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A wild circulation: High presence of *Porcine circovirus* 3 in different mammalian wild hosts and ticks

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Summary

Porcine circovirus 3 (PCV-3) has emerged as a potential threat for swine industry, being consistently reported in the presence of several clinical signs all around the world. Recently, its presence in wild boar has been demonstrated at high prevalence. This evidence is surprising since the lower density of wild populations might not be expected to sustain such efficient viral transmission. Porcine circoviruses were proven to exhibit a certain plasticity in the host tropism and were detected in unrelated species, like mice, dogs and ruminants. However, if this scenario applies also to wild animals remains to be established. Therefore, this study aimed to investigate the presence of PCV-3 in wild ungulates other than wild boar and in related hematophagous ectoparasites. One hundred and nine animals were sampled from different hilly and mountain areas of Friuli Venezia Giulia, including 9 chamois (*Rupicapra rupicapra*), 17 red deer (*Cervus elaphus*), 4 mouflons (*Ovis musimon*), 50 roe deer (*Capreolus capreolus*) and 29 wild boars (*Sus scrofa*). Additionally, host-matched ectoparasites were collected when present. *Porcine circovirus 3* was diagnosed using molecular techniques and sequencing. This study results confirmed the high PCV-3 occurrence in wild boar and reported for the first time its presence, at low prevalence, in chamois and roe deer. Moreover, two ticks (*Ixodes ricinus*), one of which non-engorged, collected from PCV-3 negative roe deer, tested PCV-3 positive. The genetic characterization of some of the strains collected from non-swine hosts allowed to prove that, albeit clearly part of PCV-3 species, they were genetically unique, demonstrating the absence of among-samples contamination and thus confirming the actual presence of PCV-3 genome in these new hosts. Therefore, this study highlights an unexpected broad PCV-3 distribution and circulation in the wild, rising further questions on porcine circoviruses infectious cycle, epidemiology and origin, which will deserve additional investigations.

KEYWORDS
Italy, molecular epidemiology, *Porcine circovirus 3*, ticks, vector-borne, wild ungulates

1 | INTRODUCTION

The Italian environment, and the Mediterranean region overall, have been affected by a major anthropic disturbance for thousands of years. However, in the last decades, a relevant change in the landscape usage has occurred. Hilly and mountain areas are being abandoned by humans, causing a progressive natural reforesting, particularly in the Apennines and Alps. Mammals and bird species

followed a similar pattern, with forest birds, ungulates and carnivores increasing over the same time period (Falcucci, Maiorano, & Boitani, 2007).

These changes were favoured by the progressive introduction of wildlife protection policies, establishment of protected areas, resettlements of extinct or near-extinction species and a rising of the public opinion about environmental issues (Barnes et al., 2016; DpR *08/09/1997*, *n. 357*, 2013; *L.R. 6/2008*, 2015; Reg *No. 157*, 1992).

While the emerging scenario can be favourably embraced from several points of view, an increase in wildlife presence comes at some drawbacks, including direct damages to agriculture, livestock and ecosystem (National Research Council (US) Committee on Agricultural Land Use and Wildlife Resources, 1970; Treves, Wallace, Naughton-Treves, & Morales, 2006).

A particularly relevant issue is the role of several avian and mammalian species as reservoirs of important infectious diseases of humans and livestock. This evidence is particularly significant because the extensive agricultural activities and recreational use of less urbanized areas, both increasing in recent years, create frequent opportunities for humans, domestic and wild animal species to interact. Additionally, the increase in wild populations has not necessarily been mirrored by a proportional expansion of available land, and their distribution remains limited to confined protected areas. Albeit not comparable with intensive farming, this condition has sometimes determined dense animal populations which can enhance infectious disease maintenance and spreading, with detrimental effects on wild animal health and welfare as well (OIE, 2010).

Several viral diseases impacting on livestock production have been recognized in wild ungulates, which can sometimes play a pivotal role in their maintenance and hinder their control and eradication (Moennig, 2015). However, the impact on wild species health, their role in the disease epidemiology and the directionality of the viral flux are less clear in most instances (Ruiz-Fons, Segalés, & Gortázar, 2008).

Despite being known since the 1970s (Tischer, Gelderblom, Vettermann, & Koch, 1982; Tischer, Rasch, & Tochtermann, 1974), the genus *Circovirus* has caught the attention of the veterinarian community particularly after the 1990s, when a new porcine circovirus, *Porcine circovirus 2* (PCV-2) was first recognized and associated with a series of clinical syndromes, now called porcine circovirus diseases (PCVD), that were rapidly reported to occur all around the world, causing severe economic losses due to mortality, reduced growth, increased secondary infection and control-associated costs (Segalés, Allan, & Domingo, 2005; Segalés, Kekarainen, & Cortey, 2013). The interest raised by this viral group coupled with the remarkable increase in research and diagnostic potential has allowed to uncover an unexpected variability of circoviruses in terms of genetic features and host distribution (Delwart & Li, 2012).

Among the latest discovered circoviruses, *Porcine circovirus 3* (PCV-3) has claimed an attention comparable to its predecessor (Palinski et al., 2017). Despite its recent discovery, it has been reported worldwide, often in the presence of clinical signs, including porcine dermatitis and nephropathy syndrome (PDNS), reproductive disorders, respiratory signs (Ku et al., 2017; Palinski et al., 2017; Shen et al., 2018) and myocarditis (Phan et al., 2016).

However, it has also been reported in asymptomatic animals (Zheng et al., 2017) and its causative role in swine disease is still debated (Franzo, Legnardi, Tucciarone, et al., 2018; Klaumann, Correa-Fiz, et al., 2018).

Remarkably, PCV-3 was proven to circulate in wild boar populations, at high prevalence (Franzo, Tucciarone, Drigo, et al., 2018; Klaumann, Dias-Alves, et al., 2018). This evidence could be considered unexpected since this virus, despite its likely ancient origin (Fu et al., 2018; Klaumann, Franzo, et al., 2018), similarly to PCV-2, emerged only recently as a global problem, in parallel with the development of intensive farming. Therefore, the high density and high turnover production system are likely to be pivotal in the rise and efficient spreading of these pathogens. Comparable conditions cannot be achieved by the sparser and long-living wild populations. So, other epidemiological cycles involving different forms of transmission and maintenance could contribute to this virus success in wildlife.

Ticks transmit more pathogen species than any other group of blood-feeding arthropods worldwide, including several viral ones (Pfäffle, Littwin, Muders, & Petney, 2013). Tick-infecting circoviruses have been described in China (Wang et al., 2018), and other arthropod species have been proven to be susceptible to Circoviridae infection (Delwart & Li, 2012). Nevertheless, the role of hematophagous arthropods in PCV-3 epidemiology has never been investigated.

The presence of other target species could also increase the available host population size, facilitating the virus maintenance in the wild.

The two main porcine circoviruses, while clearly showing a strong preference for *Sus scrofa*, have both demonstrated a certain host plasticity, being detected in other species including ruminants, rodents and dogs (Halami, Freick, Shehata, Müller, & Vahlenkamp, 2014; Kappe et al., 2010; Zhai et al., 2016; Zhang et al., 2018). Therefore, a similar PCV-3 presence in wild species can be hypothesized.

Based on these premises, the present study aims to investigate, using molecular biology assays, the presence and circulation of PCV-3 in several wild ungulate species and the potential vector role of hematophagous ectoparasites.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Samples were obtained from animals culled in hunting reserves located in the Friuli Venezia Giulia mountain area during the hunting season from May 2017 to January 2018. From each animal, hematophagous ectoparasites were collected before exsanguination to minimize potential contamination sources. Thereafter, blood was collected and the obtained serum stored at 4°C for a maximum of 4 days until delivery to the Veterinary Infectious Disease laboratory (Dept. Animal Medicine, Production and Health, Padua University,

Italy). Serum samples and parasites were stored at −80°C until processing.

For each animal, information including species, sex, estimated age, hunting site and date was recorded. Similarly, parasite species, sex and engulfment status were morphologically determined using stereomicroscopy. When more than one parasite of the same species was collected from the same host, they were merged into pools based on feeding status, avoiding the mixing of engulfed and not engulfed ticks, suspended in 500 µl of PBS and mechanically homogenized.

2.2 | PCV-3 molecular diagnosis

DNA was extracted from 200 µl of serum or parasite homogenate using the NucleoSpin®Blood extraction kit (MACHEREY-NAGEL). Prior to extraction, an exogenous internal control was added to the binding solution. DNA was tested for PCV-3 presence using the real-time PCR described in Franzo, Legnardi, Centelleghe, et al. (2018).

In order to confirm the results and characterize the viruses, a PCR was used to amplify part of the genome corresponding to the *Cap* coding gene, as described in Franzo, Tucciarone, Drigo, et al. (2018).

Briefly,thewholeCapregionwasamplified(ampliconlength:707 bp) using the primers PCV3_1303F (5-ACCGGAGGGGTCAGATTTAT-3) and PCV3_8R (5-TGCCGGGTAATACTAGCC3-3). Two microlitres of extracted DNA was added to a standard mix composed of 1X reaction buffer, 200 µM dNTPs, 0.6 µM of each primer and 0.4 µl of Phire Hot Start II DNA polymerase. Sterile nanopure water was added to bring the final volume up to 20 µl. The following thermal protocol was selected: 98°C for 30 s followed by 45 cycles of 98°C for 5 s, 60°C for 5 s and 72°C for 15 s. A final extension phase of 1 min at 72°C was also performed.

To allow the characterization of low titre samples, the partial *Rep* gene sequence was also amplified using the method described by Franzo, Legnardi, Centelleghe, et al. (2018), because of the higher sensitivity of the assay.

Two microlitres of the DNA was added to a standard PCR master mix composed of 1X Phire animal tissue PCR buffer, 0.6 µM of each primer (PCV3_rep_F 5′-AAAGCCCGAAACACAGGTGGTGT-3′ and PCV3_rep_R 5′-TTTTCCCGCATCCTGGAGGACCAAT-3′) and 0.4 µl of Phire hot start II DNA polymerase. Sterile nanopure water (Thermo Fisher Scientific) was added to bring the final volume to 20 µl. The PCR thermal protocol was 98°C for 5 min followed by 45 cycles of 98°C for 5 s, 68°C for 7 s and 72°C for 15 s. A final elongation step of 1 min at 72°C was performed.

Amplification and specificity of the bands were visualized using a SYBR safe stained 2% agarose gel.

Sequencing was performed at Macrogen (Macrogen Spain, Madrid, Spain) using the same primers. Chromatograms were visually inspected with Finch TV program 1.4.0 (2004–2006 Geospiza Inc) and consensus sequences were obtained using ChromasPro (ChromasPro Version 1.5; Technelysium Pty Ltd, South Brisbane, Australia; http://technelysium.com.au/wp/chromaspro/).

2.3 | Sequence analysis

Obtained sequences were compared to a reference dataset (one for the *Rep* and one for the *Cap* encoding gene) representative of all available PCV-3 sequences spanning the considered region.

To account for the coding nature of these sequences, they were translated at amino acid level, aligned using the MAFFT method (Standley, 2013) and then back-translated to nucleotide sequence using the TranslatorX program (Abascal, Zardoya, & Telford, 2010). To allow the comparison with the maximum number of sequences, particularly with previously submitted wild boar ones, the *Cap* gene alignment was reduced to a 402-bp region, where a full coverage could be obtained for all sequences. Phylogenetic analysis was performed using the maximum likelihood approach implemented in PhyML (Guindon et al., 2010), selecting as the best substitution model the one with the lowest AIC calculated using Jmodeltest (Darriba, Taboada, Doallo, & Posada, 2012). The robustness of the clade reliability was evaluated using the non-parametric version of the aLRT (Shimodaira–Hasegawa [SH]-aLRT), implemented in PhyML 3.0 (Anisimova & Gascuel, 2006).

To account for the phylogenetic uncertainness, the association between quantitative traits (i.e. collection host) and tree topology was tested calculating different statistics (parsimony score [PS] and association index [AI]) using BaTS (Parker, Rambaut, & Pybus, 2008) on all trees sampled during two Metropolis-coupled Markov chain Monte Carlo (MCMCMC) run. More in detail, a phylogenetic analysis was performed using the Bayesian approach implemented in MrBayes (Ronquist & Huelsenbeck, 2003) selecting as the best substitution model the one with the lowest Bayesian information criterion (BIC) calculated using Jmodeltest (Darriba et al., 2012).

Two independent runs, including one cold and three heated MCMCMC chains were run for 1,000,000 generations sampling model parameters and tree every 500 generation. The runs convergence and mixing were visually inspected and results accepted only if estimated sample size (ESS) was higher than 200 and potential scale reduction factor (PSRF) approached 1.

The posterior trees, after discarding the first 20% of the trees as burn-in, were used for BaTS analysis. The above-mentioned statistics were calculated across all these trees and the observed median (μ_{obs}) value was selected as final outcome. The distribution under the null hypothesis (i.e. no trait–phylogenesis association) was obtained by randomizing without replacement the tip–trait association 1 thousand times for each tree of the posterior distribution. Each randomized dataset was used to calculate the statistics medians (μ_{null}), which formed the null distribution. This distribution was used to achieve a *p*-value by simply evaluating the proportion of simulated values more extreme than the observed one.

2.4 | Statistical Analysis

Association between PCV-3 infection, group age and health status was evaluated using a Chi-squared or Fisher's exact test values.

The presence of statistically significant differences in the viral titre among species and groups was evaluated using the Mann–Whitney (two groups) or Kruskal–Wallis test (more than two groups), followed by post hoc Mann–Whitney tests with Bonferroni correction. The statistical significance level was set to $p < 0.05$.

3 | RESULTS

A total of 109 animals were sampled from different mountain areas of Friuli Venezia Giulia. In this study, 9 chamois (*Rupicapra rupicapra*), 17 red deer (*Cervus elaphus*), 4 mouflons (*Ovis musimon*), 50 roe deer (*Capreolus capreolus*) and 29 wild boars (*Sus scrofa*) were included (Figure 1). Overall, ticks (all belonging to *Ixodes ricinus* species) were obtained from 2 chamois, 13 red deer, 3 mouflons, 28 roe deer and 1 wild boar (Figure 1). One chamois (12.5%), 2 roe deer (4%) and 13 wild boars (44.8%) were PCV-3 positive (Figure 2). Viral titre analysis demonstrated an overall higher titre in wild boar compared to other species. All infected animals, ranging from 1 to 5 years (estimated age), were classified to be in good health status. No differences in sex and age were detected between infected and non-infected subjects. Two out of 47 (4.25%) tick pools collected from roe deer tested PCV-3 positive despite not originating from positive individuals. One pool (Z1) included engulfed ticks, while the other sample (Z3) was a single non-engulfed adult female.

Besides ticks, 13 animals (2 wild boars, 8 roe deer and 3 red deer) showed a really mild *Lipoptena* spp. infestation, which all tested PCV-3 negative.

The full *Cap* gene was sequenced from 6 wild boars only, while the partial *Rep* gene could be sequenced for 8 wild boars, 1 chamois and the Z3 tick. The *Rep* and *Cap* gene sequences obtained throughout this study are available in GenBank (accession number MK044770-MK044785). The phylogenetic analysis of the *Rep* gene demonstrated a relevant heterogeneity of the collected strains, which were amply interspersed along the tree (Figure 3). No clear geographical patterns could be observed and strains collected in the present study showed a certain relationship with others collected in different countries all over the world. However, sequences 12 and 78 were identical to PCV-3 strains collected from domestic pigs (accession number MF162298.1 and MF162299.1; Figure 3) and sequence 100 was part of a quite separate cluster including Italian sequences collected from domestic pig populations. Additionally, strain 53, collected from a wild boar, and strain Z3, obtained from a roe deer tick were closely related and part of the same cluster (Figure 3).

The analysis of ORF2-based tree provided overall comparable results and allowed the comparison with previously sequenced wild boar strains collected in the Colli Euganei Regional Park (Italy; Franzo, Legnardi, Tucciarone, et al., 2018; Figure 4). No strict clustering could be observed among the sequences collected from the two separated populations. The genetic distance among sequences from Friuli and Colli Euganei ranged from 0.2% to 3.1%. However, a comparable distance could be observed among the Friulian wild strains and domestic pigs ones (i.e. 0 to 5.2%), including a perfect identity between sequences 53 and 100 with others collected in Italy and other European, North American and Asian countries, in the considered ORF2 region.

Phylogeny–trait association demonstrated a certain clustering of the wild boar sequences, even originating from different countries (Figure 4). This evidence was also statistically confirmed by the PS score ($p = 0.005$). However, discordant results were obtained by the AI ($p > 0.05$), suggesting the mild association between viral phylogeny and host.

4 | DISCUSSION

The wild ungulates populations increased remarkably in Friuli Venezia Giulia since the WWII. Different factors have contributed to this change, including animal protection and reintroduction policies, increasing population awareness about environmental issues and mountain depopulation, causing agricultural and grazing land abandonment (Barnes et al., 2016; Carnevali, Pedrotti, Riga, & Toso, 2009; De Meneghi, 2006).

Overall, about 4,000 wild boars, 9,800 red deer, 9,600 chamois, 25,000 roe deer and 1,200 mouflons were registered, potentially creating favourable conditions for infectious diseases to spread (http://www.regione.fvg.it/rafvg/cms/RAFVG/ambiente-territorio/tutela-ambiente-gestione-risorse-naturali/gestione-venatoria/).

The examined wild boar population demonstrated a high PCV-3 frequency, which is not surprising since a similar viral prevalence was previously described in another Italian wild boar population (Franzo, Tucciarone, Drigo, et al., 2018). However, differently from the Colli Euganei Regional Park, where a large animal aggregation (about 4,000 animals) is confined in a limited and relatively isolated geographic area (i.e. 18,694 ha; Franzo, Tucciarone, Drigo, et al., 2018), the region herein considered is significantly wider (agroforestal-pastoral land of about 691,795 ha) and less confined (*Piano faunistico regionale*, 2015). Although the wild boar distribution is not uniform, a significantly lower density can be confidently stated. The detection of a comparable prevalence suggests that PCV-3 is able to circulate efficiently in wild populations and likely persist for a long time, increasing the transmission likelihood in a sparse population. Accordingly, Klaumann, Dias-Alves, et al. (2018) demonstrated the long-lasting persistence of PCV-3 in wild boars, which could be longitudinally detected for more than 5 months. Differently from Franzo, Tucciarone, Drigo, et al. (2018) and Klaumann, Dias-Alves, et al. (2018), who reported a higher frequency in adult animals, no age or sex differences could be identified in the Friulian population. However, the number of sampled subjects was relatively low compared to the previous studies and, particularly, few adults were sampled, decreasing the statistical power to detect significant differences.

The high viral prevalence demonstrated in wild boars, even higher than the average one reported in domestic populations (Faccini et al., 2017; Franzo, Legnardi, Hjulsager, et al., 2018; Klaumann, Franzo, et al., 2018; Stadejek, Woźniak, Miłek, & Biernacka, 2017), supports the role of wild populations as virus reservoirs.

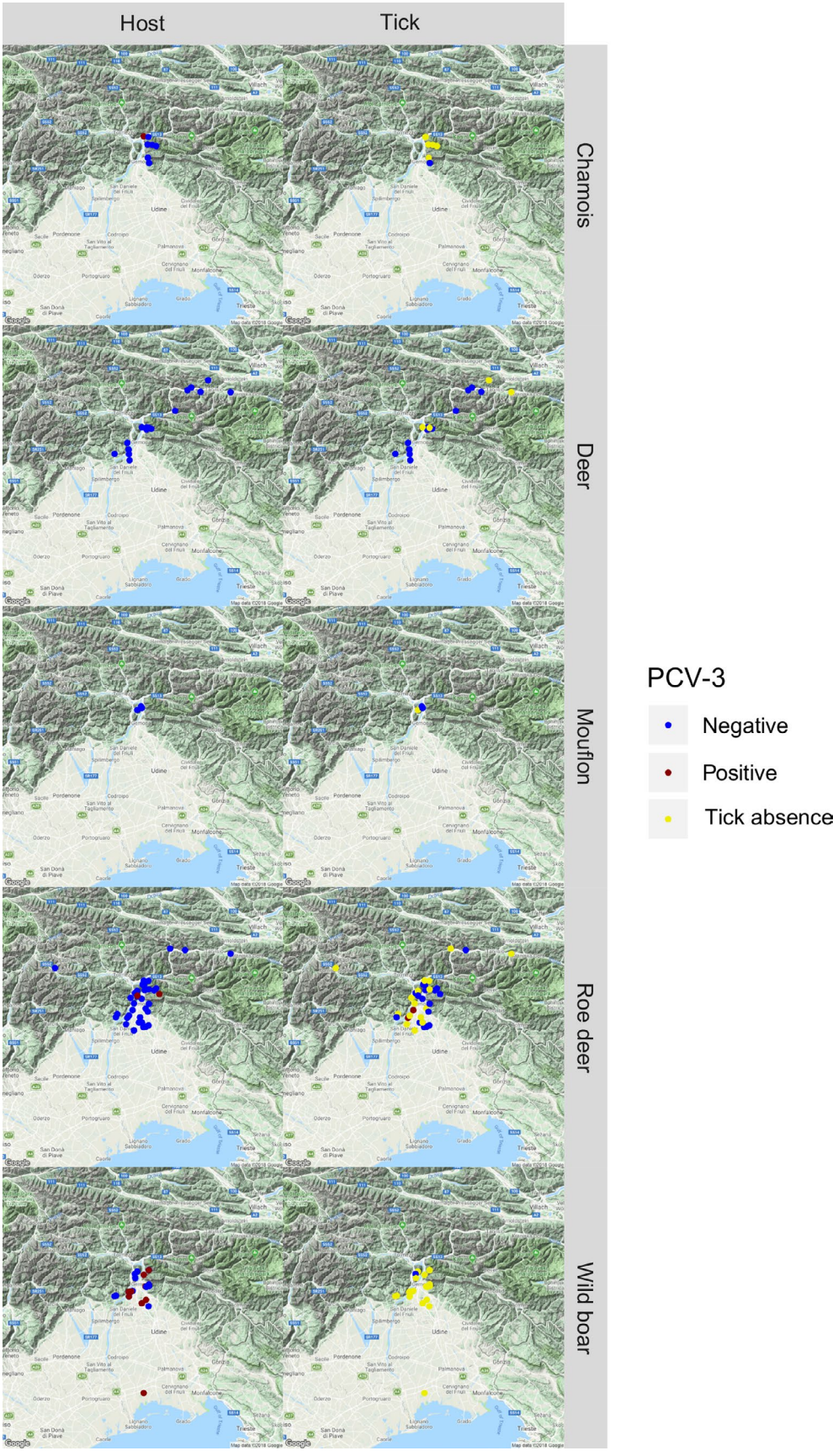

FIGURE 1 Geographical distribution of host and tick samples. Positive and negative samples have been colour coded. Additionally, hosts from which no ticks were detected have been colour coded in yellow [Colour figure can be viewed at wileyonlinelibrary.com]

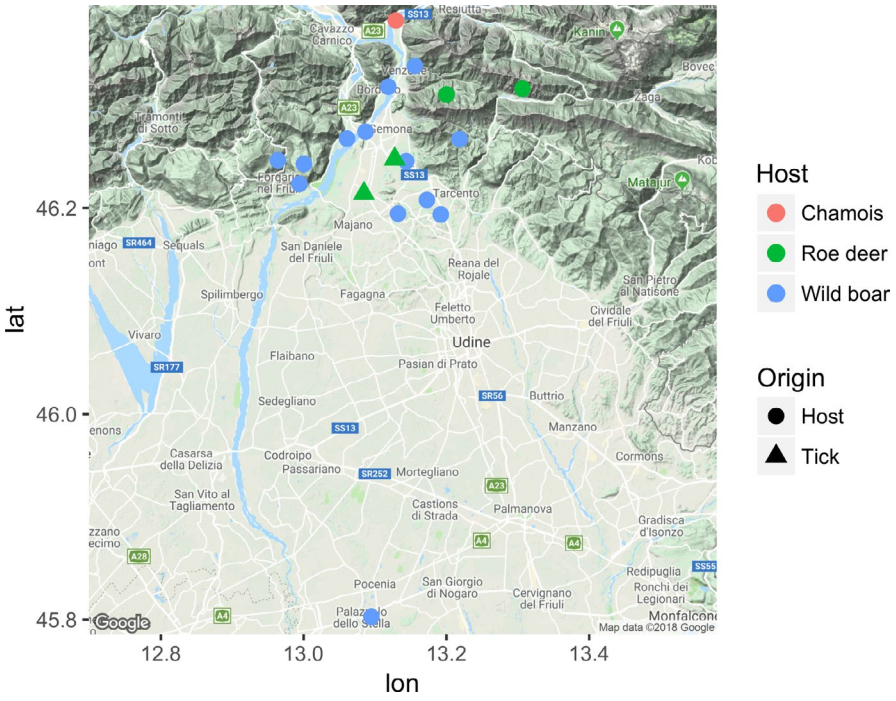

FIGURE 2 Geographical distribution of *Porcine circovirus-3* (PCV-3) positive samples in wild ungulates (full circle) and ticks (full triangle). Different hosts have been colour coded [Colour figure can be viewed at wileyonlinelibrary.com]

Remarkably, the analysis of phylogeny–trait association highlighted a certain clustering of the sequences collected in wild boar, even originating from different countries. The host–phylogeny association might confirm the effective persistence and maintenance of PCV-3 in wild boar, allowing an independent evolution, at least to a certain extent. Nevertheless, molecular epidemiology information about wild populations is currently limited to Italy and Spain (Franzo, Tucciarone, Drigo, et al., 2018; Franzo, Legnardi, Centelleghe, et al., 2018), and this could lead to a bias due to geographical, rather than host, clustering. Although spatial clustering appears not to be a feature of PCV-3 epidemiology, which is characterized by a wide mixing of strains collected in different countries (Franzo, Legnardi, Hjulsager, et al., 2018), more extensive studies, based on a representative sampling of the worldwide wild boar population, should be performed to confirm the detected association. Additionally, several legal and illegal introduction events from other Italian region and other countries (particularly from central Europe) affected both wild boar and other ungulate populations (Carnevali et al., 2009; Massei & Toso, 1993; *Piano faunistico regionale*, 2015), clearly complicating the epidemiological pattern understanding. As a matter of fact, if the clustering of wild boar PCV-3 sequences is due to a prolonged persistence followed by host-specific adaptation or to epidemiological links among the wild populations remains to be elucidated.

In spite of the detected clustering, the wild and domestic populations were not totally independent (Figures 3 and 4). In fact, the identity or close clustering of wild-derived sequences with domestic ones suggests the occurrence of epidemiologically effective contacts. However, the directionality of the viral flux and the potential menace represented by wild boar for commercial pigs remain unknown.

Remarkably, the present study demonstrated the presence of PCV-3 in other two wild ungulate species although at low titre and prevalence: one chamois and two roe deer. Even if the limited DNA amount hindered the whole genome sequencing of these strains, the genetic distance of their *Rep* sequences from those identified in wild boar excludes the occurrence of contamination events and supports an actual infection (Figure 4). Even if the main host of porcine circoviruses is indubitably the *Sus scrofa*, PCV-2 has been detected also in other species, including mice and ruminants (Halami et al., 2014; Kappe et al., 2010; Pinheiro et al., 2013). Similarly, PCV-3 has been described in phylogenetically distant species, like dogs (Zhang et al., 2018), and an extremely closely related virus (i.e. beef circovirus) was identified in bovines (Zhang, Li, Deng, Kapusinszky, & Delwart, 2014). These evidences support a certain host plasticity of porcine circoviruses, which evidently affects also wild species. If the wild species herein reported represent a dead-end infection or play an actual role in PCV-3, epidemiology will need further investigations.

More surprisingly, two ticks (*I. ricinus*) carried PCV-3 DNA, which differed genetically from all other sequences herein reported, excluding also in this case the "contamination hypothesis." While a simple accidental detection due to blood meal could justify this finding, some evidences support a different explanation. First, both ticks were collected from PCV-3 negative roe deer. Albeit titres or past viraemia below the assay limit of detection could be advocated, one of the ticks was still not engorged. Therefore, a transstadial PCV-3 (or at least its genome) transmission emerges as a potential explanation. This hypothesis brings with it a potential role of other hosts like micromammals that are typically involved in previous stages of *I. ricinus* development (Estrada-Peña & de la Fuente, 2014; Mihalca & Sándor, 2013). The evidence that related species, such as mice, are susceptible to PCV-2, further

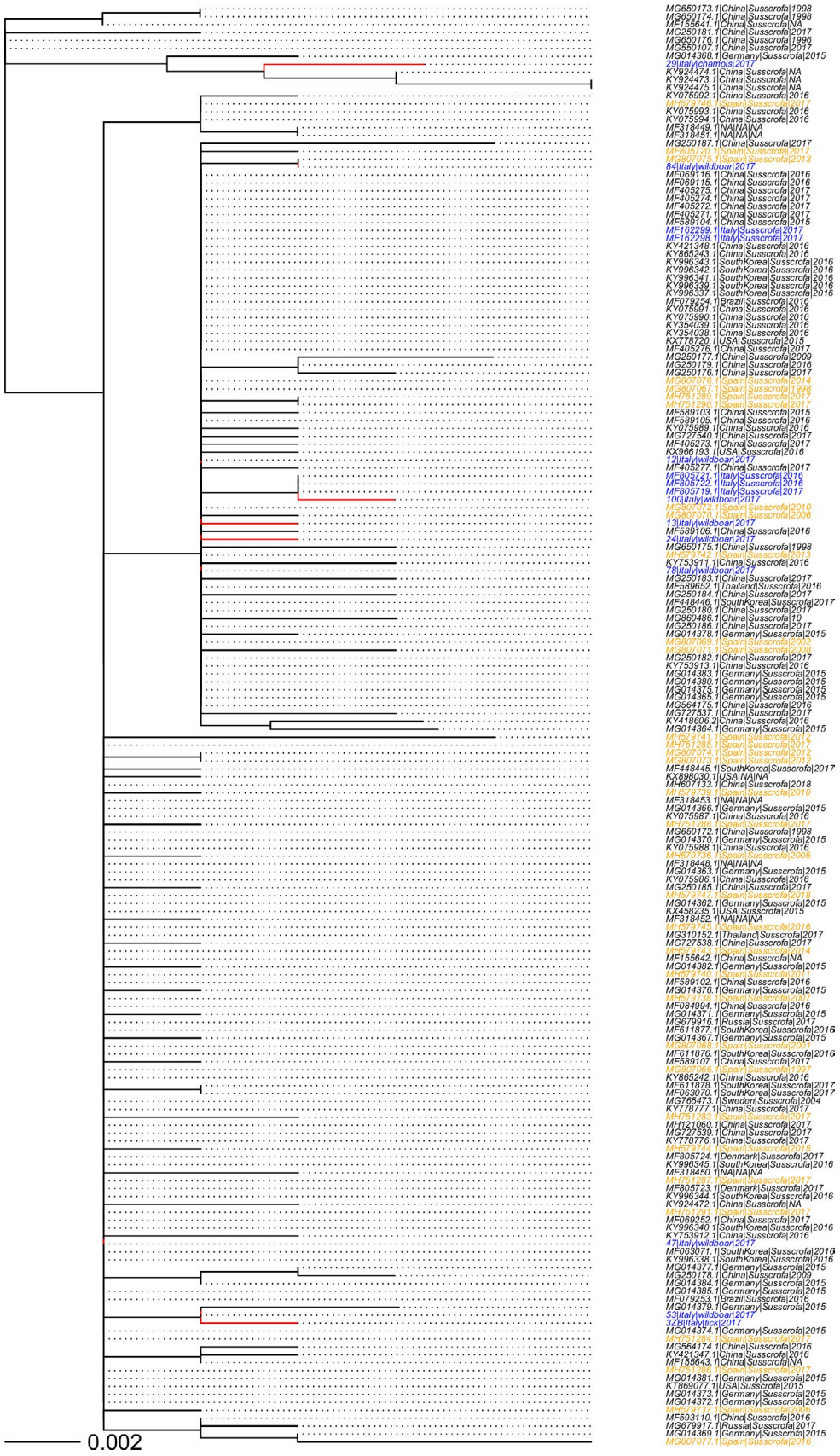

FIGURE 3 Maximum likelihood (ML) Phylogenetic tree reconstructed based on the partial *Rep* gene sequence. Branches corresponding to strains collected from wild animals and ticks are coloured in red. Labels of Italian and Spanish *Porcine circovirus-3* strains are coloured in blue and orange respectively [Colour figure can be viewed at wileyonlinelibrary.com]

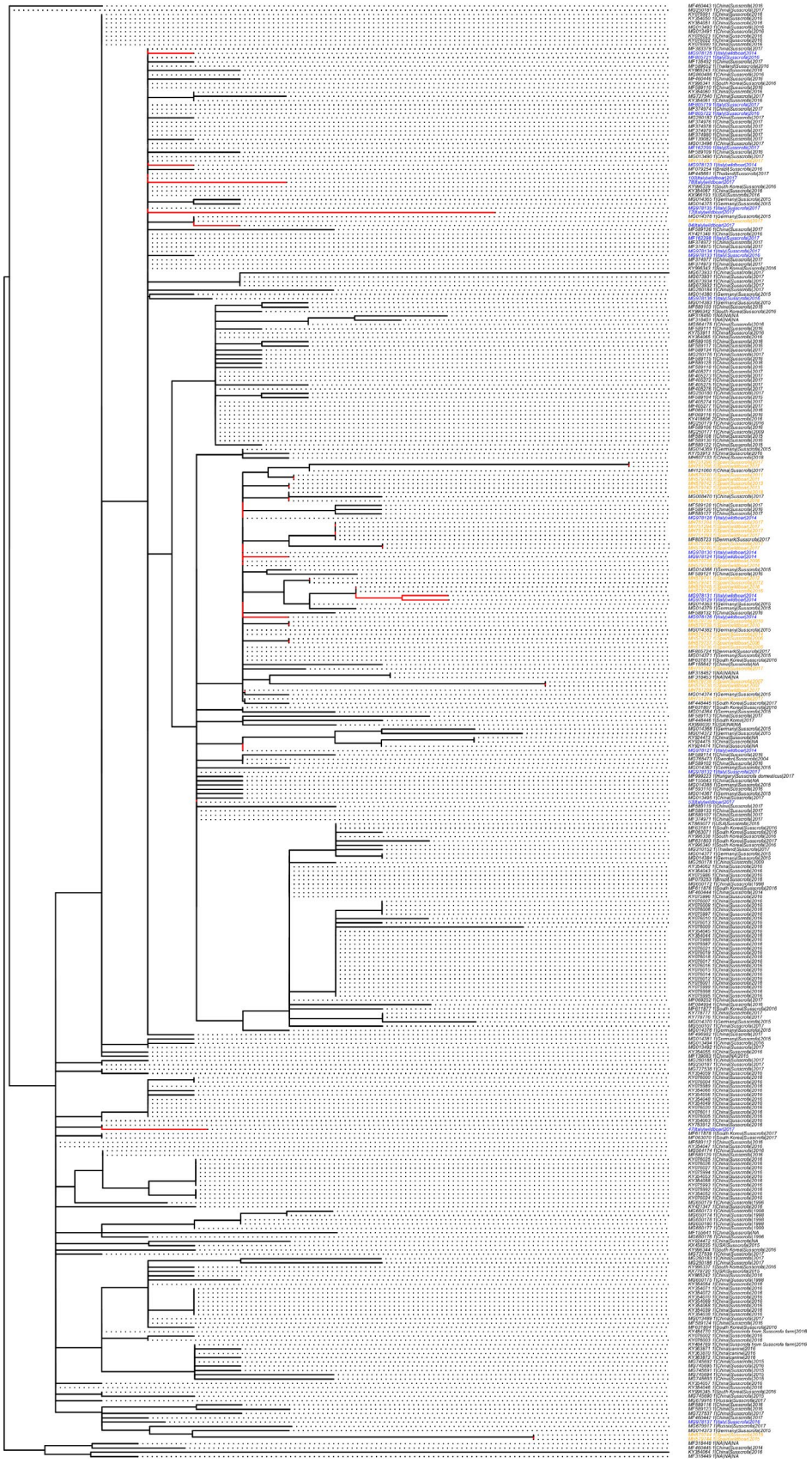

FIGURE 4 ML Phylogenetic tree reconstructed based on the partial *Cap* gene sequence. Branches corresponding to strains collected from wild boar are coloured in red. Labels of Italian and Spanish *Porcine circovirus-3* (PCV-3) strains are coloured in blue and orange respectively [Colour figure can be viewed at wileyonlinelibrary.com]

emphasizes the need to investigate more deeply the role of their wild counterparts in PCV-3 epidemiology.

Clearly, the viral genome detection in ticks is not sufficient to prove their vector competence. Whether the pathogen transmission takes place from the tick to the host or vice versa will require additional experimental studies (Estrada-Peña & de la Fuente, 2014).

The present study, besides confirming the wide PCV-3 circulation in wild boar, highlights its presence in other wild ungulates and related ticks, demonstrating an unexpected wide distribution in wildlife.

The evidences herein reported could encourage the implementation of additional investigations that could provide a further insight into the ecology, epidemiology and potential origin of PCV-3.

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