PX564519 grouped with PCV2d reference sequences; they were assigned to those genotypes accordingly.

Table 2. PCV2 and PCV3 sequences obtained in this study, with the locality of origin of each sequenced animal and the genotype assigned.

GenBank Accession	Virus	Genotype	Locality
PX564516	PCV2	PCV2b	Lalinske livade
PX564517	PCV2	PCV2b	Apatinski rit
PX564518	PCV2	PCV2b	Apatinski rit
PX564519	PCV2	PCV2d	Bački Monoštor
PX564520	PCV3	PCV3-a	Bački Monoštor
PX564521	PCV3	PCV3-a	Sonta
PX564522	PCV3	PCV3-a	Bezdan
PX564523	PCV3	PCV3-a	Lalinske livade
PX564524	PCV3	PCV3-a	Sonta

Pairwise distances supported these assignments (Tables S8 and S9). PX564516–PX564518 differed from PCV2b references by 1.40–1.84% on average, against 5.93–6.33% from the next closest genotype, and PX564519 differed from PCV2d references by 1.83%, against 6.48% from PCV2g. Maximum within-genotype divergence was 3.4% for PCV2b and 4.0% for PCV2d, well below the 13% threshold, although this comparison is only indicative (Section 2.7). The closest single sequence to PX564516–PX564518 lay at 0.8–1.2%, in each case a domestic pig strain from Germany (MW262924.1), and previously reported Serbian wild boar sequences at 1.0–2.0%; PX564519 lay 1.0% from PCV2d strains from China and Uruguay, 1.5% from Serbian wild boar sequences and 2.8% from the only Serbian domestic pig PCV2d sequence (HQ378158.1). The Serbian wild boar sequences share only 200 positions with the fragment analysed, so these distances correspond to two to four nucleotides.

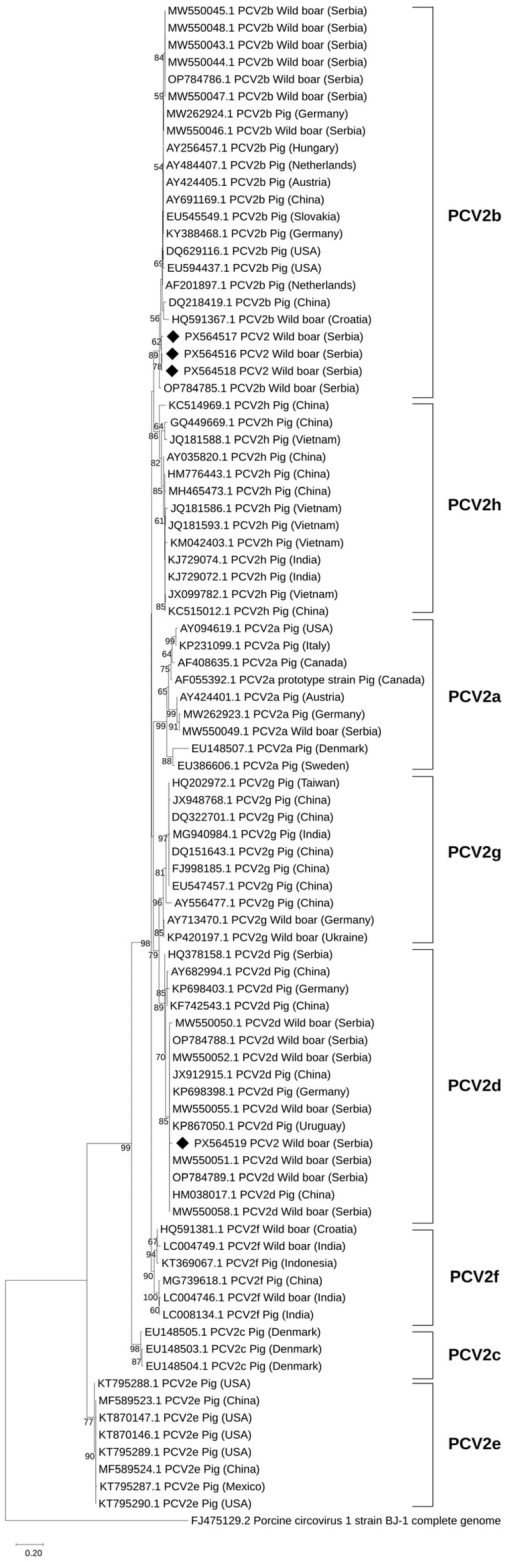


Figure 2. Maximum likelihood phylogenetic tree based on a 503 nt fragment of the PCV2 capsid gene (89 sequences; 509 alignment positions, all sites used). Sequences obtained in this study are marked with black diamonds; reference sequences representing genotypes PCV2a–PCV2h are labelled with accession number, genotype, host and country of origin. The analysis was performed in MEGA12 under the Hasegawa–Kishino–Yano model with gamma-distributed rates across five categories (shape parameter 0.4002); the tree with the highest log-likelihood (−3809.46) is shown. Node support was assessed by 1000 bootstrap replicates, and values ≥ 50 are shown. PCV1 (FJ475129.2) was used as the outgroup. The scale bar indicates nucleotide substitutions per site.

The three PCV2b sequences grouped with OP784785.1, from a wild boar in South Banat District, towards the base of the PCV2b cluster near sequences from a Croatian wild boar (HQ591367.1) and a Chinese domestic pig (DQ218419.1); other Serbian wild boar PCV2b sequences (MW550043–MW550048, OP784786.1) lay elsewhere in the cluster among domestic pig sequences from Europe, China and the United States. PX564519 fell among Serbian wild boar PCV2d sequences (MW550050–MW550052, MW550055, MW550058, OP784788.1, OP784789.1), together with a Serbian domestic pig sequence (HQ378158.1) and references from Germany, China and Uruguay.

3.3. PCV3 Complete Genome Sequences, Genetic Distances and Genotype Assignment

Complete genomes of 2000 nt were obtained from five PCV3-positive animals (PX564520–PX564524; Table 2).

Excluding MG372488.1, maximum complete-genome divergence was 2.96% between two Chinese references (KY924475.1 and MF405276.1), just within the 3% criterion [2] (Figure S1, Table S6); among the five sequences reported here, it was 1.46%. For ORF2, the maximum was 3.26%, between PX564522 and three references from Hungary (ON015889.1) and the United States (MK496272.1 and MK496294.1), and 2.79% among the sequences reported here, well within the 6% threshold (Figure S2, Table S7). MG372488.1 differed from all other sequences by 7.43–9.49% across the genome and by 12.09–13.18% within ORF2.

At the complete-genome level, PX564520, PX564521, PX564523 and PX564524 were equally close (0.50–0.75%) to MK117051.1 and ON015883.1, domestic pig sequences from Serbia and Hungary that differ from each other by 0.25%, and a Portuguese wild boar sequence (PV753268.1) was equally close to PX564523. The most divergent sequence, PX564522, was closest to ON015883.1 (1.20%). In ORF2, PX564523 was nearest to an Italian wild boar sequence (MN781187.1; 0.47%), followed at 0.62% by PV753268.1 and a German domestic pig sequence (MG014374.1), whereas the other four were nearest to domestic pig sequences from Sweden, Germany and China. As these separations amount to a few nucleotides, the single nearest sequence depends on the references included and is not interpreted further.

Over the 543 capsid-gene positions shared with the four partial sequences previously reported from wild boar in South Banat District (OP784790.1–OP784793.1) [27], the sequences reported here differed from them by 0–2.03% (0–11 nucleotides; Table S10). PX564520 was identical to OP784793.1 over this region, and each of the other four sequences lay 0.55–1.10% (three to six nucleotides) from its closest South Banat sequence; for PX564522, the most divergent of the five genomes, this was OP784790.1. Over the same region, the five sequences reported here differed from one another by 0.55–2.58%, and the South Banat sequences differed by 0–1.29%.

Complete-genome and ORF2 trees placed all five sequences within PCV3-a (Figures 3 and 4). In the genome tree, they fell within a group supported at 99% that included every sequence except MG372488.1 and KY924473.1–KY924475.1. The latter three lie within PCV3-a and correspond to the intermediate cluster (Clade 3) that Franzo et al. identified only at the genome level [2], the pattern observed here. The five sequences did not cluster together, and their positions largely differed between the two trees; only two placements were stable, PX564523 with MN781187.1 and PX564521 with a group of Chinese sequences (MG250181.1, MF318448.1, MF318449.1 and MH522791.1). With a single representative of the divergent clade, the bipartition separating it is a terminal split whose bootstrap support is uninformative, so assignment to PCV3-a rested on the distance criteria and on congruence between the two trees. MG372488.1 was separated by a long branch in both analyses.

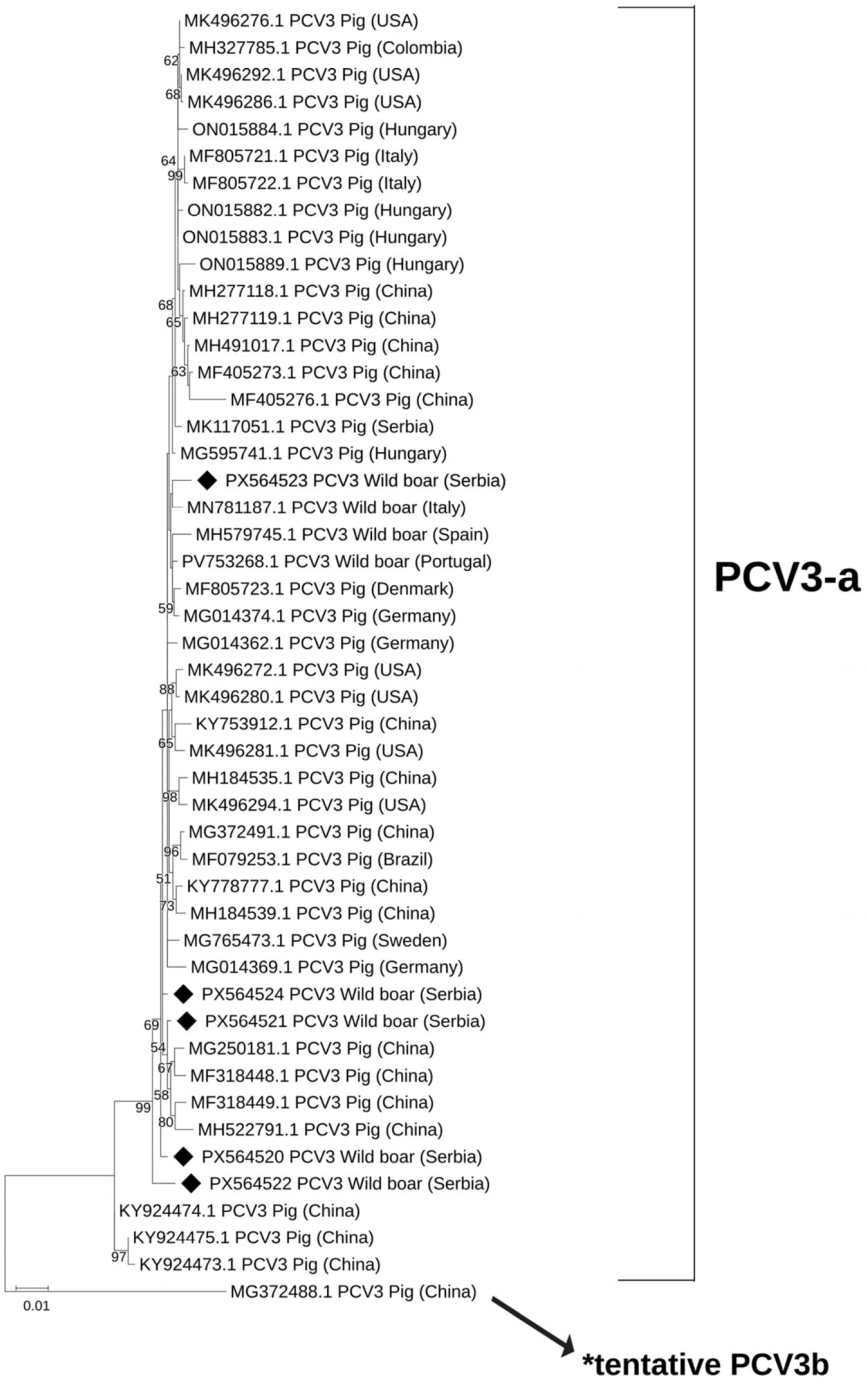


Figure 3. Maximum likelihood phylogenetic tree based on complete PCV3 genome sequences (48 sequences; 1993 of 1996 alignment positions retained after exclusion of sites with less than 95% coverage). Sequences obtained in this study are marked with black diamonds; reference sequences are labelled with accession number, host and country of origin. The analysis was performed in MEGA12 under the Tamura three-parameter model with gamma-distributed rates across five categories (shape parameter 0.1168); the tree with the highest log-likelihood (−5716.68) is shown. Node support was assessed by 1000 bootstrap replicates, and values $\geq$ 50 are shown. The tree is unrooted and displayed with midpoint rooting. The bracket indicates genotype PCV3-a; the arrow and asterisk mark MG372488.1, the sole representative of the tentative PCV3b clade of Franzo et al. [2], which has not been formally recognised as a genotype. The scale bar indicates nucleotide substitutions per site.

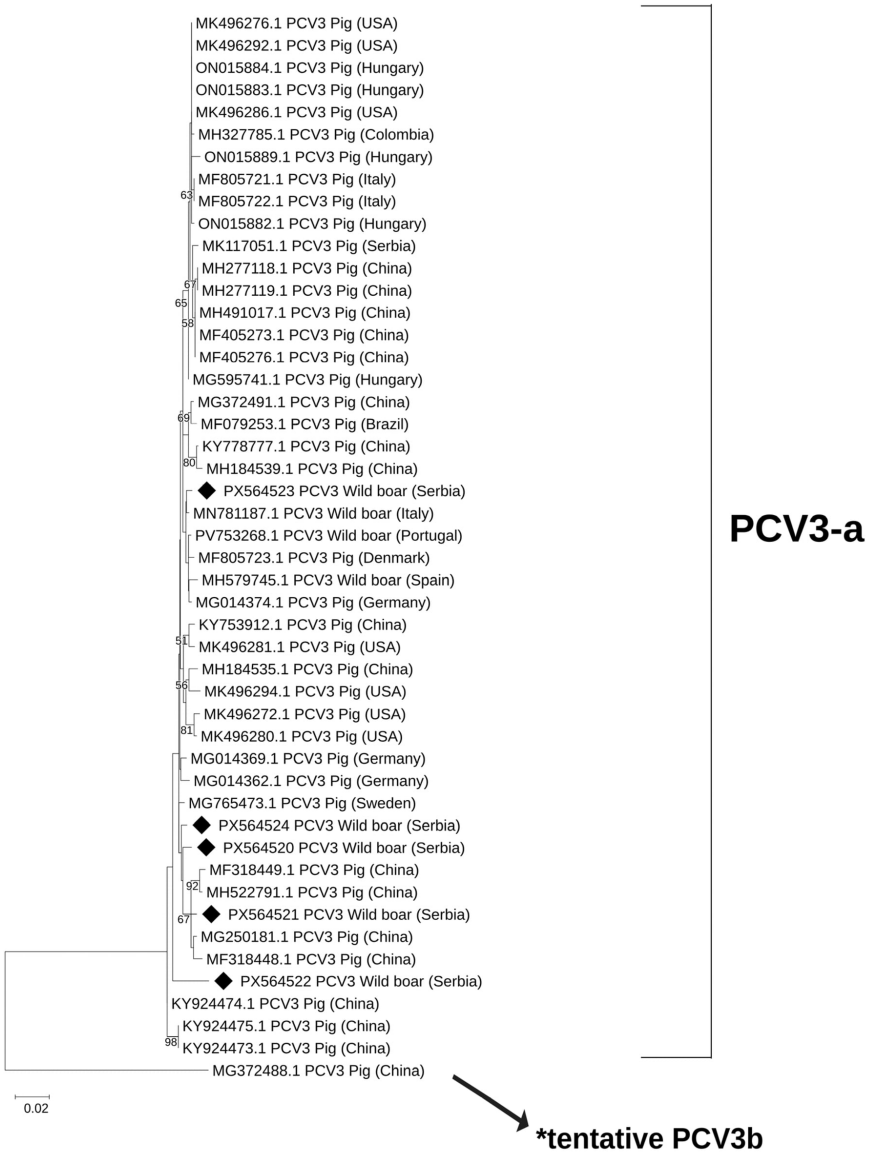


Figure 4. Maximum likelihood phylogenetic tree based on the ORF2 region of PCV3 (48 sequences; 645 alignment positions). Sequences obtained in this study are marked with black diamonds; reference sequences are labelled with accession number, host and country of origin. The analysis was performed in MEGA12 under the Hasegawa–Kishino–Yano model with gamma-distributed rates across five categories (shape parameter 0.1345); the tree with the highest log-likelihood (−1924.21) is shown. Node support was assessed by 1000 bootstrap replicates, and values ≥ 50 are shown. The tree is unrooted and displayed with midpoint rooting. The bracket indicates genotype PCV3-a; the arrow and asterisk mark MG372488.1, the sole representative of the tentative PCV3b clade of Franzo et al. [2], which has not been formally recognised as a genotype. The scale bar indicates nucleotide substitutions per site.

All five genomes encoded a 214-residue Cap and a 296-residue Rep without premature termination (Table 3). Cap varied at six positions, with one to five differences between genomes (0.47–2.34%), and Rep varied at two, both in PX564522. At the marker positions of Fux et al. [33], three genomes carried the complete group b pattern of that scheme (which is not the tentative PCV3b clade of Franzo et al. [2]). PX564521 carried lysine at Cap 27, matching the intermediate pattern of a subcluster placed by Fux et al. between their two groups [33], and PX564522 carried alanine at Rep 122, the result of a single nucleotide substitution. Although PX564522 was the most divergent sequence in the ORF2 nucleotide dataset, it differed from PX564520 and PX564524 at a single Cap residue, so most of its

divergence was synonymous. Cap positions 5 and 27 lie in the arginine-rich amino-terminal region. Position 56 lies in the BC loop and is a contact residue of the epitope of the PCV3-specific monoclonal antibody 2B5, resolved by cryo-electron microscopy; it is the only residue of that epitope reported to vary, and the aspartic acid-to-asparagine substitution, as in PX564523, was modelled to remove one salt bridge while leaving the other contacts intact [37]. Position 132 lies just outside the EF loop (residues 109–131), which contributes a further contact to that epitope [37,38]. No variable position lies in the CD loop (residues 72–79), which carries the epitope of another monoclonal antibody, 1H11 [38], and none of the genomes carries the capsid pattern 24V, 27K, 56D, 98R and 168K of a cluster from the Midwestern United States [4].

Table 3. Amino acid residues at the variable positions of the replication-associated protein (Rep) and capsid protein (Cap) of the five PCV3 genomes, together with the marker positions proposed by Fux et al. [33].

Sequence	Rep 112	Rep 122 †	Cap 5	Cap 24 †	Cap 27 †	Cap 56	Cap 77 †	Cap 98	Cap 104	Cap 132	Cap 150 †
PX564520	Q	S	A	A	R	D	S	Q	F	R	I
PX564521	Q	S	A	A	**K**	D	S	**R**	F	R	I
PX564522	**L**	**A**	A	A	R	D	S	K	F	R	I
PX564523	Q	S	**P**	A	R	**N**	S	Q	**Y**	R	I
PX564524	Q	S	A	A	R	D	S	K	F	**C**	I

† Marker positions of Fux et al. [33]. Residues found in only one of the five genomes are shown in bold.

3.4. Recombination Analysis

RDP5 returned two signals in the PCV3 genome alignment, neither of which met the acceptance criteria (Table S4). The first identified PX564522 as the putative recombinant, with KY924474.1 (domestic pig, China; 98.5% similarity) as major parent and PX564523 (100%) as minor parent, between positions 630 and 1336, and was detected by six methods. The second identified PX564521, with MG014369.1 (domestic pig, Germany; 99.3%) as major parent and PX564523 (100%) again as minor parent, between positions 384 and 1527, was detected by five methods. Neither reached $p < 0.001$ in three or more methods (corrected p-values in Table S4).

Both signals were flagged as possibly attributable to a process other than recombination and as possibly misidentifying the recombinant, with PX564523 being named in each case as a candidate actual recombinant. The beginning breakpoint of the first event had a wide confidence interval (from position 1583 through the genome origin to 1020), whereas its ending breakpoint was better constrained (1176–1347). PX564522 and PX564523 differed at only three Rep positions, all upstream of position 581 and thus outside the segment identified. Across positions 1–309, all five sequences were close to the reference genomes except PX564523, and six of the eight sites separating PX564522 from PX564523 in that window were unique to PX564523; the corresponding chromatograms were of good quality, excluding a sequencing artefact.

GARD favoured a partitioned model for the same alignment: c-AIC fell from 7674.13 with no breakpoint to 7548.99 with three (positions 550, 1337 and 1755; Table S4), and the partitioned model was strongly preferred over a single topology with variable branch lengths (7549.0 against 7711.2, an evidence ratio well above 100), indicating topological incongruence rather than rate variation. Excluding MG372488.1 and KY924473.1–KY924475.1 gave the same result (breakpoints 417, 1337 and 1755; 7266.8 against 7404.4), and in both runs position 1337 carried the highest model-averaged support (Figure 5). Because Cap is encoded on the complementary strand, its stop codon occupies positions 1336–1338,

next to the 228-position intergenic region that follows the Rep stop codon (1105–1107). Position 1337 therefore falls within the Cap stop codon, at the boundary between the Cap coding region and the intergenic region, one position from the ending breakpoint of the first RDP5 signal (1336). A second breakpoint of almost equal support, at position 1755, lies within Cap.

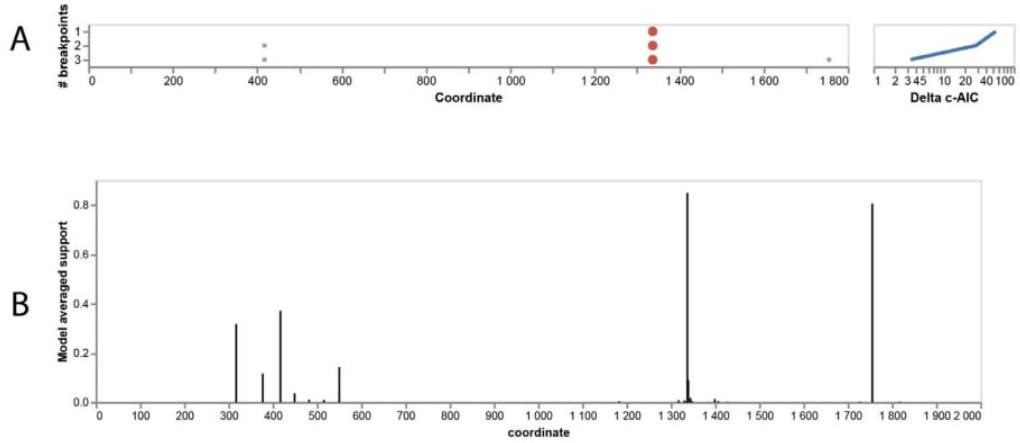


Figure 5. Detection of recombination breakpoints in the aligned PCV3 complete genome sequences using GARD, after exclusion of MG372488.1 and of the divergent sequences KY924473.1, KY924474.1 and KY924475.1 (44 sequences). (**A**) Positions of inferred breakpoints and the change in corrected AIC between models with successive numbers of breakpoints. (**B**) Model-averaged support for breakpoint position across the alignment. Two breakpoints carried comparably high model-averaged support, at positions 1337 and 1755, and are visible as the two tallest peaks in panel B; smaller peaks lie between positions 300 and 550. Position 1337 lies within the Cap stop codon (1336–1338), at the boundary between the Cap coding region and the intergenic region (Section 3.4).

To establish whether this signal was present without the sequences reported here, GARD was repeated on the reference genomes alone (43 sequences, and 39 after the divergent sequences were also removed). Both runs again preferred a model with a different topology in each partition over a single topology (Δc-AIC 164.01 and 149.14; Table S4), with the best-supported breakpoint at position 1262, in the non-coding region between the 3′ ends of Rep and Cap. The incongruence is therefore already present among the reference sequences and is not introduced by the five genomes reported here, although the exact breakpoint positions vary with the composition of the dataset.

The GARD result concerns the combined alignment as a whole and does not identify a recombination event in the five genomes reported here, for which neither RDP5 signal met the acceptance criteria. No recombination signal was detected among the PCV2 fragments by any method, and GARD retained no breakpoint: the best single-breakpoint model, at position 245, gave a c-AIC of 8543.60 against 8447.43 without a breakpoint, and model-averaged support was negligible throughout (maximum approximately 3×10^{-9}).

4. Discussion

Both viruses were detected at frequencies close to those reported from South Banat District, at the opposite end of Vojvodina, in the same seasons (17.5% for PCV2 and 15.5% for PCV3 among 103 animals [27]), but convenience samples of this size cannot establish whether circulation differs between districts. Higher figures have been published for eastern Serbia, with true prevalence estimates of 59.2% for PCV2 and 49.0% for PCV3 among 166 animals [28], and PCV2 was found in half of 66 spleens from western Serbia and the Republic of Srpska [29]. The eastern survey also reported 69.9% and 67.0% for 24 animals from South Banat, which its authors attributed to their detection method and

the inclusion of liver [28]. The present data cannot determine whether methodological, biological or sampling differences explain a difference of this size within one population.

Published rates are also rarely comparable. The eastern Serbian figures are true prevalences, whereas those reported here and for South Banat are apparent prevalences, and tissue matrix, assay chemistry and extraction platform differ between studies. Hunting-based collection is not random with respect to age or sex, and in intensive production PCV2 detection rates and viral loads rise from weaning through the finishing period and fall in adults [39], so the age structure of a sample affects detection. Finally, PCV2 was detected in 40.3% of South Banat wild boar in 2018–2019 and PCV3 in none [26], against 17.5% and 15.5% in the same hunting grounds three years later [27]; whether this reflects a change in circulation or in diagnostic sensitivity cannot be resolved from published data.

Three of the four PCV2 sequences belonged to PCV2b and one to PCV2d. In Serbian wild boar, PCV2d accounted for most characterised sequences, PCV2b for over a third and PCV2a for a small minority [26], while PCV2d has become predominant in European domestic pigs [5]. Four sequences show co-circulation but cannot establish genotype proportions, nor can they support any statement about PCV2b in wild boar in relation to vaccination of domestic pigs; that question would require larger sequence sets from both populations sampled in parallel.

Relationships with published PCV2 strains were shallow: each sequence lay within 0.8–1.2% of its closest relative and 1.0–1.5% of the nearest Serbian wild boar sequence, that is, four to six nucleotides over the 503 nt fragment and two or three over the 200 positions shared with the Serbian wild boar sequences, too few to resolve geographic structure.

The five PCV3 genomes met the criteria for PCV3-a [2], with maximum divergence of 1.46% at the genome level and 2.79% within ORF2, a narrow range typical of PCV3. Because most published PCV3 sequences fall within PCV3-a, the assignment is descriptive rather than discriminating, and the label is used only as a framework internal to this dataset [9]. Labels from other schemes, such as PCV3-1 and PCV3-3c for Serbian wild boar sequences [27] or PCV3-2a and PCV3-3g for Portuguese sequences [17], do not map onto it.

The amino acid data illustrate the same difficulty. One genome matched the intermediate pattern of Fux et al. [33] and another departed from the group pattern by a single substitution, in line with the later finding that no reliable amino acid markers separate PCV3 groups [2]. Marker-based schemes are therefore sensitive to point mutations, and two of the four capsid marker positions, 24 and 27, lie in the arginine-rich amino-terminal region rather than in a surface loop [37,38]. Whether the variation at position 56, a contact residue of the 2B5 epitope, and at position 132, next to the EF loop [37,38], affects antibody binding would require serological testing.

The five genomes were dispersed across PCV3-a, interleaved with domestic pig sequences from Europe, Asia and the Americas and wild boar sequences from Italy, Spain and Portugal. Weak phylogeographic and host structure is a recurring, though not universal, feature of PCV3 data from wild boar [11,13]: the first wild boar population examined carried considerable variability without closely related domestic pig strains [10], a divergent clade has been described in Italian rural pigs and wild boar [16], and Portuguese wild boar and domestic pigs carried different genotypes [15]. No wildlife-associated lineage was distinguished here.

PX564523 grouped with an Italian wild boar sequence (MN781187.1) in both trees, but no test of host association was carried out, so this is not evidence of host structuring. At the genome level, all five genomes were nearest to domestic pig sequences from Serbia or Hungary that differ from each other by only 0.25%; at such separations, the nearest sequence depends on the references included, so no geographic or host origin can be inferred. Over the shared capsid region, the sequences reported here differed from the

earlier South Banat sequences [27] by no more than they differed from one another, and one was identical to a South Banat sequence. Given how little PCV3 varies, this places the viruses of the two districts within a common pool of diversity but does not establish a direct link between them.

Neither RDP5 signal met the acceptance criteria: both were detected by fewer than three methods at $p < 0.001$, both were flagged as possibly spurious or as possibly misidentifying the recombinant, their breakpoint intervals were wide, and both nominated the same minor parent, PX564523, at complete similarity. In datasets of limited diversity, triplet searches tend to recover the one sequence that carries distinctive substitutions [35], and six of the eight sites underlying the stronger signal were unique to PX564523, whose chromatograms were of good quality. No recombination event can therefore be attributed to the sequences reported here.

GARD nevertheless detected topological incongruence of the kind that recombination produces, supported by an evidence ratio well above 100 in every dataset, including the reference sequences alone. The best-supported breakpoint lay at the boundary between the Cap coding region and the intergenic region, one position from the ending breakpoint of the stronger RDP5 signal, and a second lay within Cap; without the five genomes reported here, the best-supported breakpoint again lay in the intergenic region, so exact positions should not be over-interpreted. This placement is consistent with recombination in circoviruses occurring mainly in intergenic regions, and a breakpoint at a gene boundary cannot be detected within a single coding region, which, together with the low variability of PCV3, may explain why a recent analysis of curated ORF2 data found no recombination [9]. A recent Iberian whole-genome analysis likewise found consistent recombination signals among PCV2 but not PCV3 sequences [17]. The present result is therefore best read as incongruence within this alignment rather than as a general property of PCV3. The absence of a PCV2 signal carries little weight, given the 503 nt alignment.

No animal carried both viruses. With independent circulation at the observed frequencies, about two co-infected animals would have been expected among 90, so their absence provides no evidence of interference between the two viruses. Co-infection is also uncommon in European domestic pigs [5] and was infrequent in South Banat [27]; the 31.0% reported from eastern Serbia [28] follows from the much higher single-virus prevalences in that study.

In summary, both viruses circulate in wild boar in West Bačka District at levels comparable to those elsewhere in Vojvodina, with viral diversity not separable from that of European domestic pigs. Host genetic data from the province likewise show widespread past introgression from domestic pigs [23]. Sequence similarity of this magnitude cannot show whether, or in which direction, the viruses pass between wild boar and pigs, which would require both hosts from the same area to be sampled in parallel. The study does show that material from statutory African and classical swine fever surveillance can provide complete genomes and genotype data for other pathogens at little additional cost; African swine fever reached this district only in 2025, several years after the samples were collected [40].

Limitations

The study has several limitations. Sampling was confined to one district and two seasons, and localities contributed very unevenly (49 of the 90 animals came from Bački Monoštor and Lalinske livade). Because the occurrence of infectious diseases in pig populations can vary between months and years with meteorological conditions [41], detection rates describe the material examined rather than prevalence in Vojvodina, and per-locality figures are given as counts. Age, sex, body condition, and clinical and histopathological

data were not available, so neither risk factors nor disease associations could be examined. Sequencing was attempted only on samples with a Ct of 25 or below, so the sequences represent the samples with the lowest Ct values rather than the full diversity present. The PCV2 analysis rests on a 503 nt ORF2 fragment because only capsid-gene assays were established for PCV2 in our laboratory; this suffices for genotype assignment but gives limited resolution within genotypes and little power to detect recombination. Domestic pigs from the same area were not sampled and few Serbian domestic pig sequences are available, so the question of viral exchange between the two populations is raised rather than answered. Recombination inference is computational, and its participants cannot be identified from five closely similar genomes. The study is therefore descriptive: it establishes that both viruses circulate in this population and characterises the sequences recovered, but it does not support inference about prevalence, disease or transmission between wild boar and domestic pigs.

5. Conclusions

PCV2 and PCV3 circulate in wild boar in West Bačka District, Vojvodina, at frequencies close to those recorded at the same time in South Banat District, although two convenience samples of this size cannot establish whether circulation differs between districts. The PCV2 sequences belonged to PCV2b and PCV2d, and the PCV3 genomes met the distance criteria for PCV3-a, all within the diversity already documented in Serbian wild boar and European domestic pigs; genetic distances were too small to resolve geographic structure. No recombination event could be attributed to the sequences reported here; the topological incongruence detected by GARD, also present among the reference sequences alone, is exploratory and does not demonstrate recombination in these genomes. Material from statutory swine fever surveillance, collected before African swine fever reached the district, proved suitable for characterising other pathogens. Whether wild boar sustain these viruses independently, and in which direction any exchange with domestic pigs runs, would require pigs from the same area to be sampled in parallel.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci13111189/s1, Table S1, primer and probe sequences, with the real-time PCR cut-off and internal control criteria; Table S2, PCV2 reference sequences; Table S3, PCV3 reference sequences; Table S4, output of the recombination analyses (RDP5 and GARD for both viruses); Table S5, wild boar cross-tabulated by hunting ground and locality; Table S6, PCV3 complete-genome *p*-distance matrix; Table S7, PCV3 ORF2 *p*-distance matrix; Table S8, PCV2 ORF2 fragment *p*-distance matrix; Table S9, mean *p*-distances of the PCV2 sequences to each genotype reference set; Table S10, *p*-distances between the PCV3 sequences reported here and partial capsid sequences previously reported from Serbian wild boar, over 543 shared positions; Table S11, Ct values of all positive animals; Figure S1, PCV3 complete-genome *p*-distance matrix, colour scale capped at 3% with off-scale pairs in a single reserved colour; Figure S2, PCV3 ORF2 *p*-distance matrix, colour scale capped at 3.5% on the same convention, the within-genotype criterion at that level being 6%.

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Institutional Review Board Statement: The study was reviewed by the Ethics Committee for the Protection and Welfare of Experimental Animals, Faculty of Veterinary Medicine, University of Belgrade. The Committee issued Opinion No. 01-06/2023, adopted at its session of 24 June 2023 and dated 26 June 2023 (registry No. 01-602, 30 June 2023). The opinion covers the research programme "Molecular characterisation of parvoviruses and circoviruses detected in wild boar and carnivore populations in West Bačka District", from which this work derives, and confirms that it is ethically justified; no invasiveness category was assigned.

Informed Consent Statement: Not applicable.

Data Availability Statement: The nucleotide sequences presented in this study are openly available in GenBank under accession numbers PX564516–PX564519 (PCV2) [https://www.ncbi.nlm.nih.gov/nuccore/PX564519.1/] (accessed on 5 October 2026) and PX564520–PX564524 (PCV3) [https://www.ncbi.nlm.nih.gov/nuccore/PX564524.1/] (accessed on 5 October 2026). The pairwise distance matrices are provided in Tables S6–S10. The alignments and tree files, derived from the sequences in Tables S2 and S3, are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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