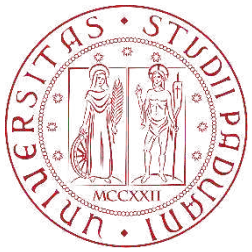

UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Comparative Biomedicine and Food Science

Second Cycle Degree (MSc)

In Biotechnologies for Food Science

Comparative Analysis of Porcine Circovirus Type 2 (PCV2) evolution in Domestic Pigs and Wild Boar

Supervisor

Prof. FRANZO GIOVANNI

Submitted by

Ruocco Luigi

Student n. 2091193

ACADEMIC YEAR 2023 – 2024

ABSTRACT

The aim of this study is to investigate the genetic aspects of PCV2 and deepen our understanding of the molecular epidemiology and evolution of the virus in pigs. All available PCV2 ORF2 sequences were collected from PubMed, considering the country of sampling, the date, and the host, to analyze viral population dynamics, dissemination, and evolutionary patterns. Specifically, selective pressures acting on circulating variants in domestic pigs and wild boars were compared. The results provide insights into viral dispersion patterns and how different hosts, based on their biological characteristics and immunity (largely influenced by vaccination), may contribute to viral evolution.

The analysis of the PCV2 capsid gene revealed several highly conserved regions, indicative of essential functions related to viral stability and replication, while less conserved regions exhibited variations likely driven by host-specific selective pressures. Comparative analyses highlighted significant differences in selection patterns: in domestic pigs, a prevalence of mutations under positive selection was observed, reflecting selective pressures potentially linked to intensive farming practices and vaccination. In contrast, wild boars exhibited a predominance of purifying selection signals, emphasizing the need to preserve protein functionality in a natural ecological context.

Phylogenetic reconstruction demonstrated the global distribution of PCV2 genotypes (PCV2a, PCV2b, and PCV2d) in both populations, with limited geographical clustering and evidence of free viral circulation facilitated by the international trade of pigs. Local clades, such as those observed in Italy, suggested possible independent evolution and specific adaptations to local contexts. The bidirectional viral flow between domestic pigs and wild boars underscored the role of the latter not only as secondary reservoirs but also as contributors to transmission, with significant epidemiological implications. Overall, the study provides crucial insights into the evolutionary dynamics of PCV2 and its interaction with different hosts. The observed differences between domestic pigs and wild boars reflect distinct selective pressures and the complexity of evolutionary processes shaping the virus's genetic diversity. These findings highlight the need for monitoring and containment strategies to limit interactions between domestic and wild populations, thereby preventing the spread and evolution of pathogens with significant health and economic impacts.

Riassunto

L'obiettivo di questo studio è investigare gli aspetti genetici del PCV2 e approfondire la comprensione dell'epidemiologia molecolare e dell'evoluzione del virus nei suini. Sono state raccolte tutte le sequenze di ORF2 di PCV2 disponibili su PubMed, considerando il paese di raccolta, la data e l'ospite, per analizzare le dinamiche della popolazione virale, la diffusione e i pattern evolutivi. In particolare, sono state confrontate le pressioni selettive che agiscono sulle varianti circolanti nei suini domestici e nei cinghiali selvatici. I risultati forniscono informazioni sui modelli di dispersione virale e su come i diversi ospiti, in base alle loro caratteristiche biologiche e all'immunità (in gran parte influenzata dalla vaccinazione), possano contribuire all'evoluzione virale.

L'analisi del gene del capside di PCV2 ha rivelato numerose regioni altamente conservate, indicative di funzioni essenziali legate alla stabilità e alla replicazione virale, mentre le regioni meno conservate hanno mostrato variazioni probabilmente guidate da pressioni selettive specifiche dell'ospite. Le analisi comparative hanno evidenziato differenze significative nei pattern di selezione: nei suini domestici è stata osservata una prevalenza di mutazioni sottoposte a selezione positiva, riflettendo pressioni selettive potenzialmente legate alle pratiche di allevamento intensivo e alla vaccinazione. Al contrario, nei cinghiali selvatici è emersa una predominanza di segnali di selezione purificante, sottolineando la necessità di preservare la funzionalità delle proteine in un contesto ecologico naturale.

La ricostruzione filogenetica ha dimostrato la distribuzione globale dei genotipi di PCV2 (PCV2a, PCV2b e PCV2d) in entrambe le popolazioni, con un clustering geografico limitato e evidenze di una libera circolazione virale favorita dal commercio internazionale di suini. Cladi locali, come quelli osservati in Italia, hanno indicato una possibile evoluzione indipendente e adattamenti specifici ai contesti locali. Il flusso virale bidirezionale tra suini domestici e cinghiali ha sottolineato il ruolo di questi ultimi non solo come reservoir secondari, ma anche come contributori alla trasmissione, con significative implicazioni epidemiologiche. Nel complesso, lo studio fornisce informazioni cruciali sulle dinamiche evolutive del PCV2 e sulla sua interazione con ospiti diversi. Le differenze osservate tra suini domestici e cinghiali riflettono pressioni selettive distinte e la complessità dei processi evolutivi che modellano la diversità genetica del virus. Questi risultati evidenziano la necessità di strategie di monitoraggio e contenimento per limitare le interazioni tra popolazioni domestiche e selvatiche, prevenendo così la diffusione ed evoluzione di patogeni con rilevanti impatti sanitari ed economici.

Table of Contents

1. INTRODUCTION

1.1. Pork consumption and the impact of circovirus on pigs: a global picture

The global pig industry has increased by 140% since the 1960s (1). The rise is related to improved socioeconomic conditions of a low-middle income countries. The increased consumption of meat regards especially pork. The top three pork producers globally are China, United States (US) and the European Union (EU). China is the most important pork meat producer with nearly 50% of the world's total production (1). Intensive farms have spread around for several years, also structured on several floors and hosting hundreds of thousands of pigs.

T skyscraper of the Zhong Xin Kai Wei Modern Breeding Company (shown in **Figure 1**) have reached 26 floors with 600 thousand pigs (The New York Times, no date).In **Figure 2** is shown an example of high-density, multi-floor pig production system.

Figure 1. *Zhong Xin Kai Wei Modern Breeding Company*

Figure 2. *Example of high-density, multy floor pig productions system*

With the increase in pig production, there has been a simultaneous increase of pathogenic virus detections. The international trade of livestock plays an important role in the dissemination of diseases. This situation takes place within a global context in which regions and countries with quite different levels of production and sanitary standards have commercial contact, making it very difficult to provide full product traceability and prevent disease transmission risks substantially (3).

The transportation of livestock is crucial because is related to the outbreak of many pathogenic viruses. Among the viral diseases that can affect pigs there are Swine Influenza, Porcine

Reproductive and Respiratory Syndrome (PRRS), Porcine Epidemic Diarrhea (PED), Classical Swine Fever (CSF), African Swine Fever (ASF), Foot-and-Mouth Disease (FMD), Pseudorabies (Aujeszky's Disease) and Porcine Circovirus diseases (PCVDs). While apparently less clinically impacting, porcine circoviruses are important pig pathogens being responsible for several syndromes that cause significant economic losses for the meat industry (4). PCV was already known as a not-pathogenic persistent contaminant of PK-15 cell line cultures when, in 1991, a new clinical condition appeared in Canada (5). This condition was marked by chronic wasting, rapid and difficult breathing, pale appearance, swollen lymph nodes, and jaundice. Based on these symptoms and the age of affected animals, it was named Post Weaning Multisystemic Wasting Syndrome (6) Initially linked to PCV, later serological and genomic analyses identified a distinct circovirus. Sequencing analysis showed differences in the DNA sequences, showing about 70% and 76% similarity to the original strain described by Tischer. (7) Therefore, the newly identified pathogenic strain was named PCV2, while the non-pathogenic strain was renamed PCV1. In the following years, report of PMWS became more frequent especially in Europe, United States, Asia, and South America. Although initially debated, the pathogenic role of this virus and the relevance became clear over time, establishing PCV2 as an emerging global pathogen (8).

Originally linked to postweaning the multisystemic wasting syndrome (PMWS, now referred to as PCV2 systemic disease or PCV2-SD) in the early 1990s, PCV2 was later implicated in other clinical conditions, including PCV2 reproductive disease (PCV2-RD) and porcine dermatitis and nephropathy syndrome (PDNS). Together, these conditions (PCV2-SD, PCV2-RD, and PDNS) are classified as porcine circovirus diseases (PCVDs)(4). While PCVDs have complex causes, most PCV2 infections remain subclinical (PCV2 subclinical infection, PCV2-SI). Interestingly, PCV2-SI exerts a greater economic burden than clinical infections by reducing average daily weight gain and increasing vulnerability to co-infections, ultimately impacting farm profitability (9). In addition to the sings and lesions directly associated to PCV2, the immunosuppressive nature of the infection can predispose to several other infections (10,11).

In fact, pigs with PCV have a significant immune system modification, as seen by the disease's histological features and the prevalence of secondary and opportunistic infections in afflicted animals (12) For many years, the main goals of PCV control have been to enhance management techniques and reduce risk factors that affect the disease's clinical manifestation. However, the development and commercialization of specific vaccines was a cornerstone in PCV2 control. Their

application in the field allowed to control the occurrence of clinical forms and has significantly decreased the prevalence of PCV2(13,14), although sporadic outbreaks still occur, likely due to improper vaccination. However, a reduced protection due to viral variability and decrease in cross-protection has sometimes been claimed (15).

1.2. Taxonomy of PCV

Porcine Circovirus 2 belongs to the *Circoviridae* family and has a circular single-stranded DNA genome enclosed in a small (20-25 nm) icosahedral capsid. It is part of the *Circovirus* genus, which distinguishes it from the *Cyclovirus* genus of the same family. The organization and orientation of protein-coding genes are the main basis for this categorical distinction. Taxonomic classification divides members of the *Circoviridae* family into species using a demarcation threshold. Viruses with less than 80% identity in the genomic sequence are assigned to a different species (16). In **Figure 3** is represented the genomic organization of PCV-1. The genomic structure of *Circoviridae* viruses is composed of two main open reading frames (ORFs) encoding for two main functional and structural proteins: Rep and Cap. Rep is composed of two domains, helicase domain and endonuclease domain. The capsid protein (Cap) has a jelly roll structure with a positively charged N-terminal arm and aggregates in 60 copies to form the capsid (**Figure 4**).

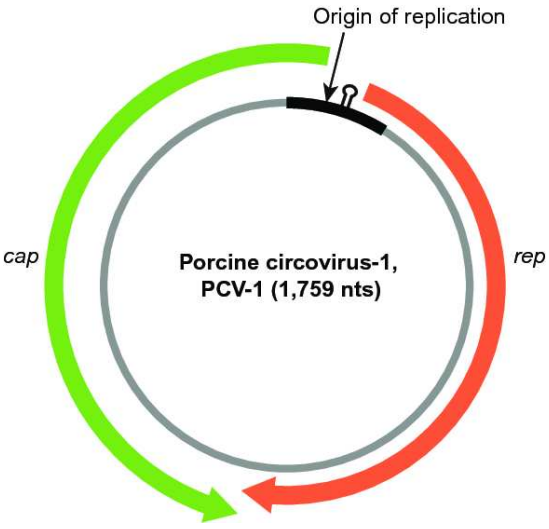

***Figure 3**. Genomic Organization of PCV-1*

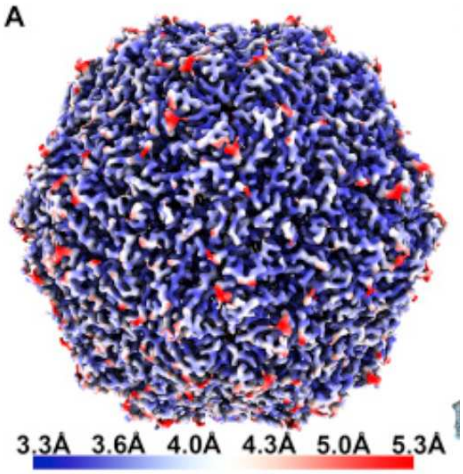

***Figure 4**. Icosahedral cryo-EM determined structure of the purified PCV2d VLP colored according to the local resolution*

The viral genome is ambisense and the two ORFs are located on complementary strands of the replicative DNA. Rolling circle replication (RCR) originates from the stem-loop structure with a highly

conserved sequence in the intergenic region between the two ORFs. Circoviruses of the genus *Circovirus* (including PCV2) primarily infect vertebrates, such as pigs and birds. Cycloviruses, on the other hand, can infect both vertebrates and invertebrates. According to ICTV guidelines, the most recent taxonomy has added binomial nomenclature for species in the family *Circoviridae*. For example, the species PCV2 is now known as *Circovirus porcine2*, but the virus is still called Porcine circovirus 2. This change was made to improve taxonomic clarity and facilitate the classification of new species as they emerge (16) Circoviruses of the genus Circovirus (including PCV2) primarily infect vertebrates, such as pigs and birds. Cycloviruses, on the other hand, can infect both vertebrates and invertebrates (**Figure 5**).

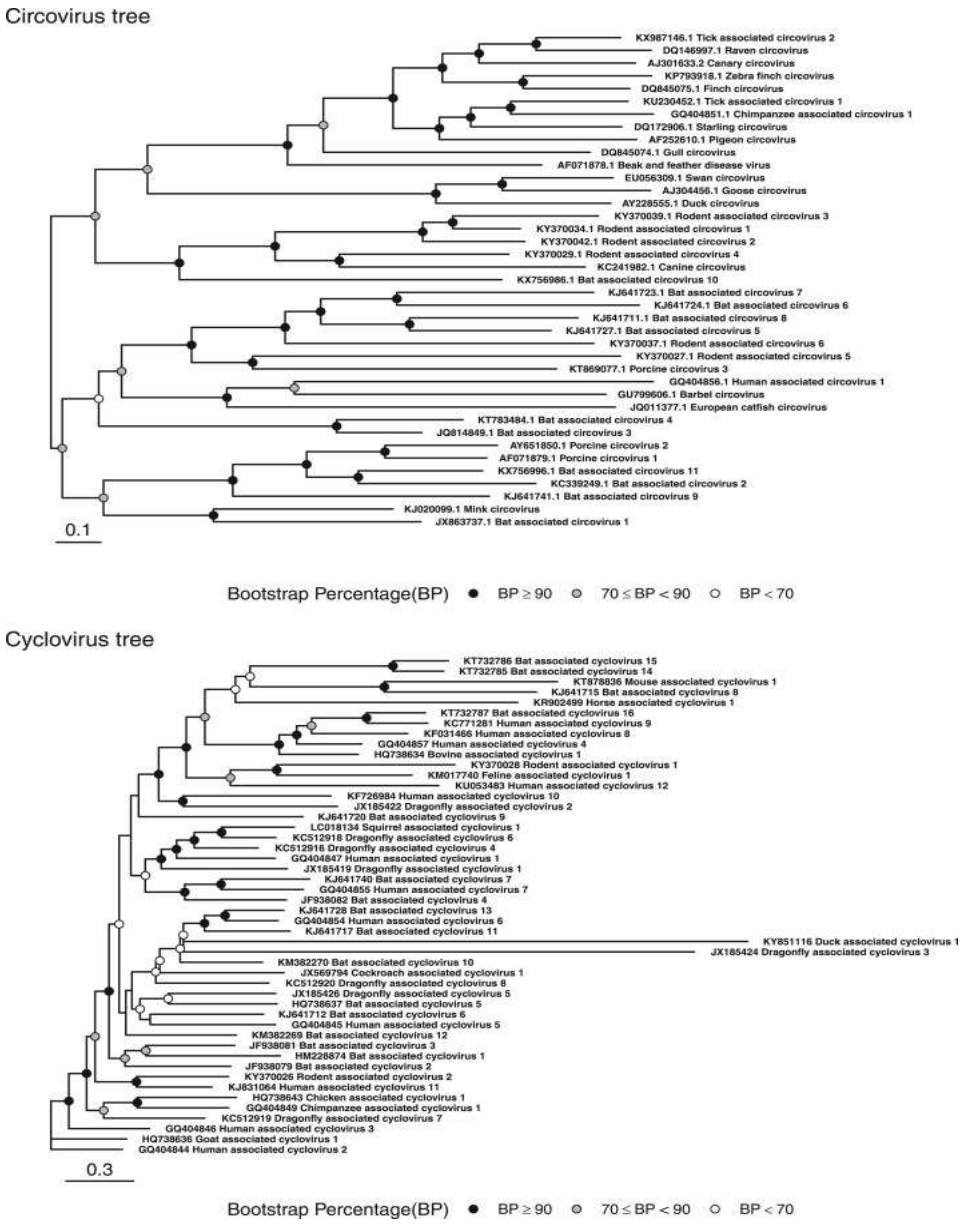

***Figure 5**. Phylogenetic trees of the main species included in the Circovirus and Cyclovirus genus*

1.3. Structure of pcv2: molecular mechanisms and genetic characteristics

PVC2 is the smallest autonomously replicating viruses in mammalian cells, with a diameter of about 17 nm. as other members of this genus have icosahedral symmetry, lack an envelope, and contain a single-stranded, circular DNA genome. It has a genome of about 1.7 kilobases. The DNA genome of the PCV1 and PCV2 was found to be 1759 and 1766-1768 nucleotides, respectively (17,18)

Their genome has circular genomic structure with a characteristic stem-loop feature at the ORI that is responsible for DNA replication termination. During replication, a rolling-circle replication, the loop stem consists of an 11-base paired palindromic sequence, and the loop consists of a stretch of 10-12 nucleotides, tending to 12 in PCV1 and 10 in PCV2, including an 8-nucleotide conserved sequence. This sequence forms a cleavage site where the Rep complex binds to initiate replication (17). The genome of PCV2 comprises eleven potential open reading frames (ORF), altohugh only for six the expression has been proven and a in deep characterization has been achieved only for three. ORF1 encodes two replication proteins, Rep and Rep'; ORF2 encodes the capsid protein Cap, while ORF3 is believed to encode proteins responsible for virus pathogenicity and apoptosis. Transcription of each of these ORFs specifically depends on viral replication; it is dependent on enzymes in the host cell, although viral Rep complex is necessary initiate the replication process (19).

The ORF1 is the most conserved region of the genome, with an identity of 79.5–85% between PCV1 and PCV2. This gene encodes two proteins, Rep, consisting of 312 amino acids, and Rep', comprising 178 amino acids, produced through differential splicing of ORF1. Rep' contains the first 122 amino acids of Rep but has a different C-terminal sequence. The characteristic amino acid domains include RC-I, RC-II to coordinate the divalent ions, RC-III with the tyrosine residue for cleavage at Ori, and a P-loop for helicase and ATPase activity. Compared with Rep, Rep' lacks the P-loop, which is dispensable for the *in vitro* cleavage at the replication origin but is required for replication in cell cultures (20)

Since PCV2 replication depend on host polymerases, replication takes place in the host cell S phase wherein the ssDNA assumes a dsDNA intermediate perhaps initiated by a minus genome initiation primer-like complex, although similar complexes have been described in other viruses, confirmation of this structure in PCVs is awaited (21)

The Rep complex unwinds the dsDNA at the replication origin, nicks the c-DNA at the loop to bind covalently to the 5' end, and exposes the 3'OH end that act as a primer for DNA polymerase synthesizing a complementary strand, completing replication when reaching the stem-loop structure. This gives rise to a positive single-stranded DNA and a double-stranded circular DNA comprising the original negative strand and the new complementary strand. The single-stranded DNA further can continue replication or be encapsidated in the viral particles (22).

The second largest reading frame is ORF2, encoding the cap gene for the capsid protein Cap. Cap is a 233-234 amino acid protein with a molecular weight of about 30kDa. The protein provides the icosahedral capsid packaging the viral genome, mediates virus attachment, and effects the transmission of viral DNA to the host cell nucleus via an N-terminal NLS or nuclear localization signal. The major epitopes have been identified in the PCV2 capsid, which is the main target of the host immune response (6,10).

Although ORF3 is not crucial for infection or replication of the virus, it has been suggested that the protein can interact with an E3 ubiquitin ligase and thus potentially be involved in the stability of tumor protein p53, linked to apoptosis. Its interaction can lead to programmed cell death, allowing this virus to spread to other cells and consequently to lymph nodes. This hypothesis was further justified by the studies of PCV2 strains infections in mice (20)

Another protein encoded by ORF4 could prevent apoptosis—a potential antagonist to the protein expressed by ORF3. During replication, the detection of ORF4 as a transcript has been detected and may play a role in the inhibition of caspase-mediated apoptosis, thus allowing for a more persistent viral replication (22)

The ORF5 and 6 have been shown to be transcribed and translated, interfering with host signaling pathways at different levels

1.4. PCV2 capsid variability: implications for virulence and virus evolution

The capsid protein of PCV2 is essential for the virus's structure and interaction with the host immune system. It plays a central role in immune recognition, as it induces responses that help in the neutralization and elimination of the virus. PCV2 has a high mutation rate, comparable to that of RNA viruses, and these characteristic favors rapid evolution and the emergence of genetic variants (23). Phylogenetic studies have identified several major genotypes (PCV2a, PCV2b, and PCV2d) that

have succeeded one another over time as dominant genotypes. For example, the PCV2a genotype, dominant until the early 2000s, was subsequently replaced by the PCV2b genotype, which was associated with outbreaks of more severe disease (24).

PCV2d has emerged as the dominant genotype in several countries, supplanting PCV2b ((25). This switch between genotypes, known as “genotype shift,” reflects the virus’s constant adaptation to changes in the host’s environment, which can be influenced by natural factors and artificial selective pressures such as vaccination (26–28).

Recent sequencing has shown that the genetic variability of the capsid is concentrated mainly in epitopic regions, which are targeted by antibody and cellular responses. Specific mutations in the Cap protein appear to enhance the virus’s ability to evade the immune system (29). This ability is particularly relevant in settings where vaccination is widespread, as the virus must adapt to a partially immunized host (19). The co-circulation of various genotypes in different geographical areas suggests that PCV2 may benefit from frequency-dependent selection, in which the fitness of a genotype decreases as it becomes more common, thus favoring periodic shifts in the dominant genotype (30).

Clinically, PCV2 capsid variability has been associated with possible differences in virulence between genotypes. Although further studies are needed to confirm this hypothesis, some research suggests that PCV2d may be associated with more severe clinical forms, such PCV2-SD and other systemic manifestations (5). The link between capsid variability and virulence is still an active area of research, as specific virulence markers for PCV2 have not yet been definitively identified.

1.5. Role of vaccination and immunity in the prevention of PCV2 infections: challenges and perspectives

One of the major steps in controlling the circovirus diseases in pigs has been the introduction of vaccines against the PCV2, which has eventually brought a sharp decline in clinical signs and economic losses for the pig industry. The vaccines used so far, based on the genotype PCV2a, which was initially the most frequent, have been highly effective against various viral strains and clearly diminished signs, both clinical and subclinical (31). However, the wide use of these vaccines has imposed evolutionary pressures that might have affected the temporal distribution of viral genotypes. In fact, of the use of PCV2a-based vaccine have been speculated as a factor driving viral

evolution and contributin to the success of other genotypes such as PCV2b and particularly PCV2d, which mat show a greater ability to evade the vaccine-induced immune response (15,28). Immune escape is the ability of a virus to evade the host's immune system and, in fact, represents a direct response to vaccine pressures (32,33). The major target of immune response is the Cap protein encoded by the ORF2 gene. However, while this protein is highly mutable, the virus's ability to 'escape' antibody recognition is more limited compared to the mechanisms of antimicrobial resistance. Indeed, some of the important antigenic regions have acquired mutations in the PCV2d genotype that reduced antibody neutralization efficiency. The most recent studies indicate that the model of frequency-dependent selection is being followed during evolution of PCV2. In other words, if a genotype is relatively rare, it usually is in an advantageous position, whereas once it gets too abundant, it loses this advantageous position because of increasing population immunity and loss of fitness. This has leasd to oscillation of PCV2a, PCV2b, and PCV2d detection frequency, each prevailing at different times, influenced by the immune pressures, both natural and vaccine induced. Such dynamics allow this virus to maintain genetic variability, making it difficult to completely eradicate the pathogen. This virus is notorious for its high propensity for recombination, an event that offers the virus a chance to generate new variants through the recombination of DNA regions from different genotypes (34). It happens quite often both in vaccinated domestic pigs and in unvaccinated wild boars, further enhancing the virus's potential to produce resistant variants that might lead to an immune- circumventing event against the vaccines currently in use. It is this genetic diversity that improves chances for resistance (12). Thr co-circulation of several genotypes, in addition to a high mutation rate, underlines the requirement for permanent surveillance, even among wild populations like those of the wild boars, since these reservoirs of genetic variability are in contact with, and may re-infect, domestic populations, thus contributing to the emergence and introduction of new viral variants. While it is of utmost importance to ensure that advances in biosecurity practices in pig farms prevent transmission between wild and domestic animals, real life scenario testify that often their efficacy is limited (35,36). Thefore, a pivotal role must be played by vaccination. In this scenario, vaccines in current use will need periodic evaluation and updating to counter the continuously arising genotypes. Mass vaccinations that are based on monovalent formulation, though affording protection against the clinical symptoms, encourage the selection of variants with evasion capability. Probably soon, polyvalent vaccines covering epitopes from different genotypes will provide broader and longer protection against various variants of PCV2 (37).

2. OBJECTIVES AND HYPOTHESIS

The present study purpose is to examine whether selective pressures act differently on wild and domesti populations to assess and quantify the impact of vaccination over time. This will allow us to evaluate the potential evolutionary divergence influenced by vaccination practices and the pattern and sites where it occurs.

As secondary objectives, we aim to investigate the genetic variability within each host, assess differences between them, and determine if any unique genetic or phenotypic traits exist. Additionally, studying viral flow between these two populations will not only clarify the role of wild populations in shaping viral evolution but also reveal their potential contribution to the introduction of the virus into domestic populations.

Conversely, we will evaluate the role of domesticated farming as a potential threat to wild populations, examining if and how it could impact the health and viability of wildlife.

3. MATERIALS AND METHODS

3.1. Sequence selection

Complete ORF2 sequences of PCV2 were retrieved from the NCBI virus. However, there is no specific requirement for those depositing sequences to include adequate metadata about the sequenced strains, including the DNA collection date, country, and host species, which can complicate the sequence selection and analysis process. We used PubMed as the main source for gathering and verifying the information needed for analysis, searching published articles reporting PCV2 sequences (i.e., relative accession number) and clearly annotated metadata.

This step provided a comprehensive overview of all available data, creating a solid basis for our study.

One of the main challenges encountered was host identification, the comparison of which was one of the main objectives of the present study. Sequences of Sus scrofa are often deposited without specifying whether they belong to Sus scrofa domesticus or wild Sus scrofa (wild boars). This lack of detailed information can make data interpretation difficult, especially because these two groups of animals can have different distributions and epidemiological characteristics. To address this issue, we carefully reviewed all available literature to find missing information, allowing us to ensure a highly curated sequence annotation. Only sequences for which such data were available were considered for further analysis.

We collected a total of 2015 sequences from wild boar and domestic pig samples after reviewing the literature and selecting the necessary data.

This sample enabled us to identify two populations for comparison. Since the number of sequences obtained from domestic pigs outnumbered the wild ones, we selected a set of domestic pig sequences with a temporal distribution comparable to that of the wild boar sequences and maintained a ratio of approximately 1.5 domestic pig sequences for every wild boar sequence.

This approach alloed us to adequately represent the geographical spread of PCV2 across the various environments inhabited by wild boars and domestic pigs.

3.2. Sequence analysis

Once the two populations have been carefully and attentively selected, ensuring that they are representative for both domestic pigs and wild boars, the alignment of the sequences was performed. For this purpose, Jalview (38) and MEGA (39)were used. A final alignmen of 702 base-pair, incliuding 331 and 223 sequences of domestic and wild boar, was obtained. To prelimanry evaluate the within and between populations genetic and amino acid variability of the selected sequences, different graphical tools were implemented. In particular, the sequence alignment quality and variability, were assessed at nucleotide and amino acid position by visual inspection, by evaluation of summary statistics generated by Jalview and by graphical tools like WebLogo (40).

3.3. Phylogentic tree

The MEGA XI program was used to create the phylogenetic tree after loading a FASTA sequence file annotated with information on wild boar and Sus scrofa domesticus. The tree was constructed using the Maximum Likelihood (ML) approach, which is especially well-suited for estimating evolutionary relationships based on probabilistic models of sequence change. The substitution model was selected based on the Bayesian information criterion (BIC), calculated through the same software.

3.4. Viral migration among populations

To jointly reconstruct the population-specific viral effective population size (Ne), strain migration between categories (i.e., wild boars and domestic pigs), and other population parameters, as well as phylogenetic trees representing viral genealogy, a Bayesian structured coalescent analysis (41) was performed using the MultitypeTree package in BEAST 2.7 (42). The structured coalescent is a statistical framework that models the genealogy of individuals sampled from a structured population evolving under a migration matrix model. In this context, each category represents an 'island' (*deme*) with a specific population size, interconnected by migration events occurring at a constant rate over time.

A Markov chain Monte Carlo (MCMC) run of 100 million generations was conducted, with model parameters and phylogenetic trees sampled every 10,000 generations. The nucleotide substitution model was chosen based on the Bayesian Information Criterion (BIC), and a relaxed lognormal molecular clock was selected (43). Results were evaluated in Tracer v1.7 (44) and considered

reliable only if mixing and convergence were adequate, with an Estimated Sample Size (ESS) exceeding 200 for all parameters. Parameter estimates were summarized as the mean and 95% Highest Posterior Density (HPD) after discarding the first 20% of the run as burn-in. Maximum clade credibility (MCC) trees were generated and annotated using TreeAnnotator from the BEAST package.

3.5. Selective pressure analysis

Selective pressures were identified using the Datamonkey website (45), implementing the SLAC (Single-Likelihood Ancestor Counting) (46) and MEME (Mixed Effects Model of Evolution) (47) methods.

SLAC is a maximum likelihood model-based method that calculates pervasive positive or negative selection at specific sites. It compares the number of expected and observed synonymous and non-synonymous substitutions at each position of the gene, determining whether there is evidence of purifying or diversifying selection quickly and efficiently. MEME, on the other hand, is a more sensitive approach that identifies episodic positive selection signals at specific sites. It uses a mixed model that allows the detection of positive selection events that occur only in some branches of the phylogenetic tree, improving the ability to detect complex evolutionary signals compared to more conservative methods. Finally, to formally assess differences in selective pressure strength among host populations, contrast-FEL was employed (48). The identified features of interest were mapped onto the capsid tertiary structure, downloading the 6lm3 PDB file from Protein Data Bank (49) and editing it using the pymol library in Python (50).

4. RESULTS and DISCUSSION.

4.1. Discussion on sequences alignments

Alignment plays a key role in investigating similarities and differences between the sequences of the two populations, facilitating in-depth understanding and providing insight into genetic relationships. The quality of the alignment was evaluated by visual inspection and by assessing the absence of poorly aligned regions, frameshift mutations and premature stop codons.

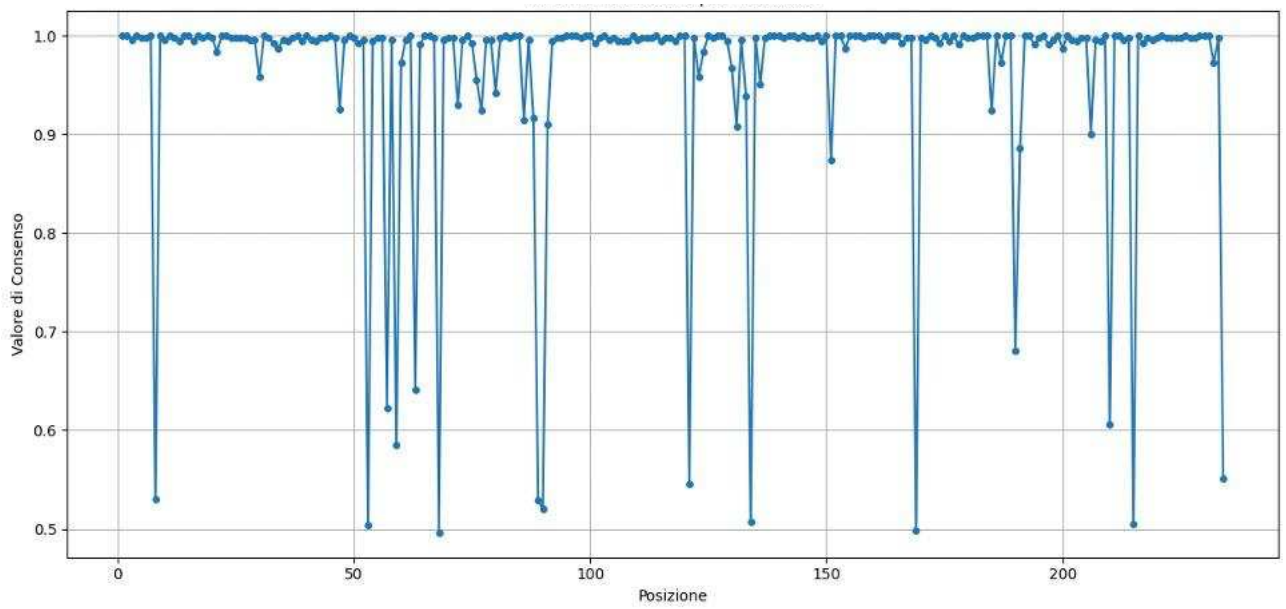

***Figure 6**: Value of consensus for each alignment position in the combined dataset including domestic pigs and wild boards.*

The variability of the aligned sequences was assessed using different statistics. Among those, the consensus and conservation statistics calculated for each position provided concordant results.

The first one (**figure 6**) emphasize the relative frequency of the consensus base (e.g., showing the percentage of sequences that possess the dominant base compared to the total). While (**figure 7**) represent a global measure such as the level of conservation or variation of each position.

A comprehensive analysis of the entire capsid protein was performed to ensure a complete representation. This approach allows for detailed interpretation of the data, highlighting both conserved and variable regions, which are critical to understanding stability, function, and evolutionary dynamics. The first aspect analyzed by the sequence alignment is nucleotide conservation, showing an overall homogeneity of the alignment, with a higher variability in specific positions.

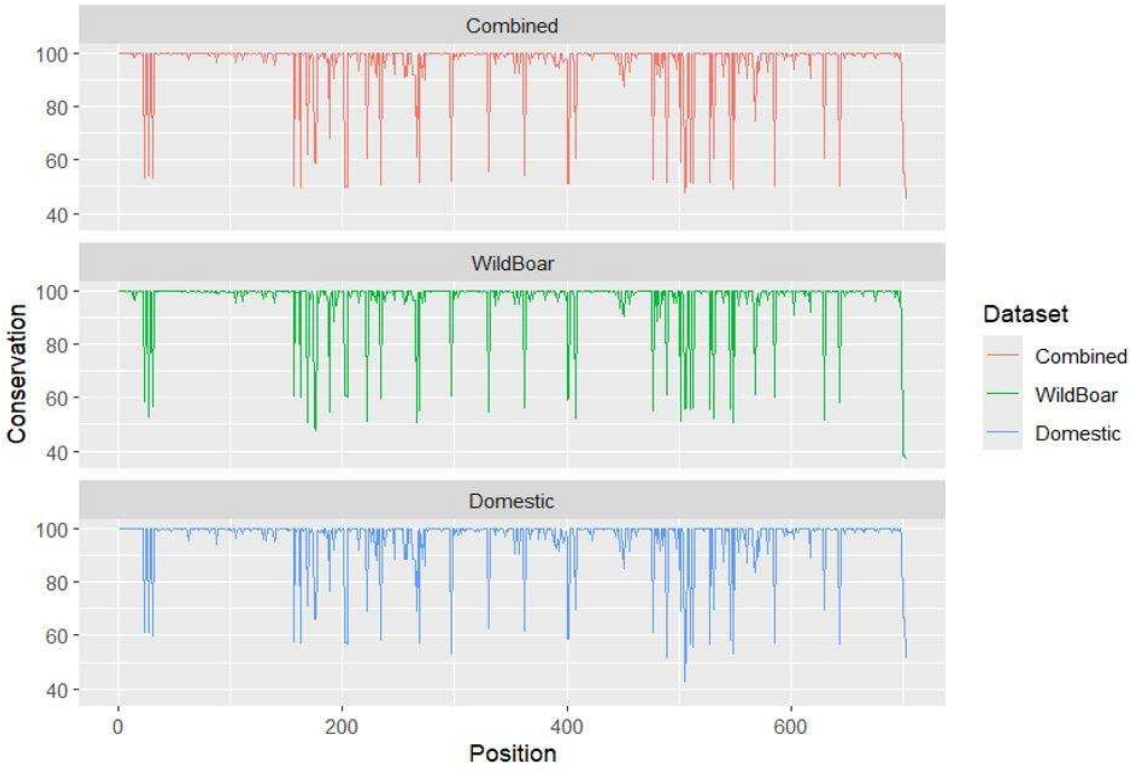

***Figure 7**. Graphical depiction of the Conservation of the diffent positions of the ORF2 DNA alignment. The results of the whole dataset, and for Wild boar and domestic pig independently are reported.*

Although **Figure 7** does not properly allow to determine whether the observed variation is due to intrinsic variations present in both categories and between the two pig populations, some preliminary conclulsion can be drawn. Generally, the 3 figures show similar conservation patterns. However, some subtle differnces can also be observd in the same regions of the sequence.

A more informative depiction can be achieved through the nucleotide projection and WebLogo analyses (**Figures 8, 9, 10, 11**), the distribution of variability appears similar between the two populations. This indicates that certain regions of the protein are inherently more prone to variation, while others remain highly conserved. Generally speaking, conserved regions correspond to genome segments encoding functional proteins or domains, such as those involved in DNA

binding, while more variable regions may reflect areas under immune selective pressure. Therefore, similar consideration could be extended to PCV2 also.

However, more subtle differences between populations were observed, which may be influenced by distinct selective pressures. The analysis of amino acid logos for both populations revealed comparable patterns, further supporting our hypothesis. These findings provide valuable insights into the genetic diversity and potential evolutionary adaptations distinguishing wild boars from domestic pigs, which suggested deeper and specific analysis to understand the genetic and evolutionary relationships among the strains circulatin these host.

Overall, the analysis of nucleotide and amino acid sequences from wild boars and domestic pigs show numerous highly conserved areas, suggesting the existence of a common genetic basis linked to essential functions. Variations observed in certain areas (with lower conservation and consensus values) could indicate specific adaptation features or functional differences. Such differences could be influenced, for example, by the prevalence of the virus in each population, which in turn determines the level of population immunity present (30). Furthermore, the epidemiological context or vaccination could play an important role in shaping these variations, imposing different selective pressures (51). Based on this evidence, we considered interesting to verify, through formal hypothesis testing and dedicated methods, whether these variations are associated with distinctive features of domestic pigs or wild boars, providing insights to better understand the interactions between host, virus and environment.

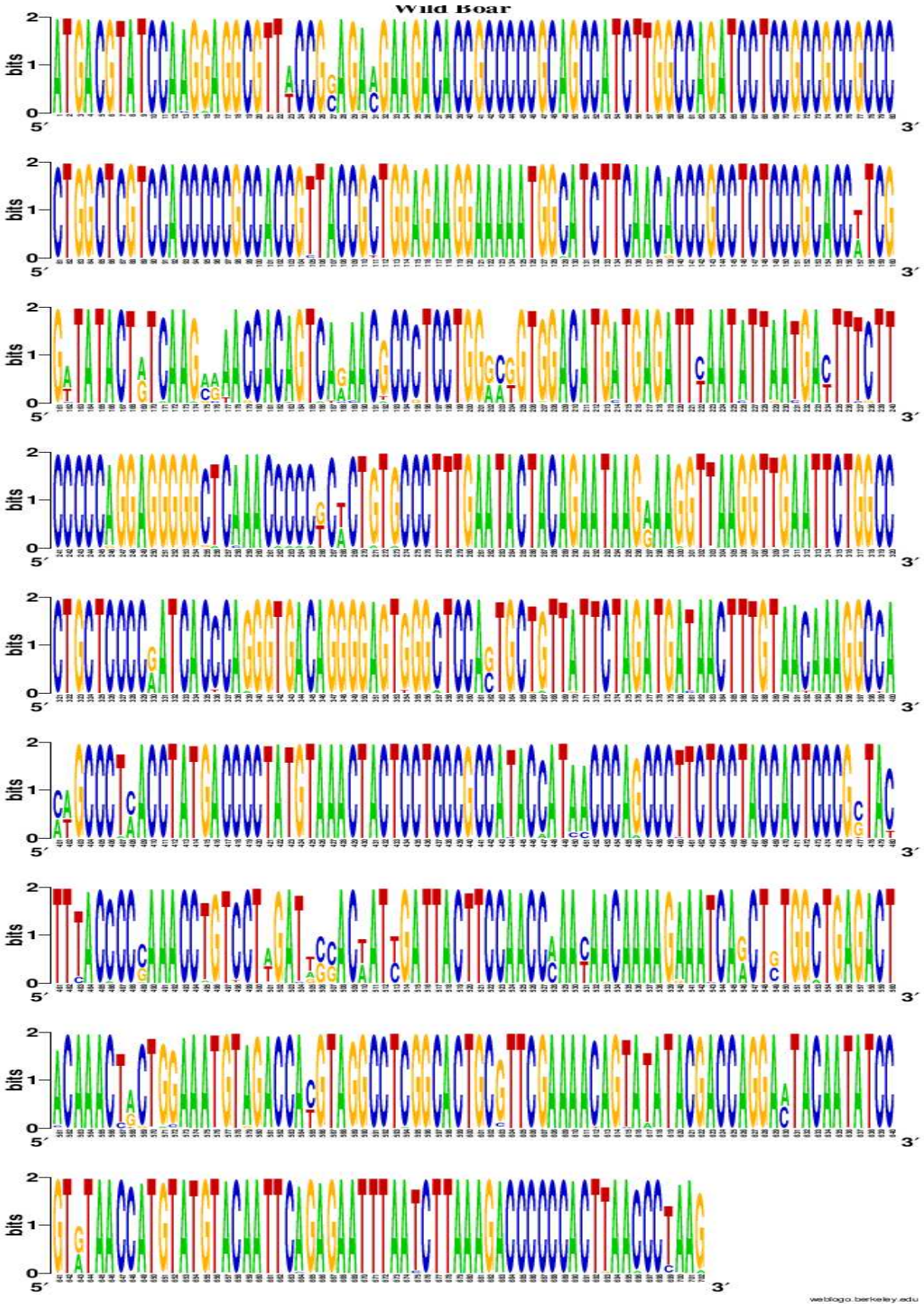

***Figure 8**: Sequence Logo Representation of PCV2 Capsid Gene Variability in Wild Boars*

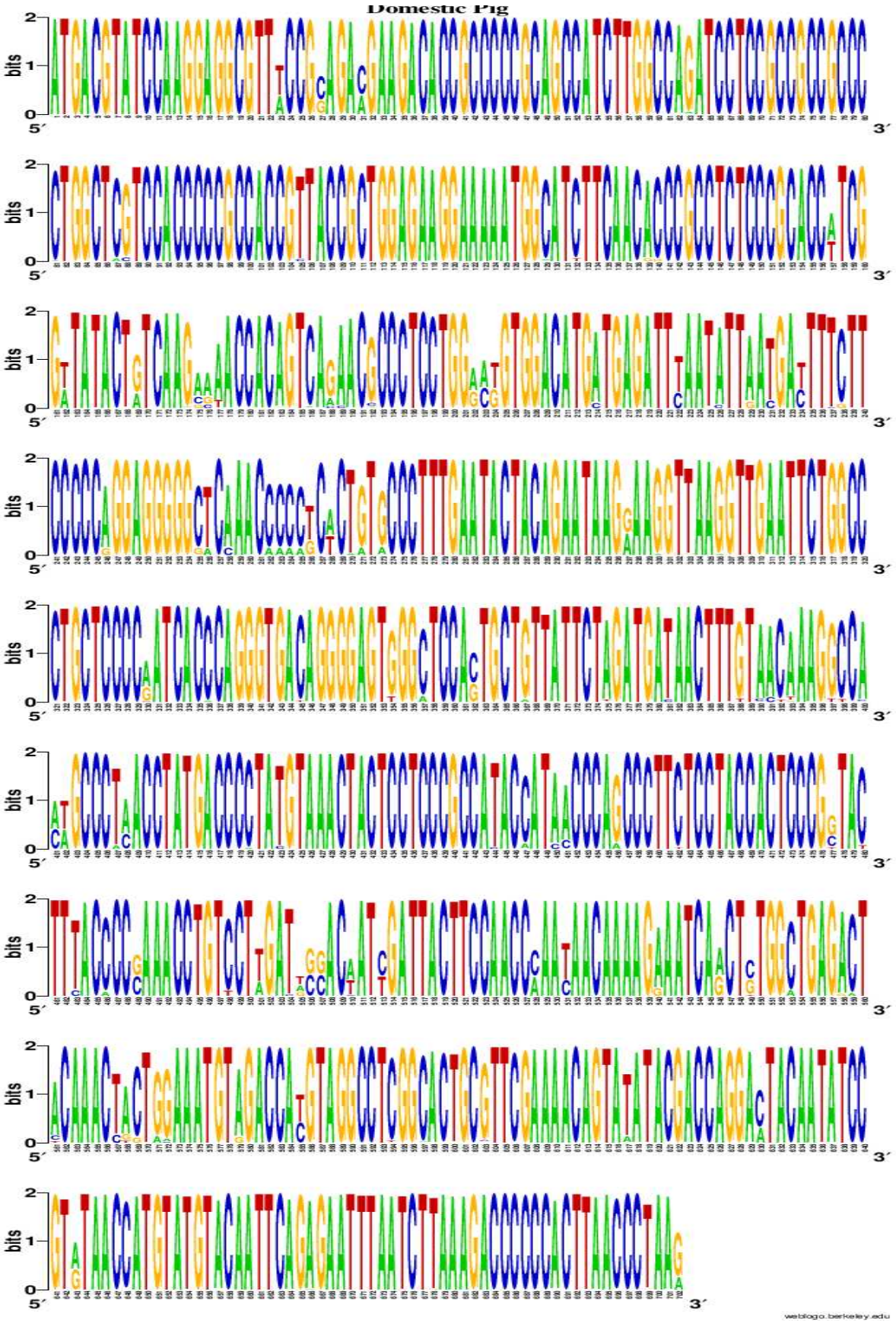

Figure 9. *Sequence Logo Representation of PCV2 Capsid Gene Variability in Domestic Pigs*

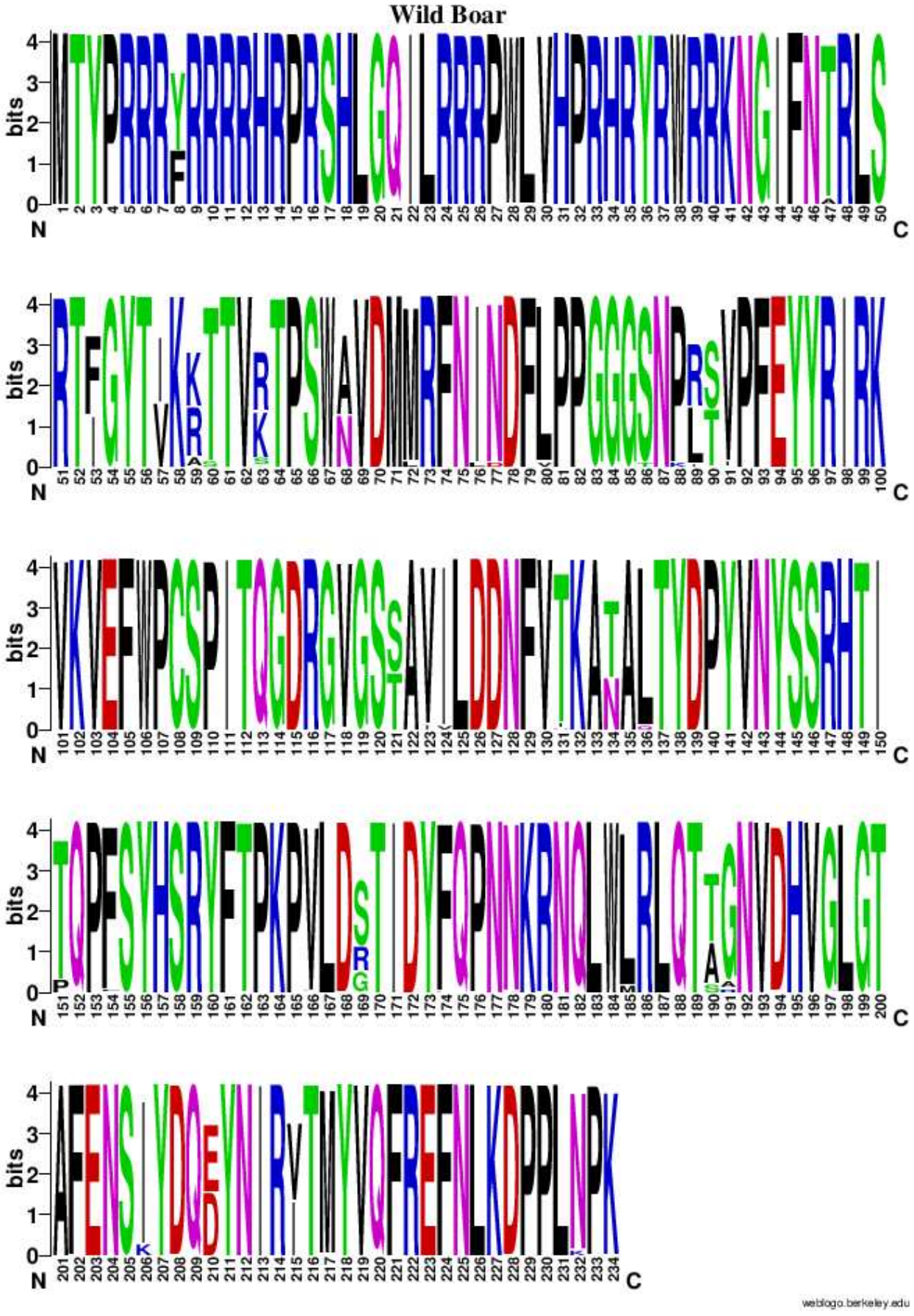

Figure 10. *Sequence Logo Representation of PCV2 Capsid Amino acid Variability in Wild Boar*

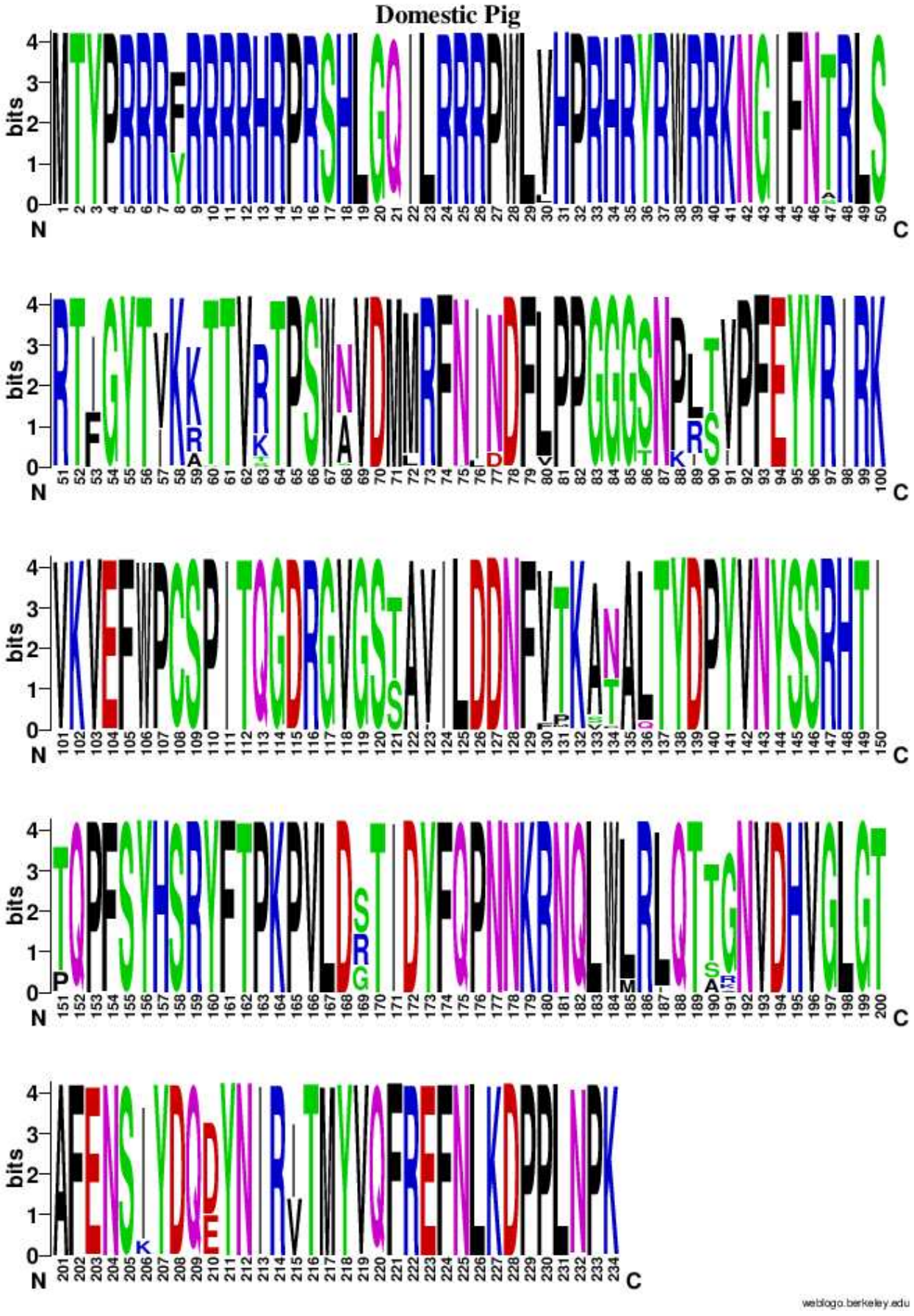

Figure 11. *Sequence Logo Representation of PCV2 Capsid Amino AcidVariability in Domestic pigs*

4.2. Phylogenetıc tree of pcv2 straıns

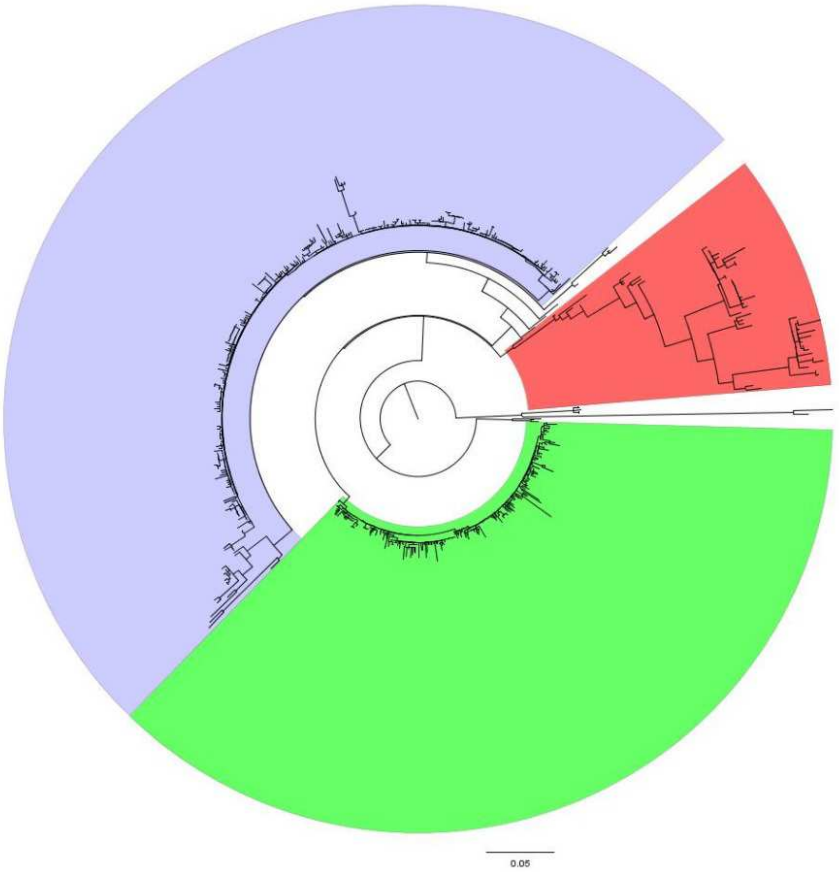

Figure 12. *Maximum likelihood phylogentic tree based on the ORF2 of strains included in the study. The three main genotypes, PCV2a, PCV2b and PVC2d have been highlighted in red, green and blue, respectively.*

The phylogentic analysis allowed to classify the vast majoroty of strains included in the study in the three main genotypes, PCV2a, PCV2b and PCV2d, mirroring the well-known epidemiological scenario (52) and thus proving the representativeness of our dataset (**Figure 12**).

Only few strains, appeared to belong to minor genotypes or are not classifiable within the three main categories. The evolutionary scale reported in the phylogenetic tree allows estimating the genetic distances between strains: shorter branches indicate greater genetic similarity, while longer ones signal more marked evolutionary divergence. The PCV2b and PCV2d genotypes, although featuring a higher number of strains, showed a lower internal variability, as indicated by the length of the branches, while the PCV2a genotype appears more heterogeneous and diversified. This can be explained by the estimated more ancient origin of PCV2a compared to the other genotypes (8,53). The analysis of the strain collection host shows that both populations are represented within

the three main genotypes. This distribution proves the absence of a genotype-host bias. Consequently, the representativenedss of strains belonging to the PCV2a, PCV2b, and PCV2d genotypes collected worldwide and in both considered populations allows to draw conclusions valid at a global level, highlighting the relevance of the study in the context of the evolutionary dynamics and genetic diversity of the virus.

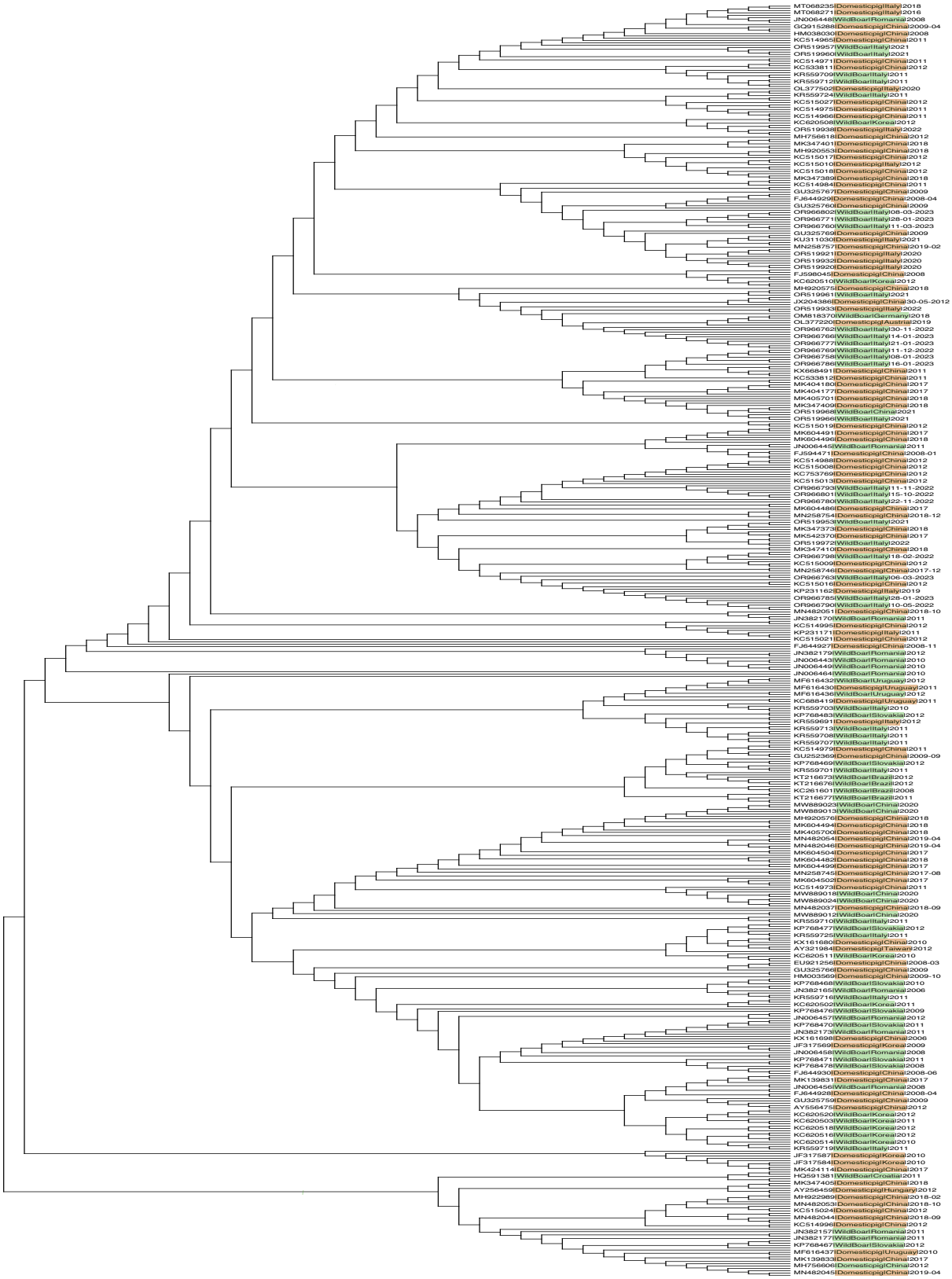

***Figure 13**. Maximum likelihhod phylogenitc tree based on the ORF2 sequences of PCV2. Tips corresponding to wild boars and domestic pigs were highlighted in green and orange, respectively.*

The structure of the phylogenetic tree shows the close relationships between the strains circualting in the two populations. More in detail, the structure of the phylogenetic tree shows the close relationships between the strains circualting in the two populations (**Figure 13**). At first, strain collected from domestic and wild populations were mixed, and wild boar sequences form small clades or even single branches dispersed among the domestic pigs collected strains. This confirms at international level what observed in previous studies based on single countries (35), the strain exchange among wild and domestic populations. Moreover, strains collected in different countries were interspersed in the phylogentic tree, highlighting the absence of a strong geographical clustering and thus the unconstrained viral circulation at worldwide level. The relationship among European wild boar collected strains suggests possible gene flow between countries. Two hypotheis could be involved, at first that geographical borders are permeable, and the virus can spread between wild populations in connected areas, modifying the genetic structure of the virus in Europe. Alternatively, trade of domesitc pig among countries may determine the geographical dispersal of PCV2 strains among intensively raised animals, which could then spill in the wild populaitons (54,55). Although non definitive conclusions can be raised, and likely a combiantion of both forces is involved, the latter phenomenon is probably dominant considering the limited homerange of wild boar population (56) and the more intense viral flow from domestic to wild boar population estimated through structured coalescent analysis (see following section).

However, smaller, local clades could be identified, as for the Italian sequences. In these Italian clusters, although with some exception, wild sequences tended to group separately, which could indicate a possible local evolution of PCV2 in different hosts. A possible hypothesis is that in Italy the virus follows more limited transmission paths in domestic and wild populations, probably due to the limited direct interaction between these two groups. The presence of numerous Italian samples collected over extended time periods also indicates a persistence of the virus in these populations, which could contribute to a continuous diversification of local variants (36). Similarly, it was noted for the Chinese sequences that a temporal trend was outlined, tracing the evolution of the virus over time. The older sequences from 2008-2010 are distinguished from the more recent ones from 2017-2020, probably due to selective pressure or epidemiological events that could have influenced the evolution of the virus, probably favoring the rise of new genotypes (57–59). It is assumed that natural selection or the application of control measures, such as vaccination, cause changes in the virus leading to more resistant or adaptable forms (33).

Overall, this could indicate the occurrence of a complex epidemiological pattern, where the introduction of strains originating from foreing countries through trade, contact among wild and domestic populations and circulation of different variants in these population potentially under heterogeneous evolutionary or selective processes, can contribute to shape the PCV2 scenario (30). Unfortunately, the avaialbility of a limited and not fully representative number of sequences can not be underemphasized and could hide links between domestic and wild populations; i.e. identical or similar strains could have been circulating in both populations, but no diagnosis and sequencing was done.

4.2 Analysis of PCV2 viral population size and migration dynamics between wild boars and domestic pigs

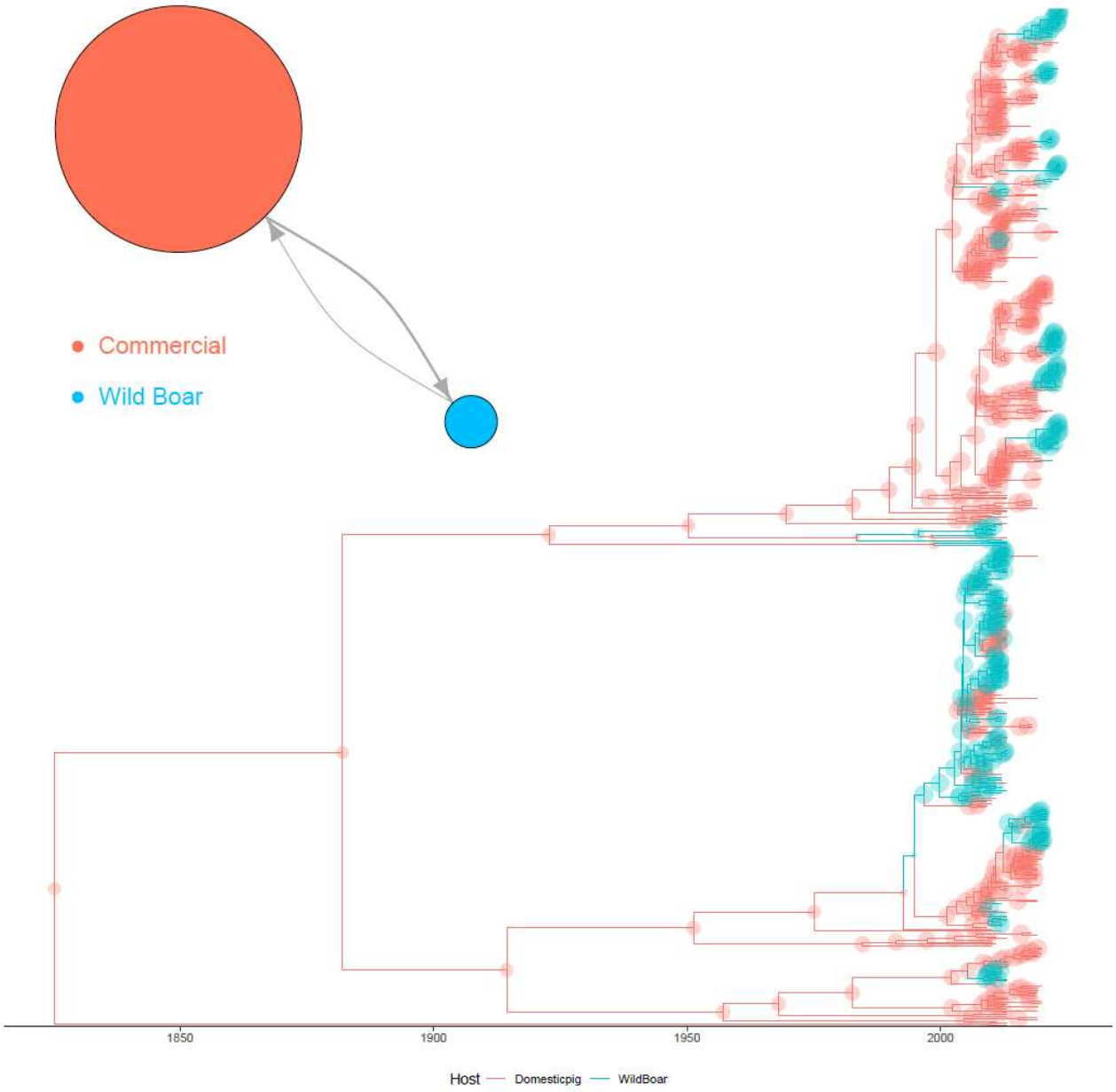

Figure 14. *Structured coalescent-based phylogenetic tree of the samples included in the present study. Branch colors, as from legend, mark the inferred animal category where the ancestral strain was circulating, while node size represents the posterior confidence of the inference. In the top right insert it is reported the network depicting the migration rate between animal categories. Arrows and circles size are proportional to the inferred migration rate and population size, respectively.*

The above-mentioned findings were further confirmed by the structured coalescent analysis (**Figure 14**). This analysis highlights that PCV2 circulates mainly in domestic pigs globally, in line with previous studies conducted in Italy (36). The size of the viral populations, represented by the circles in the network, confirms that domestic pigs host the largest viral populations, consistent with their

role as primary reservoir of the virus. Wild boars, on the contrary, host smaller viral populations, reinforcing the hypothesis that their role is mainly that of secondary hosts.

However, multiple events of introduction of the virus from domestic pigs to wild boars emerge, indicating a recurrent transmission between these two populations. A significant aspect that emerged from the migration network is the bidirectionality of viral flows. Although wild boars are predominantly "importers" of viral strains from domestic pigs, transmissions in the opposite direction have been observed, confirmed both by the rates reported in the network and by the presence of phylogenetic branches that directly connect wild boar-associated strains with those of domestic pigs. This phenomenon suggests that, although domestic pigs represent the main reservoir of PCV2, wild boars are not an epidemiological dead end but can act as a secondary source of transmission (60). Moreover, the detection of wild-only caldes, persisitig for extended time periods, demosntrates the capability of these populations to effetivly maintain PCV2 in field conditions and allow for its evolution in a differnt “environment”, whose consequences are hardly predictable.

These results provide important insights not only for the management of PCV2, but also for the understanding of the transmission dynamics of other pathogens with potential health and economic impact. In particular, the recent introduction of African swine fever (ASF) in Italy, currently confined mainly to wild boars, represents a significant risk for the pig sector. The bidirectionality of flows observed for PCV2 suggests that, should a similar epidemiological interaction occur for ASF, the introduction of the virus into domestic pigs could have disastrous consequences. We can state that wild boars, although mainly recipients of viral strains from domestic pigs, also represent a potential source. All this information, combined with the risk of pathogen transmission, underlines the importance of monitoring strategies and application of containment measures that were proved to limit interactions between the two populations and prevent the introduction of pathogens into domestic farming contexts.

5.4. Strenght of selectıve pressures

In addition to sequence alignment and analysis, we used the Datamonkey webserver to conduct several tests aiming at investigating the occurrence of selective pressures acting on the PCV2 capsid. The selected tests are based on the estimation of non-synonymous (dN) and synonymous (dS) substitution rates and are widely used in molecular evolution to evaluate evolutionary pressures acting on a protein-coding gene. These tests provide insights into whether a gene is evolving under

purifying selection, positive selection, or neutral evolution at the codon level. Genetic mutations are non-synonymous substitutions (dN) when changes in the DNA sequence alter the amino acid encoded by a codon and thus these substitutions may affect the structure and function of the resulting protein. On the other hand, synonymous substitutions (dS) do not alter the amino acid encoded by a codon due to the degeneracy of the genetic code. These are generally considered neutral because they do not impact protein function. dN/dS ratio (ω): The ratio of non-synonymous to synonymous substitution rates can be used to infer evolutionary pressures:

ω < 1: Purifying (negative) selection, where most non-synonymous changes are deleterious and are removed by natural selection.

ω = 1: Neutral evolution, where non-synonymous and synonymous substitutions occur at the same rate, implying no selection.

ω > 1: Diversifying (positive) selection, where non-synonymous changes are favored because they provide a selective advantage.

Statistical tests, mostly based on likelihood ratio tests or Bayesian inference, are used to compare different models and assess significant differences in the observed non-synonymous and synonymous substitution rates compared to what would be expected by chance.

5.4.1. Analysis with SLAC

The SLAC (Single-Likelihood Ancestor Counting) method was used to identify the presence of sites under pervasive diversifying selecion in wild boars and domestic animals, comparing the results to verify any differences. A ratio between the rate of synonymous and non-synonymous substitutions that differs from one (or a difference differing from zero), if shown to be significant by statistical testing, gives evidence of the presence of selective pressures. When the rate of non-synonymous substitutions is much higher than that of synonyms, it means that the protein "wants" to change; conversely, if the rate of synonyms is much higher, it indicates that the protein tends to remain unchanged

- If dN−dS>0dN - dS > 0dN−dS>0, the site tends to change.
- If dN−dS<0dN - dS < 0dN−dS<0, the site tends to remain unchanged.

The analyisis performed independently for the domestic and wild boar PCV2 sequences highlighted two sites in the domestic pig dataset only as statistically significant, i.e. sites 63 p.value=0.0077 and 191 p.value = 0.0288.

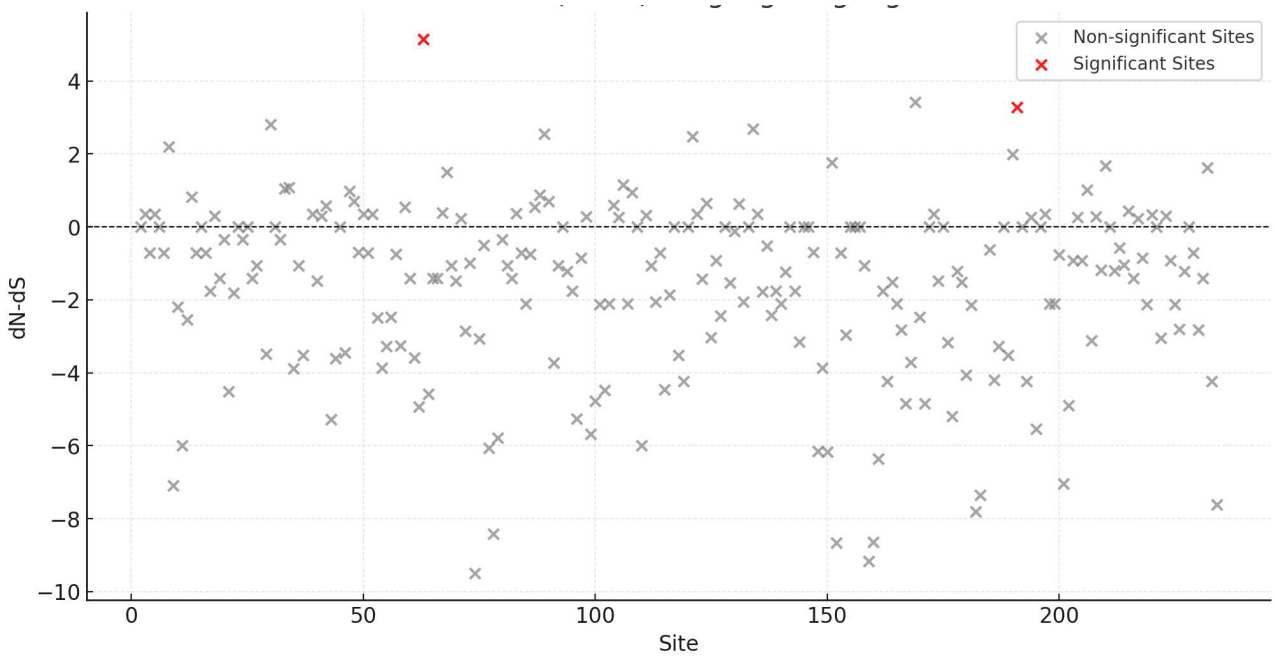

Figure 15. *dN-dS Values estimated usingSelective Pressures (SLAC) - Highlighting Significant Sites*

While a limited number of sites were found under pervasive positive selection, the evidence that they were detected only in domestic pig dataset suggests a stronger action of diversifying selection on this population, likely related to immune adaptation (**Figure 15**).

However, even if not statistically significant, several other sites showed evidence of positive selection in both datasets. The comparison of the dN-dS in the two populations shaded further light in this pattern. In **Figure 16** is reported the graph which compares the two groups (domestic vs wild animals) highlighting that in domestic pigs there is a higher freqency of mutations with positive dN −dS values. In wild boars, on the other hand, dN−dS values tend to be more negative, indicating a more intense purifying selection, probably aimed at preserving the functional stability of proteins.

The limited number of sites where statistical significance was achieved could be due to long history of PCV2 and to the emergence of different genotypes that underwent different evolutive patterns, at different time points and for limited periods. This variety could explain the presence of conflicting signals or the lack of significance in some positions. For example, one strain might show an evolutionary trend in one position, while another strain might not. Similarly, the same position in the Cap could have evolved for a limited time in one genotype only, and not in another, or even opposite selective strength could have acted on the same position depending on the environment where they were circulating of the features of co-evolving sites. This genetic diversity could "mask" or make some statistical signals less evident.

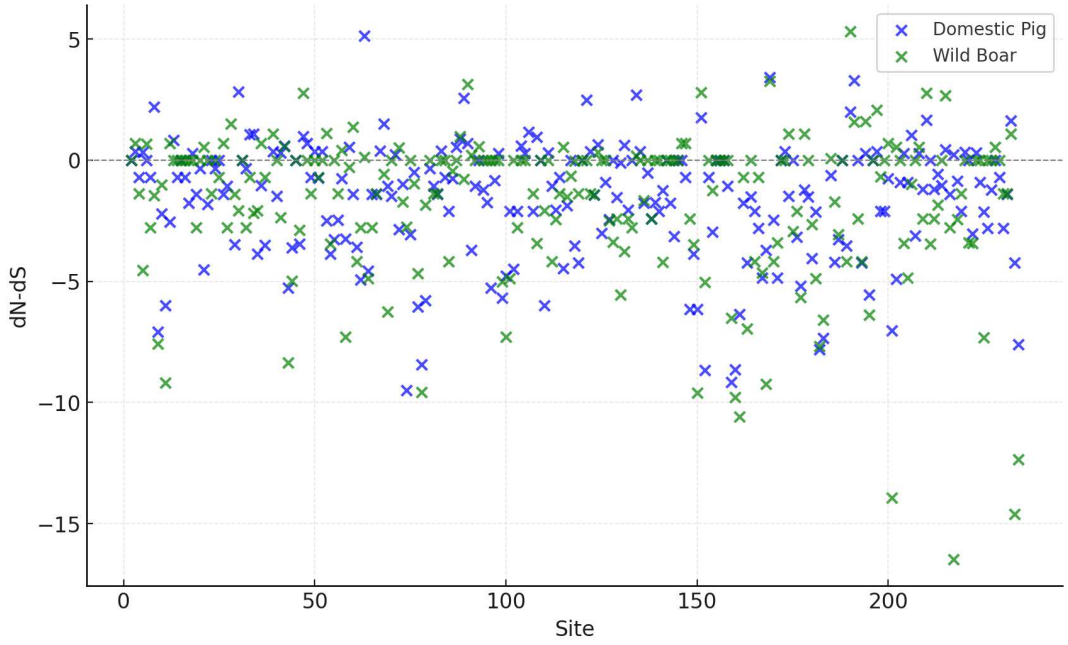

Figure 16. *dN-dS Values estimated using SLAC - Results obtained from the domestic pig ans wild boar datasets have been marked with different colors.*

5.4.2. Analysis with MEME

One of the main limitations of the previous approach was thus that it was developed to model pervasive diversifying selection, while often evolution occurs through selective burst. To account for this phenomenon, the MEME (Mixed Effects Model of Evolution) method was used to identify the presence of sites under episodic diversifying selection. MEME was applied to detect selective pressures independently on PCV2 straines sampled from wild boars and domestic animals, focusing on identifying specific sites where positive selection occurred in a subset of branches. The results were compared to evaluate potential differences in selective pressures between the two groups.

Nine capsid positions were detected under episodic diversifying selection at a p-value < 0.05) (**Table 1**). The higher number of detected sites compared to SLAC suggests that positive selection likely occurred in a subset of branches rather than being pervasive along the entire phylogeny.

Domestic Pigs		Wild boar	
Site	**p-value**	**Site**	**p-value**
59	0	**21**	0,0
63	0,01	**39**	0,0
68	0	**68**	0,0
88	0	**88**	0,0
130	0,01	**90**	0,01
134	0	**134**	0,0
137	0	**169**	0,0
169	0		
191	0,02		

Table 1*. Sites detected under statistically significant episodi diversifying selection in wild and domestic populations*

Accordingly, seven position were detected in the wild boar's dataset (**Table 1**). Interestingly, some sites are shared between the two sets of sequences, suggesting that these positions may be subject to common evolutionary pressures. One possible explanation is that these sites are the main target of the natural immune response and that are fundamental for the virus's interaction with the

immune system, an aspect shared by all hosts, regardless of species or population (29,61–63). This type of evolution could reflect the adaptation of the virus to evade or modulate overall immune responses, which are a common challenge during infection.

On the other hand, sites that are unique or specific to each group (e.g., sites present only in strains from domestic pigs or only from wild boars) could indicate selective pressures related to peculiarities of the immunity or genetic characteristics of different host populations. These sites could be the result of localized adaptations, where the virus evolves to address specific challenges related to the immune or ecological environment of a particular population. For example, wild boars, may have different evolutionary pressures than domestic pigs, which live in controlled environments, have high population density a turnover and whose immunity is largely shaperd by vaccinations based on limited number of strains (15,28,64–66).

This dualism between shared and specific sites could therefore provide important clues on the evolution of the virus, highlighting how adaptation is influenced by a combination of common and peculiar factors of the different hosts.

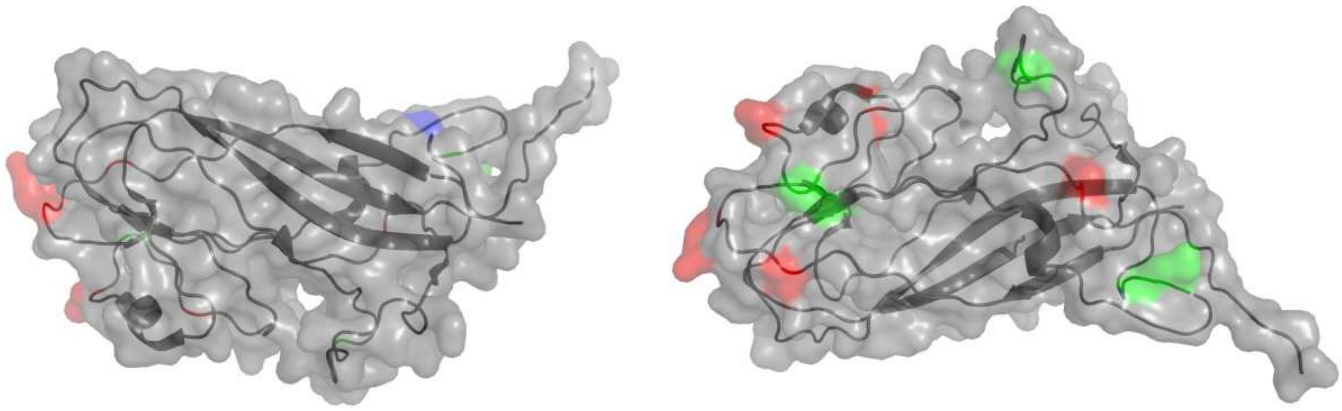

***Figure 17**. Sites detected under episodi diversifying selection with MEME in the domestic pig (red) and wild boar (blue) dataset. Shared sites are reportedin in gree. Both sides of the capsid are depicted*

Interestingly, most of the shared and domestic pig only sites under diversifying selection were located on the capsid surface, exposed to the action of the immune response (67–71), while several of the AA substitutions in wild boards were in inner sites or on other functional domains (**Figure 17**). Unfortunately, positions 21 and 39 of the protein sequence, identified as under diversifying selection in wild boars, were in portions of the protein that were not modeled during the

determination of the three-dimensional structure. This phenomenon occurs when experimental data, obtained for example by X-ray crystallography or cryoelectron microscopy, do not provide sufficient electron density to represent these regions in a definite way. Unmodeled regions, such as the one mentioned, are often associated with structural flexibility or inherent disorganization, implying that they may not take on a static and defined conformation. However, these characteristics do not exclude a significant functional importance.

Interestingly, the N-terminus of the Cap, rich in arginine (positions 1-41), has been described as a nuclear localization signal (NLS), necessary for its accumulation in the nucleus. This region could, therefore, play a key role in the functions of the virus, regulating crucial processes such as subcellular localization and interaction with host mechanisms.

In particular, the NLS affects the migration of the capsid and the DNA in the nucleus, where genome replication occurs thanks to host DNA-polymerases. Therefore, variations in this region may contribute to the replication and viral life cycle dynamics of the virus. If the changes and tendency to diversification observed in the wild populations reflects host specific adaptation, as previously suggested for other viruses (72), consisting in fine tuning to cope with the different environment, host density and contact, immune recognition etc, would require further investigations.

5.4.3. Contrast-FEL

Another approach used for the study is contrast-FEL, which allows to formally test whether the ovolution occurred with different patterns and strengths on branches of the tree leading to different hosts (i.e. wild and domestic animals). In practice, for this analysis, domestic pigs and wild boars were directly compared using a single dataset, loading the phylogenetic tree and identifying the specific branches that lead to the domestic pigs, evaluating them against the background represented by the wild boars.

Overall, several sites were detected under diversifying selection. Of those 7 sites (13, 21, 30, 68, 89, 106 and 133) were under a stronger diversifying selection in domestic pigs, while five (i.e. 39, 166, 169,190 and 197) in wild boars. In **Figure 18** is represented the significant evolutionary pressure difference between domestic pig and wild boar. Also the sites detected by contrast-FEL were especially located on the capsid surface (**Figure 19**).

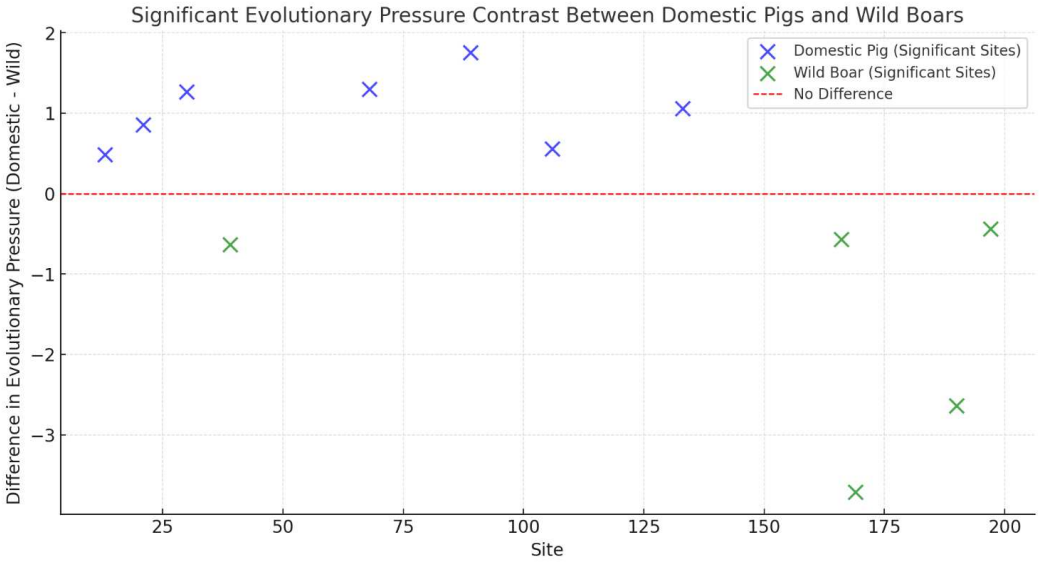

***Figure 18**: Amino acid positions where a significant difference in selective forces was proven between wild and domestic populations.*

Although with differences, all the considered methods agree in the thesis that domestic populations are under greater pressure.

While several hypotheses can be proposed, the extensive use of vaccination, posing a remarkable and less variable selective pressure is one of the most consistent. The high animal turnover might also decrease to role of natural immunity, increasing the weight of vaccine-induced one. An alternative, non conflicting hypothesis, might claim the higher population density and contact among domestic animals, favoring at the same time and higher infection frequency and thus population immunity, creating at the same time a high viral population size and high selective pressures, fundamental conditions for viral evolution to occur. However, while these studies performed at population level can provide fundamental hints of pattern and forces driving PCV2 evolution, a precise understanding of these phenomena is far from being achieved and further studies, including experimental ones, should be performed.

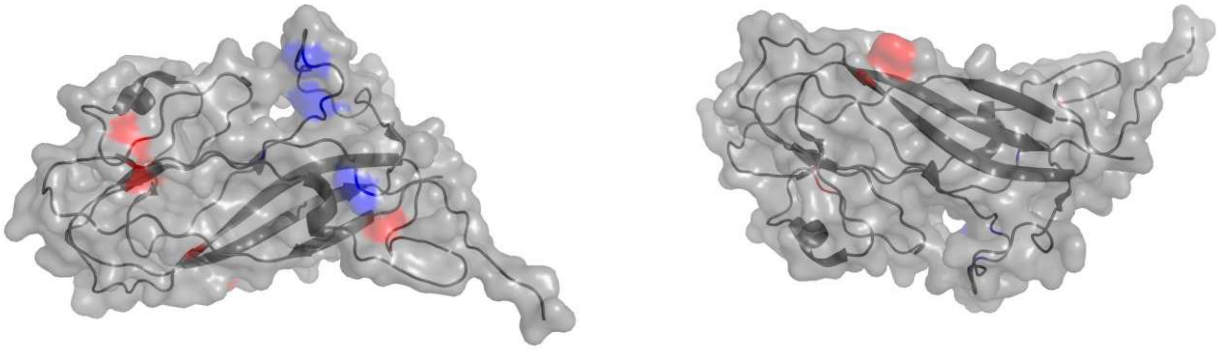

***Figura 19**. Sites detected under differential selection with contrast-FEL. Position under stronger selective pressures in the domestic pig and wild boar populations are reported in red and blue, respectively.*

6. CONCLUSION

The study of the PCV2 capsid gene provided insight into the evolutionary dynamics and genetic relationships between domestic pigs and wild boars, helping to delineate the main characteristics of genetic variability, selective pressures and virus-host interactions. First, sequence analysis identified a number of highly conserved regions indicative of essential functions related to viral stability and replication. The variations found in less conserved regions suggest specific adaptations, probably influenced by the selective pressures exerted by the host's immune responses.

Comparative analyses between domestic pigs and wild boar revealed significant differences in selection patterns. In domestic pigs, the higher frequency of mutations subject to positive selection, and their localization on the viral capsid surface, reflects selective pressure likely related to the interactions with the immune system, influenced by factors such as vaccination and intensive farming practices. In contrast, a predominance of purifying selection signals emerged in wild boars, highlighting the need to preserve the functional stability of proteins in a more natural ecological context.

From a phylogenetic point of view, the reconstructed tree showed a global distribution of the virus, with the PCV2a, PCV2b and PCV2d genotypes represented in both populations. The absence of strong geographical clustering suggested a free viral circulation at the international level, probably facilitated by the pig trade, while the presence of local clades, such as the Italian ones, indicated a possible independent evolution and specific adaptations linked to the local context. The complexity of such scenario makes challeng to disentangle the effect of selective pressures from more epidemiological patterns. However, the representiveness of the selected dataset should have limited the noise potentially caused by genotype-geographical distribution bias.

The bidirectionality of viral flows between domestic pigs and wild boars has also underlined the role of wild boars not only as a secondary reservoir but also as a source of transmission, with significant implications for epidemiological management. Because of the different evolutive pattern shown in the two poulations, and the unpredictability of its future directions, such contacts might represent a threat facilitating the emergence of new variants or genotypes with different biological features.

Overall, the results of this study provide important insights into the evolutionary dynamics of PCV2 and its interactions with different hosts. The differences observed between domestic pigs and wild

boars reflect not only distinct selective pressures, but also the complexity of the evolutionary processes that shape the genetic diversity of the virus. These results have relevant epidemiological implications, suggesting the need for monitoring and containment strategies aimed at limiting interactions between domestic and wild populations, thus preventing the introduction and spread of pathogens with potentially devastating health and economic impacts.

7. FUTURE PROSPECTIVE

Future studies should be aimed at deepening the knowledge of the mechanisms of pathogenesis of PCV2, with a specific focus on the dynamics of interaction between the virus and host immunity, as well as on the impact of vaccinations in the control and evolution of the virus. It is essential to further investigate the role of different genotypes, to understand their differences in transmissibility, ability to evade immune response and pathogenicity. In parallel, improving epidemiological monitoring strategies is crucial to understand spreading patterns and risk factors and thus limit the transmission of the virus between domestic pigs and wild boars, helping to prevent the introduction and spread of viral variants. Finally, the transmission and evolution model observed for PCV2 represents a useful paradigm for the study of other emerging pathogens, underlining the importance of an integrated and multidisciplinary approach in health and epidemiological management.

8. BIBLIOGRAFY

1. Kim SW, Gormley A, Jang KB, Duarte ME. — Invited Review — Current status of global pig production: an overview and research trends. Vol. 37, Animal Bioscience. Asian-Australasian Association of Animal Production Societies; 2024. p. 719–29.

2. The New York Times. https://www.nytimes.com/2023/02/08/business/china-pork-farms.html. [cited 2024 Oct 31]. The New York Times. Available from: The New York Times

3. VanderWaal K, Deen J. Global trends in infectious diseases of swine. Proc Natl Acad Sci U S A [Internet]. 2018 Nov 6 [cited 2022 Jan 12];115(45):11495–500. Available from: https://www.pnas.org/content/115/45/11495

4. Segalés J, Sibila M. Revisiting Porcine Circovirus Disease Diagnostic Criteria in the Current Porcine Circovirus 2 Epidemiological Context. Vol. 9, Veterinary Sciences. 2022.

5. Franzo G, Cortey M, Segalés J, Hughes J, Drigo M. Phylodynamic analysis of porcine circovirus type 2 reveals global waves of emerging genotypes and the circulation of recombinant forms. Mol Phylogenet Evol. 2016 Jul 1;100:269–80.

6. Kekarainen T, Segalés J. Porcine circovirus 2 immunology and viral evolution. Vol. 1, Porcine Health Management. BioMed Central Ltd.; 2015.

7. Shulman LM, Davidson I. The Annual Review of Virology is online at virology.annualreviews.org. Annu Rev Virol [Internet]. 2017;4:159–80. Available from: https://doi.org/10.1146/annurev-virology-

8. Segalés J, Kekarainen T, Cortey M. The natural history of porcine circovirus type 2: From an inoffensive virus to a devastating swine disease? Vet Microbiol. 2013;165(1–2):13–20.

9. Franzo G, Segalés J. Porcine circovirus 2 genotypes, immunity and vaccines: Multiple genotypes but one single serotype. Vol. 9, Pathogens. MDPI AG; 2020. p. 1–12.

10. Meng XJ. Porcine Circovirus Type 2 (PCV2): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci [Internet]. 2013 Jan 25 [cited 2019 Oct 22];1(1):43–64.

Available from: http://www.annualreviews.org/doi/10.1146/annurev-animal-031412-103720

11. Darwich L, Segalés J, Mateu E. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: An immune riddle. Vol. 149, Archives of Virology. 2004. p. 857–74.

12. Wang N, Zhan Y, Wang A, Zhang L, Khayat R, Yang Y. In silico analysis of surface structure variation of PCV2 capsid resulting from loop mutations of its capsid protein (Cap). Journal of General Virology. 2016 Dec 1;97(12):3331–44.

13. Franzo G, Tinello S, Grassi L, Tucciarone CM, Legnardi M, Cecchinato M, et al. Free to circulate: An update on the epidemiological dynamics of porcine circovirus 2 (PCV-2) in Italy reveals the role of local spreading, wild populations, and Foreign countries. Pathogens. 2020 Mar 1;9(3).

14. Afghah Z, Webb B, Meng XJ, Ramamoorthy S. Ten years of PCV2 vaccines and vaccination: Is eradication a possibility? Vet Microbiol [Internet]. 2017 Oct [cited 2017 May 15];206:21–8. Available from: http://dx.doi.org/10.1016/j.vetmic.2016.10.002

15. Franzo G, Segalés J. Porcine circovirus 2 genotypes, immunity and vaccines: Multiple genotypes but one single serotype. Pathogens [Internet]. 2020;9(12):1–12. Available from: https://www.scopus.com/inward/record.uri?eid=2-s2.0-85097884300&doi=10.3390%2Fpathogens9121049&partnerID=40&md5=0734e78726fa105c6cacf2ad04ea5af9

16. Varsani A, Harrach B, Roumagnac P, Benkő M, Breitbart M, Delwart E, et al. 2024 taxonomy update for the family Circoviridae. Arch Virol. 2024 Sep 1;169(9).

17. Varsani A, Harrach B, Roumagnac P, Benkő M, Breitbart M, Delwart E, et al. 2024 taxonomy update for the family Circoviridae. Arch Virol. 2024 Sep 1;169(9).

18. Finsterbusch T, Mankertz A. Porcine circoviruses-Small but powerful. Vol. 143, Virus Research. 2009. p. 177–83.

19. Franzo G, Faustini G, Legnardi M, Berto G, Dal Maso M, Genna V, et al. Wilder than intense: higher frequency, variability, and viral flows of porcine circovirus 3 in wild boars and rural farms compared to intensive ones in northern Italy. Front Microbiol. 2023;14.

20. Opriessnig T, Karuppannan AK, Castro AMMG, Xiao CT. Porcine circoviruses: current status, knowledge gaps and challenges. Vol. 286, Virus Research. Elsevier B.V.; 2020.

21. Khayat R, Wen K, Alimova A, Gavrilov B, Katz A, Galarza JM, et al. Structural characterization of the PCV2d virus-like particle at 3.3 Å resolution reveals differences to PCV2a and PCV2b capsids, a tetranucleotide, and an N-terminus near the icosahedral 3-fold axes. Virology. 2019 Nov 1;537:186–97.

22. Ssemadaali MA, Ilha M, Ramamoorthy S. Genetic diversity of porcine circovirus type 2 and implications for detection and control. Vol. 103, Research in Veterinary Science. Elsevier; 2015. p. 179–86.

23. Franzo G, Cortey M, Segalés J, Hughes J, Drigo M. Phylodynamic analysis of porcine circovirus type 2 reveals global waves of emerging genotypes and the circulation of recombinant forms. Mol Phylogenet Evol [Internet]. 2016;100:269–80. Available from: http://www.elsevier.com/inca/publications/store/6/2/2/9/2/1/index.htt

24. Franzo G, Segalés J. Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology. PLoS One [Internet]. 2018 Dec 1 [cited 2020 Aug 22];13(12):e0208585. Available from: /pmc/articles/PMC6283538/?report=abstract

25. Faustini G, Poletto F, Baston R, Tucciarone CM, Legnardi M, Dal Maso M, et al. D for dominant: porcine circovirus 2d (PCV-2d) prevalence over other genotypes in wild boars and higher viral flows from domestic pigs in Italy. Front Microbiol. 2024;15.

26. Kekarainen T, McCullough K, Fort M, Fossum C, Segalés J, Allan GM. Immune responses and vaccine-induced immunity against Porcine circovirus type 2. Vol. 136, Veterinary Immunology and Immunopathology. 2010. p. 185–93.

27. Kekarainen T, Segalés J. Porcine circovirus 2 immunology and viral evolution. Porcine Health Manag [Internet]. 2015;1(1):17. Available from: http://www.porcinehealthmanagement.com/content/1/1/17

28. Franzo G, Tucciarone CM, Cecchinato M, Drigo M. Porcine circovirus type 2 (PCV2) evolution before and after the vaccination introduction: A large scale epidemiological study. Sci Rep [Internet]. 2016 Dec 19 [cited 2020 Apr 7];6(May):39458. Available from: http://www.nature.com/articles/srep39458

29. Saha D, Lefebvre DJ, Ooms K, Huang L, Delputte PL, van Doorsselaere J, et al. Single amino acid mutations in the capsid switch the neutralization phenotype of porcine circovirus 2. Journal of General Virology [Internet]. 2012 Jul [cited 2020 Aug 22];93(PART 7):1548–55. Available from: https://pubmed.ncbi.nlm.nih.gov/22492916/

30. Franzo G, Tucciarone CM, Legnardi M, Drigo M, Segalés J. An updated phylogeography and population dynamics of porcine circovirus 2 genotypes: are they reaching an equilibrium? Front Microbiol [Internet]. 2024 Oct 29 [cited 2024 Nov 16];15. Available from: https://pubmed.ncbi.nlm.nih.gov/39534503/

31. Beach NM. Efficacy and future prospects of commercially available and experimental vaccines against porcine circovirus type 2 (PCV2). Virus Res [Internet]. 2012 Mar 1 [cited 2019 Aug 9];164(1–2):33–42. Available from: https://www.sciencedirect.com/science/article/pii/S0168170211003959?via%3Dihub

32. Gandon S, Mackinnon M, Nee S, Read A. Imperfect vaccination: Some epidemiological and evolutionary consequences. Proceedings of the Royal Society B: Biological Sciences. 2003;270(1520):1129–36.

33. Read AF, Mackinnon MJ. Pathogen evolution in a vaccinated world. Evolution in Health and Disease. 2010;2:139–52.

34. Lefeuvre P, Lett JM, Varsani A, Martin DP. Widely Conserved Recombination Patterns among Single-Stranded DNA Viruses. J Virol [Internet]. 2009 Mar;83(6):2697–707. Available from: http://jvi.asm.org/cgi/doi/10.1128/JVI.02152-08

35. Franzo G, Faustini G, Legnardi M, Berto G, Dal Maso M, Genna V, et al. Wilder than intense: higher frequency, variability, and viral flows of porcine circovirus 3 in wild boars and rural farms compared to intensive ones in northern Italy. Front Microbiol. 2023 Jul 31;14:1234393.

36. Faustini G, Poletto F, Baston R, Tucciarone CM, Legnardi M, Dal Maso M, et al. D for dominant: porcine circovirus 2d (PCV-2d) prevalence over other genotypes in wild boars and higher viral flows from domestic pigs in Italy. Front Microbiol. 2024 Jun 17;15:1412615.

37. Buapaichit K, Assavacheep P, Wattanaphansak S. PCV2 on Updated Genetics, Epidemiology and Control [Internet]. Vol. 46, Thai J Vet Med. Available from: https://cabidigitallibrary.org

38. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics [Internet]. 2009 May 1 [cited 2024 Nov 26];25(9):1189–91. Available from: https://dx.doi.org/10.1093/bioinformatics/btp033

39. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol [Internet]. 2021 Jul 1 [cited 2022 Aug 9];38(7):3022–7. Available from: https://pubmed.ncbi.nlm.nih.gov/33892491/

40. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res [Internet]. 2004 Jun [cited 2024 Nov 26];14(6):1188–90. Available from: https://pubmed.ncbi.nlm.nih.gov/15173120/

41. Vaughan TG, Kühnert D, Popinga A, Welch D, Drummond AJ. Efficient Bayesian inference under the structured coalescent. Bioinformatics. 2014;30(16):2272–9.

42. Bouckaert R, Vaughan TG, Barido-Sottani J, Duchêne S, Fourment M, Gavryushkina A, et al. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. PLoS Comput Biol [Internet]. 2019 [cited 2021 Mar 2];15(4):e1006650. Available from: https://journals.plos.org/ploscompbiol/article?id=10.1371/journal.pcbi.1006650

43. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. Penny D, editor. PLoS Biol [Internet]. 2006 Mar;4(5):699–710. Available from: http://dx.plos.org/10.1371/journal.pbio.0040088

44. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Susko E, editor. Syst Biol [Internet]. 2018 Sep 1 [cited 2019 Feb 7];67(5):901–4. Available from: https://academic.oup.com/sysbio/article/67/5/901/4989127

45. Weaver S, Shank SD, Spielman SJ, Li M, Muse S V., Kosakovsky Pond SL. Datamonkey 2.0: A Modern Web Application for Characterizing Selective and Other Evolutionary Processes. Mol Biol Evol [Internet]. 2018 Mar 1 [cited 2021 Nov 12];35(3):773–7. Available from: https://academic.oup.com/mbe/article/35/3/773/4782511

46. Kosakovsky Pond SL, Frost SDW. Not so different after all: A comparison of methods for detecting amino acid sites under selection. Mol Biol Evol [Internet]. 2005;22(5):1208–22. Available from: http://dx.doi.org/10.1093/molbev/msi105

47. Murrell B, Wertheim JO, Moola S, Weighill T, Scheffler K, Kosakovsky Pond SL. Detecting individual sites subject to episodic diversifying selection. Malik HS, editor. PLoS Genet [Internet]. 2012 Jul [cited 2020 Apr 7];8(7):e1002764. Available from: http://dx.plos.org/10.1371/journal.pgen.1002764

48. Kosakovsky Pond SL, Wisotsky SR, Escalante A, Magalis BR, Weaver S. Contrast-FEL - A Test for Differences in Selective Pressures at Individual Sites among Clades and Sets of Branches. Mol Biol Evol. 2021;38(3):1184–98.

49. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The Protein Data Bank [Internet]. Vol. 28, Nucleic Acids Research. 2000. Available from: http://www.rcsb.org/pdb/status.html

50. Janson G, Paiardini A. PyMod 3: a complete suite for structural bioinformatics in PyMOL. Bioinformatics [Internet]. 2021 Jun 16 [cited 2024 Nov 26];37(10):1471–2. Available from: https://dx.doi.org/10.1093/bioinformatics/btaa849

51. Franzo G, Legnardi M, Tucciarone CM, Drigo M, Martini M, Cecchinato M. Evolution of infectious bronchitis virus in the field after homologous vaccination introduction. Vet Res [Internet]. 2019;50(1). Available from: https://www.scopus.com/inward/record.uri?eid=2-

s2.0-85074742231&doi=10.1186%2Fs13567-019-0713-4&partnerID=40&md5=87428710d047f13780803438d2383bfc

52. Franzo G, Segalés J. Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology. PLoS One [Internet]. 2018;13(12):e0208585. Available from: https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0208585&type=printable

53. Firth C, Charleston MA, Duffy S, Shapiro B, Holmes EC. Insights into the Evolutionary History of an Emerging Livestock Pathogen: Porcine Circovirus 2. J Virol [Internet]. 2009;83(24):12813–21. Available from: http://jvi.asm.org/cgi/doi/10.1128/JVI.01719-09

54. Cartn-Rojas A. Transboundary Animal Diseases and International Trade. International Trade from Economic and Policy Perspective [Internet]. 2012;(OCTOBER 2012):143–66. Available from: http://www.intechopen.com/books/international-trade-from-economic-and-policy-perspective/transboundary-animal-diseases-and-international-trade

55. Franzo G, Tucciarone CM, Dotto G, Gigli A, Ceglie L, Drigo M. International trades, local spread and viral evolution: The case of porcine circovirus type 2 (PCV2) strains heterogeneity in Italy. Infection, Genetics and Evolution [Internet]. 2015 Jun;32:409–15. Available from: https://linkinghub.elsevier.com/retrieve/pii/S156713481500129X

56. Miettinen E, Melin M, Holmala K, Meller A, Väänänen VM, Huitu O, et al. Home ranges and movement patterns of wild boars (Sus scrofa) at the northern edge of the species' distribution range. Mamm Res [Internet]. 2023 Oct 1 [cited 2024 Nov 26];68(4):611–23. Available from: https://link.springer.com/article/10.1007/s13364-023-00710-5

57. Zhai SL, Chen SN, Xu ZH, Tang MH, Wang FG, Li XJ, et al. Porcine circovirus type 2 in China: An update on and insights to its prevalence and control. Virol J. 2014;11(1):88.

58. Xu T, Zhang YH, Tian RB, Hou CY, Li XS, Zheng LL, et al. Prevalence and genetic analysis of porcine circovirus type 2 (PCV2) and type 3 (PCV3) between 2018 and 2020 in central China. Infection, Genetics and Evolution [Internet]. 2021;94(April):105016. Available from: https://doi.org/10.1016/j.meegid.2021.105016

59. Yao J, Qin Y, Zeng Y, Ouyang K, Chen Y, Huang W, et al. Genetic analysis of porcine circovirus type 2 (PCV2) strains between 2002 and 2016 reveals PCV2 mutant predominating in porcine population in Guangxi, China. BMC Vet Res [Internet]. 2019 Apr 25 [cited 2021 Jul 27];15(1). Available from: https://pubmed.ncbi.nlm.nih.gov/31023307/

60. Fanelli A, Pellegrini F, Camero M, Catella C, Buonavoglia D, Fusco G, et al. Genetic Diversity of Porcine Circovirus Types 2 and 3 in Wild Boar in Italy. Animals [Internet]. 2022 Apr 1 [cited 2024 Nov 26];12(8):953. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC9031215/

61. Lekcharoensuk P, Morozov I, Paul PS, Thangthumniyom N, Wajjawalku W, Meng XJ. Epitope mapping of the major capsid protein of type 2 porcine circovirus (PCV2) by using chimeric PCV1 and PCV2. J Virol [Internet]. 2004 Aug 1 [cited 2020 Oct 4];78(15):8135–45. Available from: http://jvi.asm.org/

62. Ssemadaali MA, Ilha M, Ramamoorthy S. Genetic diversity of porcine circovirus type 2 and implications for detection and control. Res Vet Sci. 2015 Dec 1;103:179–86.

63. Saha D, Huang L, Bussalleu E, Lefebvre DJ, Fort M, Van Doorsselaere J, et al. Antigenic subtyping and epitopes' competition analysis of porcine circovirus type 2 using monoclonal antibodies. Vet Microbiol. 2012 May 25;157(1–2):13–22.

64. Reiner G, Hofmeister R, Willems H. Genetic variability of porcine circovirus 2 (PCV2) field isolates from vaccinated and non-vaccinated pig herds in Germany. Vet Microbiol. 2015 Oct 22;180(1–2):41–8.

65. Shen HG, Halbur PG, Opriessnig T. Prevalence and phylogenetic analysis of the current porcine circovirus 2 genotypes after implementation of widespread vaccination programmes in the USA. Journal of General Virology [Internet]. 2012 Jun 1 [cited 2020 Oct 1];93(6):1345–55. Available from: https://www.microbiologyresearch.org/content/journal/jgv/10.1099/vir.0.039552-0

66. Kekarainen T, McCullough K, Fort M, Fossum C, Segalés J, Allan GM. Immune responses and vaccine-induced immunity against Porcine circovirus type 2. Vol. 136, Veterinary Immunology and Immunopathology. 2010. p. 185–93.

67. Huang LP, Lu YH, Wei YW, Guo LJ, Liu CM. Identification of one critical amino acid that determines a conformational neutralizing epitope in the capsid protein of porcine circovirus type 2. BMC Microbiol [Internet]. 2011 Aug 22 [cited 2020 Oct 4];11(1):188. Available from: https://link.springer.com/articles/10.1186/1471-2180-11-188

68. Ge M, Yan A, Luo W, Hu YF, Li RC, Jiang DL, et al. Epitope screening of the PCV2 Cap protein by use of a random peptide-displayed library and polyclonal antibody. Yan A, Luo W, Hu YF, Li RC, Jiang DL, Yu XL, et al., editors. Virus Res [Internet]. 2013 Oct 1 [cited 2020 Oct 4];177(1):103–7. Available from: http://gateway.webofknowledge.com/gateway/Gateway.cgi?GWVersion=2&SrcApp=METALIBSearch&SrcAuth=EXLIBRIS&DestApp=WOS&DestLinkType=FullRecord&UT=WOS:000324974100012

69. Segalés J. Best practice and future challenges for vaccination against porcine circovirus type 2. Expert Rev Vaccines [Internet]. 2015 Mar 4 [cited 2016 Nov 1];14(3):473–87. Available from: http://www.tandfonline.com/action/journalInformation?journalCode=ierv20%5Cnhttp://dx.doi.org/10.1586/14760584.2015.983084

70. Fort M, Animals S, Aut U, Mateu E, Segal J. Characterization of immune responses to porcine circovirus type 2 (PCV2) infection and vaccination in pigs. PhD Thesis. 2009;2.

71. Patterson EI. Homology Modelling and Structural Comparisons of Capsid-Associated Proteins from Circoviruses Reveal Important Virus-Specific Surface Antigens. Crystal Structure Theory and Applications [Internet]. 2012;01(02):9–16. Available from: http://dx.doi.org/10.4236/csta.2012.12002

72. Komorizono R, Sassa Y, Horie M, Makino A, Tomonaga K. Evolutionary Selection of the Nuclear Localization Signal in the Viral Nucleoprotein Leads to Host Adaptation of the Genus Orthobornavirus. Viruses [Internet]. 2020 Nov 1 [cited 2024 Nov 26];12(11):1291. Available from: https://pmc.ncbi.nlm.nih.gov/articles/PMC7698282/

RINGRAZIAMENTI

Concludo questo percorso accademico in un momento particolarmente doloroso della mia vita. Continuare a lottare ogni giorno per ciò in cui credo e sogno è qualcosa che ho imparato da te, mamma. Anche se non ci sei più, ti rivedo in ogni cosa che vivo. Un sentito ringraziamento va alla mia famiglia e a Lorenzo, che mi sono stati accanto, ai miei amici, alla mia squadra di volley e a tutte le persone che mi hanno sostenuto in questo momento di lutto. Ringrazio tanto il mio relatore Prof. Franzo che è stato paziente e mi ha seguito davvero con attenzione.

Mamma, spero di poterti riabbracciare presto e che tu possa essere al mio fianco anche in questo traguardo, come lo sei sempre stata.