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Atypical porcine pestivirus—A widespread virus in the Swedish wild boar population

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Abstract

The recently identified causative agent of congenital tremor in domestic piglets, atypical porcine pestivirus (APPV), was detected in serum from Swedish wild boar. A previous study from Sweden described APPV in domestic piglets suffering from congenital tremor, but the APPV situation in the wild boar population was unknown. In this study, 595 serum samples from wild boar originating from 13 counties in the south and central parts of Sweden, collected between 2000 and 2018, were analysed for the presence of the APPV-genome and for antibodies against the APPV-glycoprotein E^{rns}. The results revealed that APPV is highly abundant in the Swedish wild boar population; 12% (73/595) were APPV-genome positive in serum and 72% (433/595) of the tested wild boars displayed APPV-specific antibodies. The present study also shows that APPV has been present in the Swedish wild boar population since at least the year 2000. The viral sequences obtained from the wild boars were highly similar to those obtained from Swedish domestic pigs positive for APPV and suffering from congenital tremor, suggesting a viral exchange between wild boars and domestic pigs. The high proportion of viraemic and seropositive wild boar is indicative of wild boar being an important reservoir for APPV.

KEYWORDS
APPV, atypical porcine pestivirus, genome detection, reservoir host, serology, wild boar

1 | INTRODUCTION

Atypical porcine pestivirus (APPV) was discovered in healthy pigs in the United States by high-throughput sequencing in 2014 (Hause et al., 2015). It is a ssRNA+ virus of the family Flaviviridae. The APPV is classified as the species *pestivirus K* in the genus *Pestivirus* (Hause et al., 2015; Smith et al., 2017). The virus is genetically diverse and the genome varies both within and between countries (Postel, Meyer, Cagatay,

et al., 2017; Sutton et al., 2019). It possesses three envelope proteins; E^{rns}, E1 and E2 (Hause et al., 2015). The protein E^{rns} induces a humoral immune response following an infection and has been used in previous studies to diagnose APPV in wild boars (Cagatay et al., 2018, 2019).

Shortly after its discovery in 2015, the presence of APPV was correlated with congenital tremor in newborn piglets (Arruda et al., 2016, Postel et al., 2016). Although experimental infection with a virus isolate is pending and the Koch's postulates have not been fulfilled yet,

experimental inoculation of APPV genome positive sample material can induce congenital tremor in piglets (Arruda et al., 2016; de Groof et al., 2016). Congenital tremor or *myoclonia congenita* is a congenital disease occurring in piglets characterized by tremor and ataxia (Stenberg et al., 2020b). Moreover, several studies have indicated that APPV might induce splay leg in addition to congenital tremor in newborn piglets (Arruda et al., 2016; de Groof et al., 2016; Stenberg et al., 2020b). A recent study revealed that APPV is also frequently found in Swedish piglets with signs of congenital tremor type A-II (Stenberg et al., 2020a).

Since its first detection in the United States of America, APPV has been detected in domestic pigs in Canada and South America (Dessureault et al., 2018; Hause et al., 2015; Possatti, De Oliveira, et al., 2018), Europe (Postel, Meyer, Cagatay, et al., 2017; Schwarz et al., 2017; Stenberg et al., 2020a), and Asia (Dessureault et al., 2018; Hause et al., 2015; Possatti, Headley, et al., 2018; Postel, Meyer, Cagatay, et al., 2017; Schwarz et al., 2017; Shen et al., 2018; Stenberg et al., 2020b; Yuan et al., 2017). Despite this global spread of APPV, the prevalence varies between geographic areas. In a study including 1460 serum samples from healthy domestic pigs from Germany, Italy, Serbia, Great Britain, Switzerland, China, and Taiwan, the APPV-genome prevalence was 8.9% in total, in Europe varying from 2.3% in Great Britain to 17.5% in Italy (Postel, Meyer, Cagatay, et al., 2017). In the same study, the seroprevalence of APPV-specific antibodies was 60%; 27% (394/1460) of the pigs displayed high antibody levels whereas 33% (486/1460) had intermediate antibody levels. A study on the seroprevalence of APPV-specific antibodies on German pig farms revealed that approximately 37% of the 62 farms tested in 2018 were antibody-positive (Michelitsch et al., 2019).

There is a very small body of literature that is concerned with wild boar and APPV, and no studies have been conducted in the Nordic countries. A high APPV seroprevalence has been demonstrated in wild boars in Germany (52%, 237/456) and Serbia (67%, 10/15) (Cagatay et al., 2019). Interestingly, studies on the prevalence of APPV-genome in wild boar present a large discrepancy in the proportion of viraemic animals in different countries. A low occurrence of APPV-PCR positive wild boars was found in South Korea (18/2297), Spain (1/437) and Italy (3/430), but a higher proportion of APPV-PCR positive wild boars was detected in Germany (87/465) (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Sozzi et al., 2019). These studies reveal that the prevalence of APPV differs between geographic regions and that the wild boar population in some areas might play an important role as a reservoir for APPV. Overall, these studies highlight the need for further research.

In Sweden, the wild boar population has increased substantially in the last 40 years from about 100 in 1980 to an estimate of 300 000 individuals in 2019 (Naturvårdsverket, 2020). In the 1970s several wild boars escaped or were released from enclosures in the south and central parts of Sweden. Escapees quickly established a permanent population and have since spread predominantly in the southern parts of Sweden. Now, due to global warming, the wild boars are also expanding north along the Swedish east coast, though in the northern counties the wild boar population is still sparse (Naturvårdsverket, 2020).

Although the Swedish wild boar is free-ranging, they cluster in the central and southern parts of Sweden, especially in the counties of Skåne and Södermanland, the same counties where wild boars first became established (Naturvårdsverket, 2020).

Wild boar is an important reservoir for pathogens that may infect domestic pigs, as well as a reservoir for pathogens of zoonotic potential. Therefore, the Swedish National Veterinary Institute performs yearly surveillance of specific pathogens in wild boars. At present, Sweden has a disease-free status for several epizootic pathogens, for example, classical and African swine fever virus, porcine reproductive and respiratory syndrome virus (PRRSV), and Pseudorabies virus (National Veterinary Institute, 2020). But a recent study shows that other viruses such as porcine parvovirus and porcine circovirus type 2 are highly abundant in Swedish wild boars with a seroprevalence of 78% and 99%, respectively (Malmsten et al., 2018). Despite the increasing wild boar population and the wild boars' importance as a reservoir for significant pathogens in other countries, the awareness of viral infections in the Swedish wild boar population is mainly limited to those included in the national surveillance programs.

This is the first study on APPV in the wild boar population in Sweden and any of the Nordic countries. The primary aim was therefore to determine whether APPV is present in the Swedish wild boar population by testing serum samples for the presence of APPV genome and APPV-specific antibodies, respectively. Any detected APPV genomes were then used to evaluate the genetic relatedness of APPV in Swedish wild boar and domestic pigs.
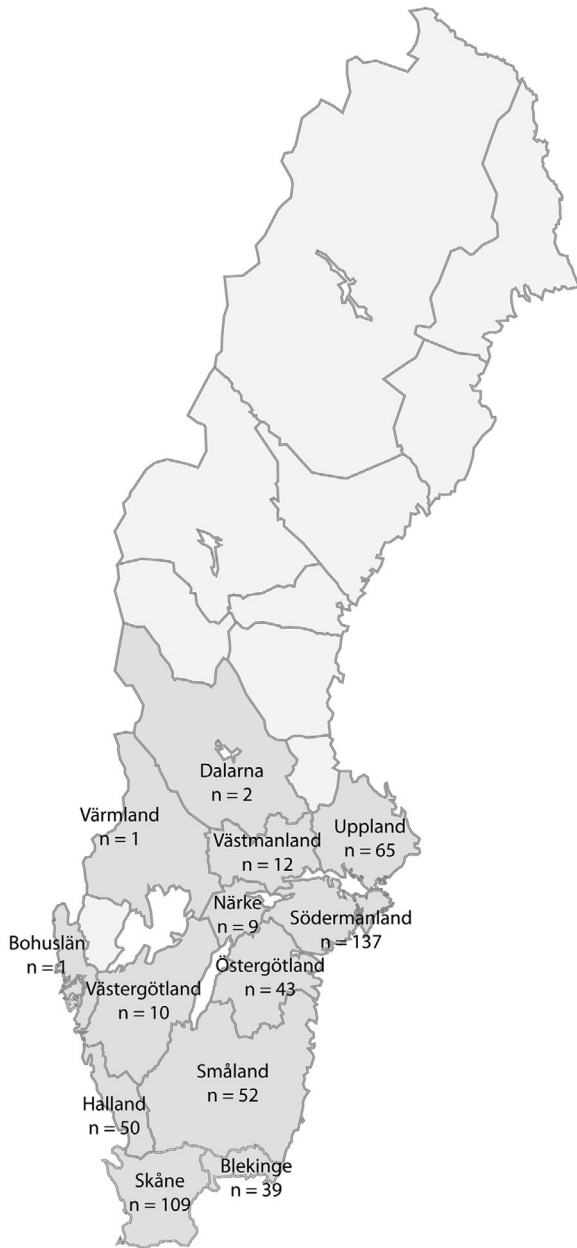
2 | MATERIALS AND METHODS

2.1 | Serum samples

Serum samples from 595 Swedish wild boars were collected between 2000 and 2018 and tested for APPV-specific antibodies against the APPV envelope glycoprotein E^{rns} and for the presence of the APPV-genome. All sampled wild boars originated from the central and southern parts of Sweden (Figure 1).

The majority of the samples (*n* = 464) were collected by hunters from wild boars after culling using sterile blood sampling tubes. The blood was then sent to the National Veterinary Institute, Uppsala, Sweden for separation of serum and storage at −80°C in the bio-bank. These serum samples were collected for the wild boar surveillance program, run by National Veterinary Institute and financed by the Swedish Board of Agricultures. The serum samples from 2000 (*n* = 19) lacked information on sampling location, and samples from 2000 to 2001 (*n* = 13) lacked information both on the sampling date and sampling location.

An additional 131 serum samples from female wild boars were collected in the years 2013–2014 by Malmsten et al. (2017). Here, blood was collected from the jugular vein or the thoracic cavity using sterile blood sampling tubes. The blood was clarified by centrifugation and the serum was separated and stored in a −20°C freezer. The reproductive status (gilt or sow) of 117 of the 131 female wild boars was recorded (Table 1).

FIGURE 1 A map of Sweden highlighting the counties where wild boars were sampled. 'N' represents sample size for the specific county. The counties with the densest wild boar populations, Skåne and Södermanland, are also the counties where the highest number of wild boars have been sampled

TABLE 1 The number of samples collected each year[a] and, if known, whether the sampled wild boar was a gilt or a sow

Year	Number of samples	Unknown gender (n)	Gilts (n)	Sows (n)
2000	19	19	–	–
2000/2001	13	13	–	–
2001	23	23	–	–
2002	43	43	–	–
2005	100	100	–	–
2009	100	100	–	–
2013	116	14	41	61
2014	15	–	10	5
2017	84	84	–	–
2018	82	82	–	–
Total	595	472	51	66

[a]The total number of samples is 595, of them, 472 were of unknown gender, 51 were recorded as gilts and, 66 were recorded as sows.

ing 0.05% Tween-20 at 37°C for 1 h. Specific binding of antibodies was detected by peroxidase-labelled rabbit anti porcine IgG (A5670, Sigma-Aldrich, MO, USA, diluted 1:35,000) and 3,3′,5,5t′Tetramethyl-benzidine (TMB, Sigma-Aldrich, MO, USA) according to the manufacturer's protocol. Porcine serum, confirmed positive for APPV E^{rns}-specific antibodies by Western blot, was used as positive control.

Initially, 88 randomly selected serum samples from the years 2000 ($n = 9$), 2001 ($n = 5$), 2002 ($n = 4$), 2005 ($n = 18$), 2009 ($n = 18$), 2017 ($n = 18$), and 2018 ($n = 16$) were tested in duplicates to assess the reliability of the assay. After establishing that the ELISA provided consistent results for the duplicates, the remaining serum samples were run as singles. To ensure a reliable inter-assay comparability, the serological values are presented as 'S/P-values' (sample/positive control). To facilitate the between-study comparability, the serological values were classified into low (S/P ≤ 0.5), intermediate (0.5 < S/P < 1.0), or high reactivity (S/P ≥ 1.0) as described previously (Cagatay et al., 2018, 2019; Grahofer et al., 2020; Postel, Meyer, Petrov, et al. , 2017). Consistent with the other studies using the same ELISA, the cut-off threshold for a positive serum sample was set to S/P > 0.5, consequently wild boars with a low serum reactivity (S/P ≤ 0.5) were regarded as serologically negative.

2.3 | RNA purification and real-time RT-PCR

For the detection of APPV genome in the serum samples, the 595 samples were first tested in pools of five containing 40 μl of each serum sample. To ensure the efficacy of the RNA isolation and the RT-PCR, in vitro-transcribed EGFP-RNA was added to each serum pool as internal control (Hoffmann et al., 2005). RNA extraction was performed according to an accredited protocol of the EU and OIE Reference Laboratory for Classical Swine Fever using the KingFisher™ Duo Prime Purification System (ThermoFisher Scientific, Waltham, USA) and the IndiMag® Pathogen Kit (Indical Bioscience, Leipzig, Germany) as

2.2 | Indirect APPV-specific ELISA

For antibody detection, an indirect APPV-specific enzyme-linked immunosorbent assay (ELISA) based on the glycoprotein E^{rns} (Postel, Meyer, Petrov, et al. , 2017) was used. Briefly, the APPV E^{rns} antigen was expressed in *Leishmania tarentolae*, purified by fast protein liquid chromatography (FPLC) and coated onto ELISA plates. Blocking was performed with PBS containing 0.05% Tween-20 and 4% skim milk powder at room temperature for 2 h. The E^{rns} ELISA assay was performed by incubating the serum samples diluted 1:25 in PBS contain-

TABLE 2 Primers used from detection of the APPV genome

Primer name	Sequence (5′ → 3′)	Region	Use	Reference
APPV-7001fw	GTGTCCAATTTTTGGGGCGTGTC	NS4B	Real-time RT-PCR	This study
APPV-7125rev	GCRACYACCACTGCTGATTCCAT	NS4B	Real-time RT-PCR	This study
APPV_5030-fw	CCCAGGCAATACCTCACAAC	NS3	Conventional RT-PCR and sequencing	(Cagatay et al., 2018)
APPV_5835-rev	TTCCTCTGGCCCTGTTCTTC	NS3	Conventional RT-PCR and sequencing	(Cagatay et al., 2018)

recommended by the manufacturers. Purified RNA pools were directly submitted to RT-PCR or stored at −80°C.

Given the growing number of available APPV sequences, a multiple sequence alignment was performed to identify a conserved region suitable for detection of genetically distinct APPV variants by real-time PCR. Using representative sequences belonging to APPV clade I that comprises all European sequences known so far (Folgueiras-González et al., 2020), a highly conserved region in the NS4B encoding sequence was identified and used to design the primer pair, APPV-7001fw / APPV-7125rev (Table 2). Validation of the new primer pair using serial dilutions of genomic RNA obtained from a cell culture infected with the German APPV isolate L277 (GenBank MF157291) as well as diagnostic samples sent to the Institute for Virology in Hannover demonstrated a sensitivity comparable to the previously used primer pair APPV-5587fw / APPV-5703rev (Cagatay et al., 2018). Furthermore, the primer pair showed less unspecific amplifications, making it particularly suitable for testing of wild boar samples. Different annealing temperatures were tested to optimize PCR conditions.

For the detection of APPV-genomes, a one-step SYBR-Green real-time RT-PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Feldkirchen, Germany) using the QuantiTect SYBR® Green RT-PCR Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Each reaction contained 12.5 μl RT-PCR mastermix, 10 pmol of each primer, 0.25 μl RT-Mix, 5.25 μl nuclease-free water and 5 μl RNA amplicons. The thermal profile was applied as follows: 50°C for 30 min, 95°C for 15 min and 40 cycles of 95°C for 15 s, 56°C for 30 s and 72°C for 30 s, followed by a melting curve analysis (from 65°C to 95°C, 0.5°C increment). PCR amplicons with a melting temperature (T_m) between 75.5 and 78.5°C were regarded as APPV positive.

Subsequently, individual samples from APPV positive pools were tested. For most samples a limited amount of serum was left (40–70 μl). When the serum volume was limited, 150 μl phosphate-buffered saline (PBS) was added prior to RNA preparation. RNA purification and real-time RT-PCR were conducted as described above. Selected samples were subjected to agarose gel electrophoresis to confirm specificity of the amplification.

2.4 | Conventional RT-PCR and nucleotide sequencing

Among the APPV-genome positive samples, differences in the melting curves could be observed, indicating genetical diversity. To obtain representative wild boar APPV sequences, 14 sera resulting in real-

time RT-PCR amplicons with different melting temperatures were selected for generation of a larger genome fragment located in the NS3 encoding region. The selected samples included sera from Västmanland, Södermanland and Blekinge since from these counties, APPV sequences from domestic pigs are available for comparison and phylogenetic analysis (Stenberg et al., 2020a).

To generate amplicons for sequencing, a conventional two-step RT-PCR was performed. For cDNA-synthesis, M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, USA) and random hexamer primers (Invitrogen, Carlsbad, USA) were used. Amplification of an 806 bp fragment within the NS3 encoding region was obtained using the primers APPV_5030-fw and APPV_5835-rev (Cagatay et al., 2018) and the DreamTaq™ Hot Start Green PCR Master Mix (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Each reaction contained 45 μl master mix, 20 pmol of each primer and 3 μl cDNA. The following thermal profile was applied: 95°C for 2 min, 40 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min, and final extension at 72°C for 5 min. The PCR amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Sanger sequencing was conducted by LGC Genomics (Berlin, Germany) applying both primers that were used to generate the amplicons. All sequences generated in this study are available on ENA, Accession numbers: ERS6287711-15.

2.5 | Phylogenetic analysis

A phylogenetic analysis was performed based on the NS3 region of APPV. Five sequences of APPV genome fragments from Swedish wild boar were obtained from the PCR amplicons (806 nt).

All available APPV NS3 sequences were mined from GenBank and aligned using MUSCLE (Edgar, 2004) implemented in Genious prime (https://www.geneious.com). Maximum likelihood trees were estimated implementing the best nucleotide substitution model in PhyML (Guindon et al., 2010). Support values were calculated using aBayes, a bayesian approach for rapid support estimation (Anisimova et al., 2011). The tree was analysed using the TempEst software for temporal signals and 'clocklikeness' of the molecular phylogenies (Rambaut et al., 2016).

2.6 | Statistical analysis

A statistician was consulted, and the statistical analysis was performed using the JMP® Pro 15.2.0 software. Statistics are reported
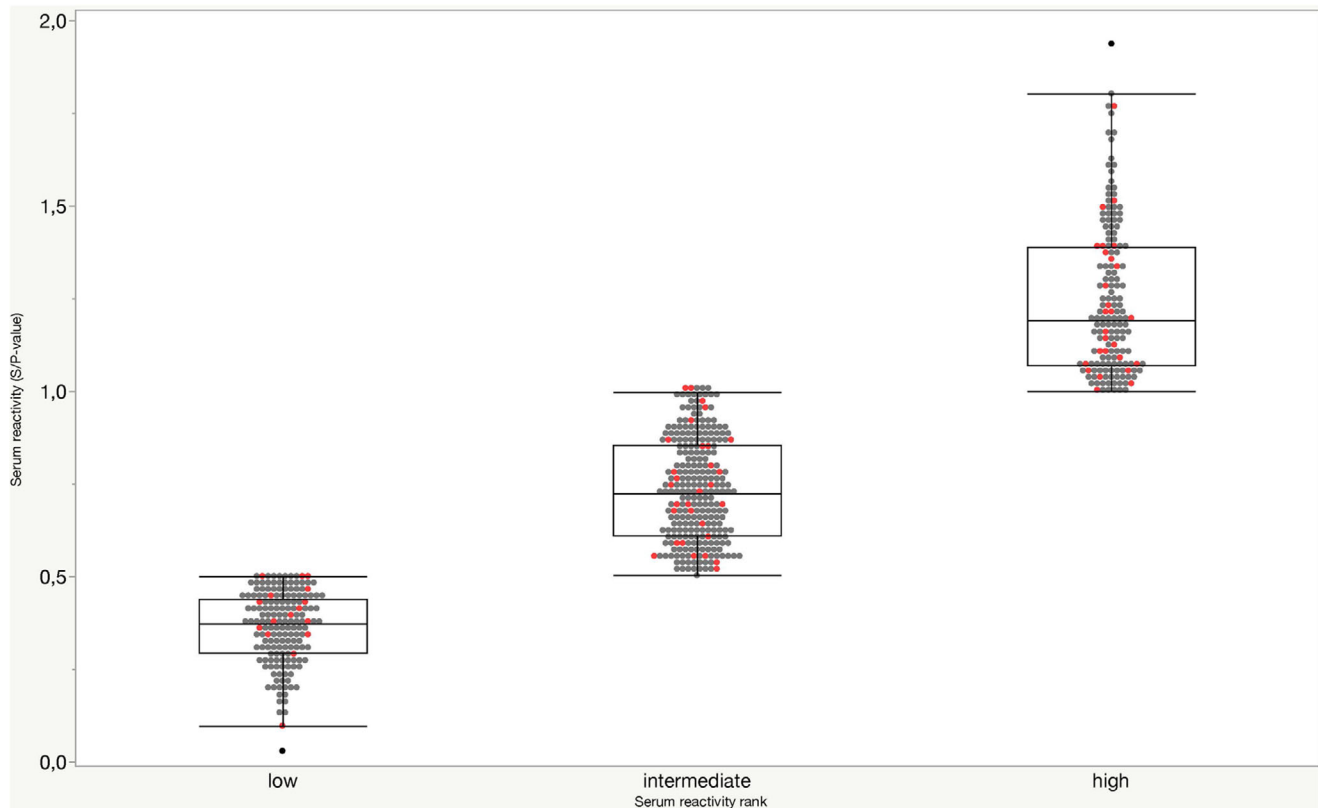
FIGURE 2 Boxplots showing the individual S/P-value (sample/positive control) for each of the 595 wild boars and how they allocate into the three subgroups of serum reactivity; low, intermediate, and high. The cut-off for a sero-positive sample is a S/P-value > 0.5. Each dot represents an individual wild boar and the red dots represent the APPV PCR-positive individuals. Low (S/P ≤ 0.5), 28%, $n = 169$, mean = 0.36, APPV PCR-positive = 9% (16/169). Intermediate (0.5 < S/P < 1.0), 44%, $n = 264$, mean = 0.73, APPV PCR-positive = 11% (30/264). High (S/P ≥ 1.0) 27%, $n = 162$, mean = 1.25. APPV PCR-positive = 17% (27/162))

as means and percentages. The dataset was analysed for differences in antibody reactivity between gilts and sows, annual differences, seasonality, and differences between the counties. To analyse the seasonality the months were subdivided in to: winter (December–February), spring (March–May), summer (June–August), and autumn (September–November).

The serum reactivity (S/P-value) was analysed by a general linear model including sampling year, county and season as fixed factors. This model included 395 of the total 595 observations. The excluded 200 observations could not be included in the model due to a lack of metadata concerning one or several of the fixed factors. The reproductive status (gilt, sow) was excluded as a factor from the general linear model since it could only include 116 of the total 595 observations, and all of these samples were collected in the years 2013–2014 in four counties (Blekinge $n = 35$, Skåne $n = 23$, Södermanland $n = 57$ and, Uppland $n = 1$). Hence, reproductive status was included in a separate general linear model. Pairwise comparisons for each factor, that is, year, county and season, were made by Tukey's honest significant difference test with the significance level set to $\alpha = 0.05$.

No statistical test was performed on the PCR-data because the number of positive samples was limited.

3 | RESULTS

3.1 | APPV in serum from Swedish wild boar

Sera from 595 wild boars sampled in Sweden in years between 2000 and 2018 were tested for the presence of APPV-genome and for the presence of APPV-specific antibodies. Overall, 72% (433/595) of the wild boars displayed APPV specific antibodies with an intermediate to high reactivity, and 12% (73/595) of the wild boars were APPV-genome positive (Figure 2). The proportion of wild boar with an intermediate serum reactivity was relatively consistent throughout the years, whereas the proportion of animals with high serum reactivity fluctuated. The largest proportion of APPV-genome positive wild boars was found among wild boars with a high antibody reactivity.

The general linear model described in Material and Methods was fitted to the data with the serum reactivity (S/P-value) as the response and year, county and season as the explanatory variable. Both year and county were found to be significant predictors of the serum reactivity (S/P-value). The season, however, was not a significant predictor. The model had an R^2 of 0.27 ($F(19, 394) = 7.385, p < .0001$). The model included 395 of the total 595 observations but all the 595 serum

samples from the years 2000 to 2018 are presented in the boxplots shown in Figures 2 and 3.

3.2 | Serum reactivity and APPV-genome detection in years between 2000 and 2018

The statistical model found the year to be a significant predictor of the serum reactivity ($F(6, 394) = 7.589$, $p < .0001$). The serum reactivity had the appearance of a sine wave, peaking in the years 2013–2014 (Figure 3, Panel A).

The pairwise comparisons made by Tukey's honest significant difference test showed that there was significantly higher serum reactivity ($n = 104$, S/P-mean $= 0.977$; $p < .05$) in 2013 than in 2002 ($n = 42$, S/P-mean $= 0.489$) and 2005 ($n = 95$, S/P-mean $= 0.612$).

The APPV-genome was detected by PCR in the wild boar serum from each year with a varying rate of PCR-positive animals. When comparing the years with > 80 observations, that is, the years 2005, 2009, 2013, 2017, and 2018, the proportion of APPV-genome positive animals seemed to increase over the years, varying from 6% (6/100) in the year 2005 to 24% (20/82) in the year 2018 (Figure 3, Panel A).

3.3 | Serum reactivity and APPV-genome detection in the sampled counties

The statistical model found the county to be a significant predictor of the serum reactivity ($F(10, 394) = 3.636$, $p = .0001$). It included 395 of the total 530 observations; the results from the 530 samples where the county was recorded are presented in the boxplot (Figure 3, Panel B).

The pairwise comparisons made by Tukey's honest significant difference test showed that Södermanland, a county situated in the central parts of Sweden, stands out with higher serum reactivity among the wild boars compared to samples from other counties. This serum reactivity ($n = 94$, S/P-mean value $= 0.97$) was significantly different ($p < .05$) from that in Halland ($n = 32$, S/P-mean $= 0.58$), Skåne ($n = 101$, S/P-mean $= 0.7$) and Blekinge ($n = 39$, S/P-mean $= 0.77$).

The APPV-genome was detected by PCR in serum from wild boars sampled in: Halland (6%, $n = 3/50$), Skåne (13%, $n = 14/109$), Småland (13%, $n = 7/52$), Södermanland (12%, $n = 16/137$), Uppland (9%, $n = 6/65$), Västergötland (40%, $n = 4/10$), Västmanland (8%, $n = 1/12$), and Östergötland (7%, $n = 3/43$) (Figure 3, Panel B).

3.4 | Serum reactivity and APPV-genome detection during summer, autumn, winter and spring

The statistical model showed that the season is not a significant predictor of the serum reactivity (F ($3,394$) $= 0.669$), $p = .573$). It included 395 of the total 530 observations; the results from the 399 samples where season was recorded are presented in the boxplot (Figure 3, Panel C).

The APPV-genome was detected by PCR with a varying prevalence in the wild boar sera from each season. Autumn (September–November) 13% (17/133). Winter (December–February) 10% (13/125). Spring (March–May) 5% (4/77). Summer (June–August) 3% (2/69) (Figure 3, Panel C).

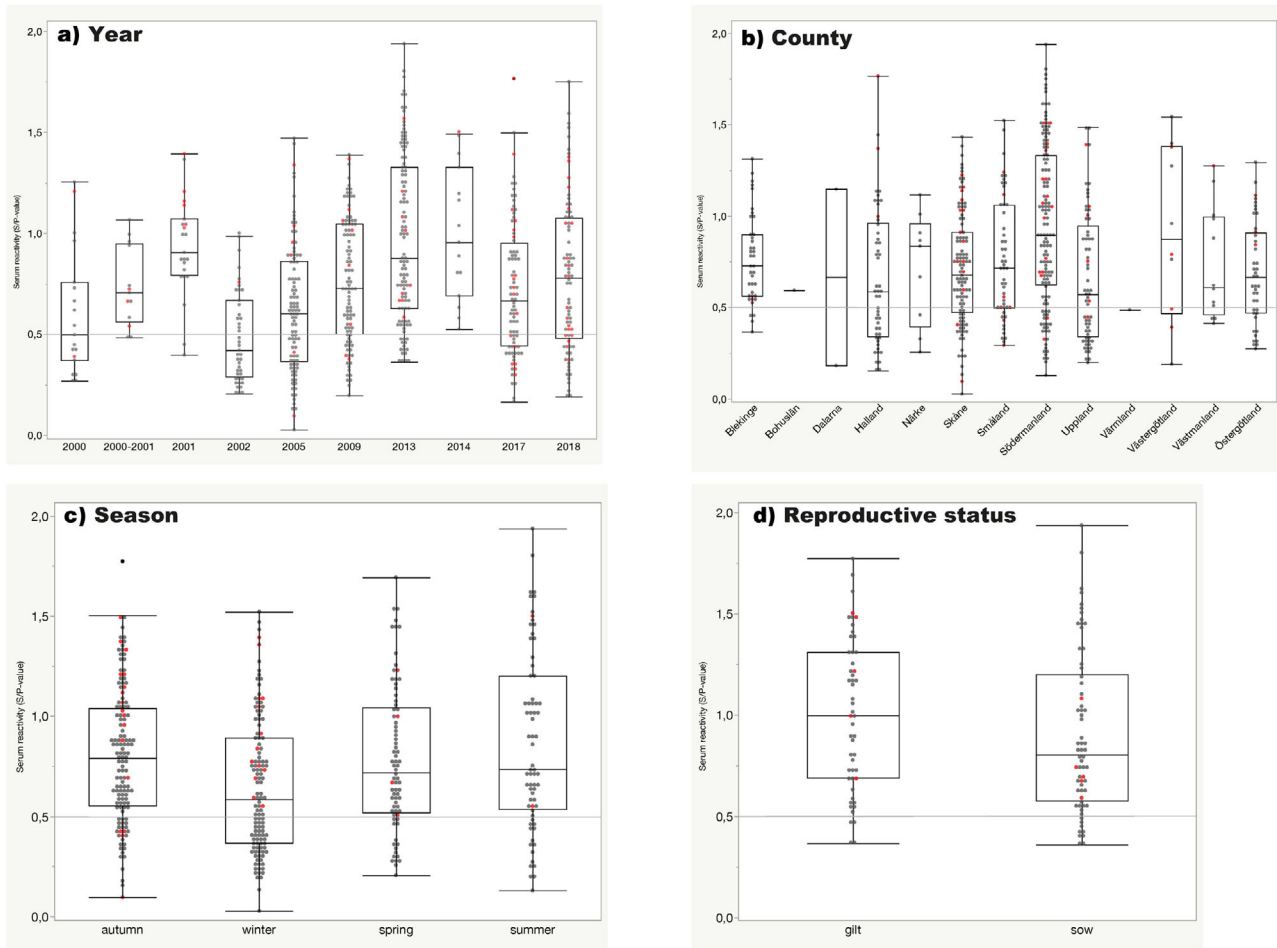## 3.5 | Serum reactivity and APPV-genome detection in female wild boars

The reproductive status of 117 female wild boars originating from four counties (Blekinge $n = 35$, Skåne $n = 23$, Södermanland $n = 57$ and, Uppland $n = 1$) was recorded, out of the total number of sampled wild boar (595). A general linear model was fitted with the serum reactivity (S/P-values) as the response, and reproductive status, year and county as the explanatory variable. The model showed that the county is a significant predictor of the serum reactivity in the female wild boar dataset ($F(8, 107) = 5.303$, $p < .0001$). The pairwise comparisons made by Tukey's honest significant difference test showed that the wild boars in Södermanland had a higher serum reactivity than the wild boars in Blekinge ($p < .05$). No statistically significant difference in the serum reactivity between gilts and sows was detected. However, there was a tendency to a lower antibody reactivity in the serum from sows compared to gilts (Figure 3, Panel D). The APPV-genome was detected by PCR in serum from both gilts and sows. The APPV-genome detection rate was 10% in gilts (5/51) and 8% in sows (5/66) (Figure 3, Panel D).

3.6 | Phylogenetic analysis

Conventional RT-PCR of 14 selected sera originating from different Swedish counties resulted in five amplicons suitable for sequencing. These were three samples from Södermanland and one from Blekinge, counties from which APPV sequences obtained from domestic pigs have been determined previously (Stenberg et al., 2020a). Additionally, it was possible to determine an APPV sequence from Uppland, from where no genetic information about APPV circulating in domestic pigs has been available before. Two distinct clusters of APPV in Swedish wild boar were detected in the phylogenetic analysis (Figure 4). The largest number of sequences were those falling into Cluster I, where all sequences from Sweden (both those generated in this study and sequences from domestic pigs described previously) fell into one discrete clade. This clade is largely dominated by sequences from Asia. A single Swedish wild boar sequence fell into Cluster II, which is largely dominated by sequences from European pigs and wild boars. The sequences from Sweden did not cluster into a single clade, and the sequence in cluster II generated from this study was more closely related to a sequence from Wild boars in Italy than to any sequences generated from pigs in Sweden. We were unable to make a definite estimate of the introduction date of these viruses into Sweden due to limitations in the temporal signal of the available data but the TempEst analysis put the introduction time of APPV to Sweden > 100 years ago.

4 | DISCUSSION

This is the first study on APPV genome- and seroprevalence in Swedish wild boar. The selection of archived samples from the national biobank

FIGURE 3 Serum reactivity and genome detection rate in Swedish wild boars from years between 2000 and 2018. Boxplot A). Distribution of the antibody reactivity or S/P-values (sample/positive control) in 595 serum samples from the year 2000 to 2018. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots represents the APPV PCR-positive individuals. There is a weak trend to increasing serum reactivity (S/P-values) from 2000 to 2018. The serum reactivity has the appearance of a light wave-like motion, gradually increasing from 2000 to 2018, peaking in 2013. Year 2000, n = 19, S/P-mean = 0.601, Std dev. = 0.309, APPV PCR-positive = 11% (2/19). Year 2000–2001, n = 13, S/P-mean = 0.733, Std dev. = 0.197, APPV PCR-positive = 23% (3/13). Year 2001, n = 23, S/P-mean = 0.933, Std dev. = 0.245, APPV PCR-positive = 26% (6/23). Year 2002, n = 43, S/P-mean = 0.483, Std dev. = 0.224, APPV PCR-positive = 2% (1/43). Year 2005, n = 100, S/P-mean = 0.622, Std dev. = 0.318, APPV PCR-positive = 6% (6/100). Year 2009, n = 100, S/P-mean = 0.765, Std dev. = 0.308, APPV PCR-positive = 8% (8/100). Year 2013, n = 115, S/P-mean = 0.977, Std dev. = 0.410, APPV PCR-positive = 8% (9/116). Year 2014, n = 15, S/P-mean = 0.998409, Std dev. = 0.330, APPV PCR-positive = 7% (1/15). Year 2017, n = 84, S/P-mean = 0.720, Std dev. = 0.332 APPV PCR-positive = 20% (17/84). Year 2018, n = 82, S/P-mean = 0.789, Std dev. = 0.376, APPV PCR-positive = 24% (20/82).) Boxplot B). Serum reactivity and genome detection rate in 530 wild boars from the different counties. Mean and individual S/P-values. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. Blekinge: n = 39, S/P-mean = 0.77, Std dev = 0.24, APPV PCR-positive = 3% (1/39). Bohuslän: n = 1, APPV PCR-positive = 0. Dalarna: n = 2, APPV PCR-positive = 0. Halland: n = 50, S/P-mean = 0.66, Std dev = 0.38, APPV PCR-positive = 6% (3/50). Närke: n = 9, S/P-mean = 0.72, Std dev = 0.31, APPV PCR-positive = 0. Skåne: n = 109, S/P-mean = 0.71, Std dev = 0.31, APPV PCR-positive = 13% (14/109). Småland: n = 52, S/P-mean = 0.76, Std dev = 0.33, APPV PCR-positive = 13% (7/52). Södermanland: n = 137, S/P-mean = 0.95, Std dev = 0.44, APPV PCR-positive = 12% (16/137). Uppland: n = 65, S/P-mean = 0.66, Std dev = 0.36, APPV PCR-positive = 9% (6/65). Värmland: n = 1, APPV PCR-positive = 0. Västergötland: n = 10, S/P-mean = 0.92, Std dev = 0.49, APPV PCR-positive = 40% (4/10). Västmanland: n = 12, S/P-mean = 0.74, Std dev = 0.31, APPV PCR-positive = 8% (1/12). Östergötland: n = 43, S/P-mean = 0.69, Std dev = 0.28, APPV PCR-positive = 7% (3/43).) Boxplot C). Serum reactivity and genome detection rate in 399 wild boars during the different seasons. Mean and individual S/P-values. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. Summer (June–August): n = 69, S/P-Mean: 0.87, Std dev = 0.446, APPV PCR-positive = 3% (2/69). Autumn (September–November): n = 133, S/P-Mean: 0.81, Std dev = 0.336, APPV PCR-positive = 13% (17/133). Winter (December–February): n = 125, S/P-Mean 0.65, Std dev = 0.332, APPV PCR-positive = 1% (13/123). Spring (March–May): n = 77, S/P-Mean: 0.79, Std dev = 0.363, APPV PCR-positive = 5% (4/77).) Boxplot D). Serum reactivity and genome detection rate in gilts and sows. Mean and individual S/P-values for 117 female wild boars. The cut-off for a sero-positive sample is a S/P-value > 0.5, marked with a grey line in the figure. Each dot represents one individual wild boar and the red dots are the APPV PCR-positive individuals. No significant difference was found in the serum reactivity between gilts and sows. Gilts: n = 51, S/P-mean = 1.012, Std dev = 0.370, APPV PCR-positive = 10% (5/51). Sows: n = 66, S/P-mean = 0.908, Std dev = 0.398, APPV PCR-positive = 8% (5/66).)
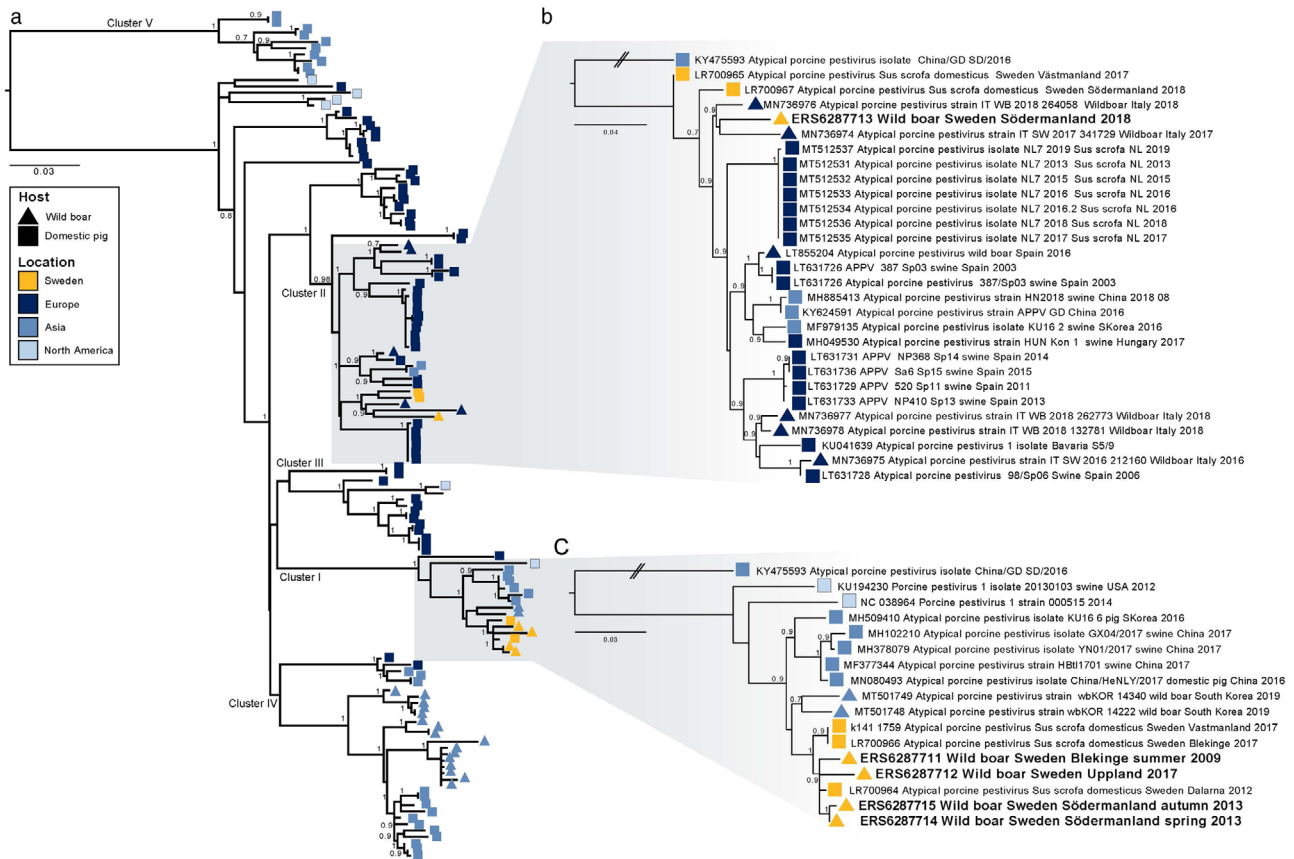
FIGURE 4 Phylogenetic Tree. Maximum likelihood tree of the NS3 region of Atypical Porcine Pestivirus. (a) Phylogeny of all NS3 sequences in GenBank. Cluster information from Gatto et al. (2019). Clade 5 was selected as the outgroup based on Folgueiras-González et al. (2020) and Gatto et al. (2019). (b,c) Expansion of select clusters to illustrate genetic relationship of sequences generated in this study. Cluster trees were rooted against KY475593, a sequence in Cluster V. Tips are coloured based on host and geographic location. Scale bar indicates number of substitutions per site. Bayesian support values (as calculated using aBayes; Guindon et al., 2010) are indicated on relevant nodes. Sequences names honour those in GenBank, such that a number of sequences are labelled 'Porcine Pestivirus 1' rather than 'Atypical Porcine Pestivirus.' Sequences generated in this study are in bold

provided an opportunity to advance the understanding of APPV in the Swedish wild boar population. The analyses revealed changes in the APPV-antibody and -genome prevalence during the past two decades as well as a difference in the occurrence of APPV between counties. Furthermore, an opportunity was obtained to genetically characterize the APPV isolates circulating in the Swedish wild boar population and to determine their genetic relatedness to APPV-sequences from Swedish domestic pigs suffering from congenital tremor.

Since APPV was only recently discovered, knowledge of the natural transmission, immunity and antibody kinetics is limited. A previous study evaluating the humoral immune response induced after infection with APPV showed that E^{rns}-specific antibodies were declining in a majority of the studied pigs after about 160 days whereas the E2-specific antibody levels did not decline significantly over the same period of time (Cagatay et al., 2019). Because of the more transient immune response to E^{rns} than to the E2 protein, the former was chosen to follow the dynamics of APPV infection in the Swedish wild boar population over time using an E^{rns} specific ELISA. Furthermore, because the APPV genome is highly variable, a new primer pair targeting a con-

served sequence in the APPVs' NS4B encoding region was designed to ensure a high detection rate. Using these methods, 12% of the wild boars (73/595) were PCR-positive for APPV genome in serum and 72% (433/595) displayed APPV specific antibodies, indicating that APPV is abundant among wild boars in Sweden.

The high seroprevalence is in line with the results from another study where the APPV antibody prevalence in wild boar was determined using the same indirect APPV-specific ELISA (Cagatay et al., 2018). Hence, the results obtained from Germany and Serbia, where 52% of the tested wild boars had APPV specific antibodies, are comparable to the present Swedish results. Interestingly, a study from Switzerland also using the same indirect APPV-specific ELISA presented an occurrence of APPV antibodies of 93% in a closed APPV endemic herd of domestic pigs (Grahofer et al., 2020), suggesting that APPV has the potential to cause an extensive herd immunity.

The overall APPV genome detection rate of 12% in Swedish wild boar is also similar to the findings in German wild boar (18%) (Cagatay et al., 2018). But the proportion APPV-genome positive wild boars in the Swedish and German population are considerably higher

compared to South Korea, Spain and Italy, where the genome detection rate is < 1% (Cagatay et al., 2018; Choe et al., 2020; Colom-Cadena et al., 2018; Sozzi et al., 2019). Although the studies are not completely comparable, since primer pairs targeting different part of the genome has been used, they clearly demonstrate that the proportion of viraemic wild boars varies between countries. The cause of the variation in the APPV-genome detection rate between studies and countries is not known but viral spread in the wild boar population can be attributed to several factors differing between countries such as the population density, climate, fencing, agriculture, foresting and, hunting (Bertelloni et al., 2020; Malmsten et al., 2018; Morelle et al., 2020; Petit et al., 2020).

Based on the outcomes from this study, it is evident that APPV is common and has been present in the Swedish wild boar population since at least the year 2000. The seroprevalence in the years between 2000 and 2018 showed a wave-like pattern, similar to the epidemiology of other viral diseases (Stegmaier et al., 2020) (Figure 3, Panel A). In the current study, the year 2013 stands out with significantly higher antibody reactivity than the other years. The high antibody reactivity caused by an extensive spread of APPV is most likely attributed to the exceptionally mild winter of the years 2012/2013 (Wern, 2015). A mild winter would have led to a higher survival rate of piglets and consequently more yearlings in 2013, increasing the spread of pathogens (Podgórski et al., 2018). The high antibody reactivity detected in wild boar sera from the year 2013 might also be confounded by the high proportion of wild boars collected in Södermanland during the years 2013/2014.

The serum reactivity (S/P-values) of wild boars sampled in Södermanland was significantly higher than in wild boars sampled in Halland, Skåne and Blekinge, indicating a higher circulation of APPV in wild boars in Södermanland than in the other counties. The county of Södermanland is located in the central parts of Sweden, whereas, Halland, Skåne and, Blekinge are counties bordering each other in the south of Sweden (Figure 1). Interestingly, the proportion of APPV-genome positive wild boars in Södermanland and Skåne were very similar, 12% and 13% respectively, whereas the proportion of genome positive wild boars in Blekinge and Halland was lower, 3% and 6% respectively. The high genome detection rate in the wild boar population of Södermanland and Skåne was somewhat expected, since Södermanland and Skåne were the first counties in Sweden to be colonized by wild boar in the 1970s and have the densest wild boar populations in Sweden today (Naturvårdsverket, 2020).

Despite the limited number of samples, there is a trend to an increase in the proportion of APPV-genome positive animals over the years. The increasing genome detection rate and seroprevalence of APPV-specific antibodies from 2000 to 2018 could be attributed to the natural degradation of RNA and antibodies in serum during storage, but there are other more plausible explanations. The number of wild boars in Sweden has increased massively in the last decades, causing denser populations and more frequent interactions between groups. The increased wild boar population has also led to an increased interest in hunting but also in supplementary feeding (Naturvårdsverket, 2020). Hunting, as well as supplementary feeding, increases mobility

and interactions between groups of wild boars and is known to escalate the spread of viruses within a wild boar population (Morelle et al., 2020). This human intervention may partly explain the higher proportion of viraemic wild boars during autumn and winter than during summer and spring but it also implicates transmission of APPV as being the most intense during the mating-season in autumn.

Horizontal as well as vertical transmission of APPV infection between domestic pigs is indeed very efficient (Cagatay et al., 2019) and pigs may shed virus in saliva, faeces, urine and semen for several months after infection, making mating a possible route of infection with APPV (Arruda et al., 2016; de Groof et al., 2016). The idea of APPV spread via semen as one possible route of infection is supported by a study on domestic pigs in which young sows presented with the highest E^{rns} antibody reactivity, and this reactivity decreased with time (Grahofer et al., 2020). If it is assumed that wild boar has the same shedding and spreading pattern as domestic pigs, it could be speculated that wild boar sows, which have been mated, should have a higher antibody reactivity than non-mated gilts. Nonetheless, no significant difference in antibody reactivity between Swedish wild boar gilts and sows was detected. There was, however, a weak trend of slightly higher antibody reactivity in gilts than in sows. This trend that could be due to the fact that younger animals (0.5–2 year) have more interaction with other wild boars, both within and between groups, making them more likely to contract infectious diseases (Podgórski et al., 2018).

At present, there are no reports of signs of congenital tremor in wild boar piglets but it cannot be excluded that APPV infection in wild boar is associated with congenital tremor. It seems plausible however, that it would be hard to find wild boar piglets with signs of congenital tremor since the sows keep newborn wild boar piglets hidden. In domestic pigs, however, there are regular reports of outbreaks of piglets born with signs of congenital tremor (Stenberg et al., 2020a). The routes of introduction of APPV into a closed swine herd is not clear but wild boars could be speculated to be a reservoir. The present study reveals that sequences from wild boar and domestics pigs from the same area are clustering together, indicating a transmission of APPV between domestic pigs and wild boars, as described for other viruses (Meng et al., 2009).

The phylogenetic analysis also revealed two clusters of APPV in Sweden, reflecting the diversity thus far described in Swedish pigs (Stenberg et al., 2020a). The clustering, where the major part of the Swedish sequences from wild boar and domestic pigs fell into one cluster (Cluster I), suggests a single introduction into Sweden and subsequent continuous circulation. Since wild boars were introduced to the Swedish fauna in the 1970s, it can be speculated that APPV was first introduced and circulated in the domestic pig population and then transmitted to the wild boar population but that wild boars today serve as the main reservoir for APPV. Although the TempEst analysis could not conclude on a set time of introduction of APPV to Sweden, it suggests that the virus has been circulating in Sweden for > 100 years. This is plausible since congenital tremor type A-II was described in Swedish domestic pigs in the 1950s (Larsson, 1955; Stenberg et al., 2020a).

Further, the clustering illuminated the need for further research on APPV in wild boar. The APPV belonging to the previously reported

phylogenetic cluster I is largely dominated by sequences from Asia, either suggesting hidden diversity in Europe, or representing the movement of pigs or wild boars from Asia to Scandinavia through anthropogenic assistance. Since the import of live pigs to Sweden is, and has been, very limited, an anthropogenic introduction of APPV to Sweden from Asia through live pigs is unlikely (Svenska djurbönders smittskyddskontroll, 2018; Swedish Board of Agriculture, 2021). The sequence generated from this study in cluster II was more closely related to sequences from wild boars in Italy than to any sequences generated from domestic pigs in Sweden. The close similarity between APPV from Swedish wild boar and APPV genomes detected in wild boar from Italy and South Korea demonstrates the knowledge gap concerning the APPV epidemiology in wild boar. Genetic characterization of APPV from wild boar originating from different countries should be performed to establish reliable epidemiological links. In general, epidemiology of viral pathogens circulating in wild animal species is still poorly understood and should be more strongly in the focus of future research.

5 | CONCLUSION

APPV was found to be highly abundant in the Swedish wild boar population. Of the 595 sampled wild boars, 12% (73/595) were viraemic and 72% (433/595) displayed APPV-specific antibodies. This study proves that APPV has been present in the Swedish wild boar population since at least the year 2000. The high proportion of viraemic and seropositive wild boar and the genetical closeness of APPV in wild boar and domestic pigs is indicative of wild boar being an important reservoir for APPV and calls for further research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Hedvig Stenberg, Elena Leveringhaus, Alexander Postel and Maja Malmberg contributed to the conception and design of the study. Hedvig Stenberg analysed the data and drafted the manuscript. Elena Leveringhaus carried out the lab work. Anna Malmsten and Anne-Marie Dalin collected the serum samples from the female wild boars. All authors have approved the submitted version of the manuscript.

DATA AVAILABILITY STATEMENT

Sequence data have been submitted to the EMBL databases under accession number ERS6287711-15.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The animal samples used in this study were archived sera that had been collected by hunters in years between 2000 and 2018 as part of a national Swedish surveillance programme or collected post mortem at abattoirs for other studies. No ethical permit was required for collecting or using these samples.

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