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Research paper

Phylogenetic analysis of porcine circovirus type 2 in Sardinia, Italy, shows genotype 2d circulation among domestic pigs and wild boars

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ABSTRACT

Porcine circovirus type 2 (PCV2) is associated with multi-factorial syndromes, commonly known as porcine-circovirus–associated diseases, which cause severe economic losses in the swine industry worldwide. Four genotypes (PCV2a, PCV2b, PCV2c, and PCV2d) have been identified. Lately, the prevalence of PCV2d has been increasing in many countries, thereby prefiguring a global replacement of PCV2b. Wild boars are also susceptible to PCV2 infection, with virus prevalence similar to that of domestic pigs. This work was aimed at expanding the knowledge about the molecular epidemiology of PCV2 in Italy. For this purpose, we analysed 40 complete ORF-2 sequences from PCV2 strains isolated from domestic pigs and wild boars in Sardinia (Italy) over a period of 5 years (2009–2013). Phylogenetic and Bayesian analyses were performed on three data sets compiled from DNA sequences over a large geographical area. PCV2b was found to be dominant in Sardinia, whereas no PCV2a and PCV2c were found. This study indicates the presence of genotype PCV2d-2 infecting both domestic and wild pigs, thus confirming its circulation in Italy. Sardinian sequences clustered mostly with Italian isolates and with strains from China, Belgium, Croatia, Taiwan, Korea, and Portugal. Genetic variability of PCV2 in Sardinia appears to be a result of both local viral evolution and different epidemic introduction events.

1. Introduction

Porcine circovirus type 2 (PCV2) is a small non-enveloped DNA virus linked with several syndromes, commonly known as porcine-circovirus–associated diseases. These diseases include post-weaning multisystemic wasting syndrome recognised as a cause of severe economic losses in the swine industry worldwide (Segalés et al., 2005; Segalés, 2012). PCV2, a member of the genus *Circovirus* of the family *Circoviridae*, has a single-stranded circular genome of 1766–1768 nucleotides containing two major open reading frames (ORFs): ORF1, also called the *rep* gene, which encodes two proteins associated with replication (Mankertz et al., 1998), and ORF2, or the *cap* gene, which encodes the immunogenic capsid protein (Nawagitgul et al., 2000). PCV2 has the highest genetic variability among single-stranded-DNA viruses and is characterised by a rate of nucleotide substitution on the order of 1.2×10^{-3} per site per year (Firth et al., 2009), close to that of RNA viruses. In 2008, the EU consortium on porcine circovirus diseases proposed a standardised nomenclature for the PCV2 definition (Segalés et al., 2008) based on pairwise sequence comparison analysis applied to ORF2 nucleotide sequences. By means of a distance threshold of 0.035 to differentiate isolate sequences, three PCV2 genotypes were defined and renamed as PCV2a, PCV2b, and PCV2c. Afterwards, a fourth genotype, named PCV2d, has been described (Guo et al., 2010; Segalés et al., 2013). Recently, isolates previously classified as the PCV2b genotype were included in the PCV2d clade (Xiao et al., 2015), which in turn has been divided in two subtypes: PCV2d-1 and PCV2d-2. PCV2d prevalence seems to have increased so much, especially in Asia (Wei et al., 2013), that a new global genotype shift was suggested (Xiao et al., 2015). In 2015, the method based on genetic distance (pairwise sequence comparison analysis) was questioned (Xiao et al., 2015; Franzo et al., 2015b), and a novel approach based on reference sequences and identification of marker positions was proposed (Franzo et al., 2015b). In 2015, PCV2d in domestic pigs in Italy was reported for the first time and classified as PCV2d subtype 2 (Franzo et al., 2015a).

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Table 1
The year of isolation, host, municipalities, genotype, and Beast IDs of the PCV2 strains analysed in this study. DP: domestic pig; WB: wild boar.

Strain	Year	Host	Municipality/Province	Genotype	Accession number	Beast ID
DP4	2012	Domestic pig	Ala' dei Sardi (OT)	CPV2d	KR559686	142IT@12D
DP6	2011	Domestic pig	S. Gavino Monreale(VS)	CPV2b	KR559687	344IT@11
DP10	2010	Domestic pig	Sinnai (CA)	CPV2b	KR559688	345IT@10
DP11	2012	Domestic pig	Oschiri (OT)	CPV2b	KR559689	346IT@12
DP12	2012	Domestic pig	Burgos (SS)	CPV2b	KR559690	347IT@12
DP13	2012	Domestic pig	Burgos (SS)	CPV2b	KR559691	348IT@12
DP14	2012	Domestic pig	Burgos (SS)	CPV2b	KR559692	349IT@12
DP17	2012	Domestic pig	Ala' dei Sardi (OT)	CPV2d	KR559693	143IT@12D
DP18	2010	Domestic pig	Gergei (CA)	CPV2b	KR559694	350IT@10
DP19	2011	Domestic pig	Benetutti (SS)	CPV2b	KR559695	351IT@11
DP20	2012	Domestic pig	Ala' dei Sardi (OT)	CPV2d	KR559696	144IT@12D
DP21	2013	Domestic pig	Villasor (CA)	CPV2b	KR559697	353IT@13
WB1	2009	Wild boar	Bultei (SS)	CPV2b	KR559698	354IT@09
WB2	2009	Wild boar	Bultei (SS)	CPV2b	KR559699	355IT@09
WB4	2009	Wild boar	Pattada (SS)	CPV2b	KR559700	356IT@09
WB8	2011	Wild boar	Porto Conte (SS)	CPV2b	KR559701	357IT@11
WB9	2010	Wild boar	Porto Conte (SS)	CPV2b	KR559702	358IT@10
WB10	2010	Wild boar	Porto Conte (SS)	CPV2b	KR559703	359IT@10
WB11	2010	Wild boar	Porto Conte (SS)	CPV2b	KR559704	360IT@10
WB12	2010	Wild boar	Porto Conte (SS)	CPV2b	KR559705	361IT@10
WB13	2012	Wild boar	Villanova Monteleone (SS)	CPV2b	KR559706	362IT@12
WB16	2011	Wild boar	Anela/Bultei (SS)	CPV2b	KR559707	363IT@11
WB17	2011	Wild boar	Anela (SS)	CPV2b	KR559708	364IT@11
WB18	2011	Wild boar	Benetutti (SS)	CPV2d	KR559709	145IT@11D
WB19	2011	Wild boar	Benetutti (SS)	CPV2b	KR559710	365IT@11
WB20	2011	Wild boar	Benetutti (SS)	CPV2d	KR559711	146IT@11D
WB21	2011	Wild boar	Benetutti (SS)	CPV2d	KR559712	147IT@11D
WB22	2011	Wild boar	Loiri S.Paolo (OT)	CPV2b	KR559713	366IT@11
WB23	2011	Wild boar	Padru (OT)	CPV2b	KR559714	367IT@11
WB24	2011	Wild boar	Padru (OT)	CPV2b	KR559715	368IT@11
WB25	2011	Wild boar	Santu Lussurgiu (OR)	CPV2b	KR559716	369IT@11
WB27	2011	Wild boar	Trinita' d'Agultu (OT)	CPV2b	KR559717	370IT@11
WB28	2011	Wild boar	Trinita' d'Agultu (OT)	CPV2b	KR559718	371IT@11
WB29	2011	Wild boar	Trinita' d'Agultu (OT)	CPV2b	KR559719	372IT@11
WB30	2011	Wild boar	Aglientu (OT)	CPV2b	KR559720	373IT@11
WB31	2011	Wild boar	Anela/Bultei (SS)	CPV2b	KR559721	374IT@11
WB33	2011	Wild boar	Ala' dei Sardi (OT)	CPV2b	KR559722	375IT@11
WB34	2011	Wild boar	Ala' dei Sardi (OT)	CPV2b	KR559723	376IT@11
WB35	2011	Wild boar	Ala' dei Sardi (OT)	CPV2d	KR559724	148IT@11D
WB36	2011	Wild boar	Padru (OT)	CPV2b	KR559725	377IT@11

PCV2 infection is widespread in pig-producing countries due to a high rate of virus circulation observed in North America, China, and Europe (Segalés et al., 2005). Besides domestic pigs, wild boars are susceptible to infection with PCV2 as confirmed by elevated virus prevalence observed in many European countries (Belgium: Sanchez et al., 2001; Spain: Vicente et al., 2004; Ruiz-Fons et al., 2006; Czech Republic: Sedlak et al., 2008; Italy: Morandi et al., 2010; Germany: Knell et al., 2005; Hungary: Cságola et al., 2006).

Because of the high genetic variability of PCV2, it seems important to study heterogeneity of PCV2 strains to update the information on virus circulation and evolutionary dynamics. Few studies have been published on PCV2 phylogeny in Italy (Franzo et al., 2015a).

The present study was aimed at the analysis of molecular epidemiology of PCV2 strains isolated in Sardinia (Italy) from domestic pigs and wild boars over a period of 5 years (2010 − 2013). To our knowledge, this is the first report of circulation of PCV2 genotypes among wild boars in Italy.

2. Materials and methods

Samples from the spleen, kidneys, and lymph nodes were collected from 120 slaughtered pigs between 2011 and 2013 in different areas of Sardinia. A total of 127 wild boars were sampled during 2009/2010, 2010/2011, and 2011/2012 hunting seasons throughout the Sardinian territory. No clinical data were available for these animals.

Viral DNA was extracted from tissue samples using the High Pure PCR Template Preparation Kit (Roche Diagnostic, Basel, Switzerland) and stored at −20 °C until further analysis. The samples were screened for PCV2 presence by real-time PCR as previously described (Opriessnig et al., 2003). Forty positive samples (domestic pigs, $n = 12$, and wild boars, $n = 28$) were randomly selected, and the complete ORF2 nucleotide sequences were amplified as described by Grau-Roma et al. (2008).

Sequencing was performed on both strands with the same primers that were used for PCR on an ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems, Germany) with a DNA sequencing kit (dRhodamine Terminator Cycle Sequencing Ready Reaction; Applied Biosystems). The consensus sequences were assembled and edited in the BioEdit software, version 7.0.0 (Hall, 1999). ClustalW was utilised for multiple sequence alignment; editing and sequence translation were performed in BioEdit.

Three ORF2 data sets were built. The first data set, for typing, was composed of 40 Sardinian strains (12 domestic pigs and 28 wild boars), 66 non-recombinant Italian sequences (Franzo et al., 2015a), and 63 reference sequences retrieved from GenBank.

The second data set was compiled from 138 and 200 sequences belonging respectively to subtypes PCV2d and PCV2b. This data set was composed of Italian and Sardinian strains and sequences chosen from a previously created database (Franzo et al., 2015a), by selecting the reference strains and variants representative of the temporal and geographic distributions of PCV2b and PCV2d and by reducing the number of clones to improve the phylogenetic signal. This data set was employed for time-scaled phylogenetic reconstruction. The third data set comprised only the 40 Sardinian strains and was used both for time-

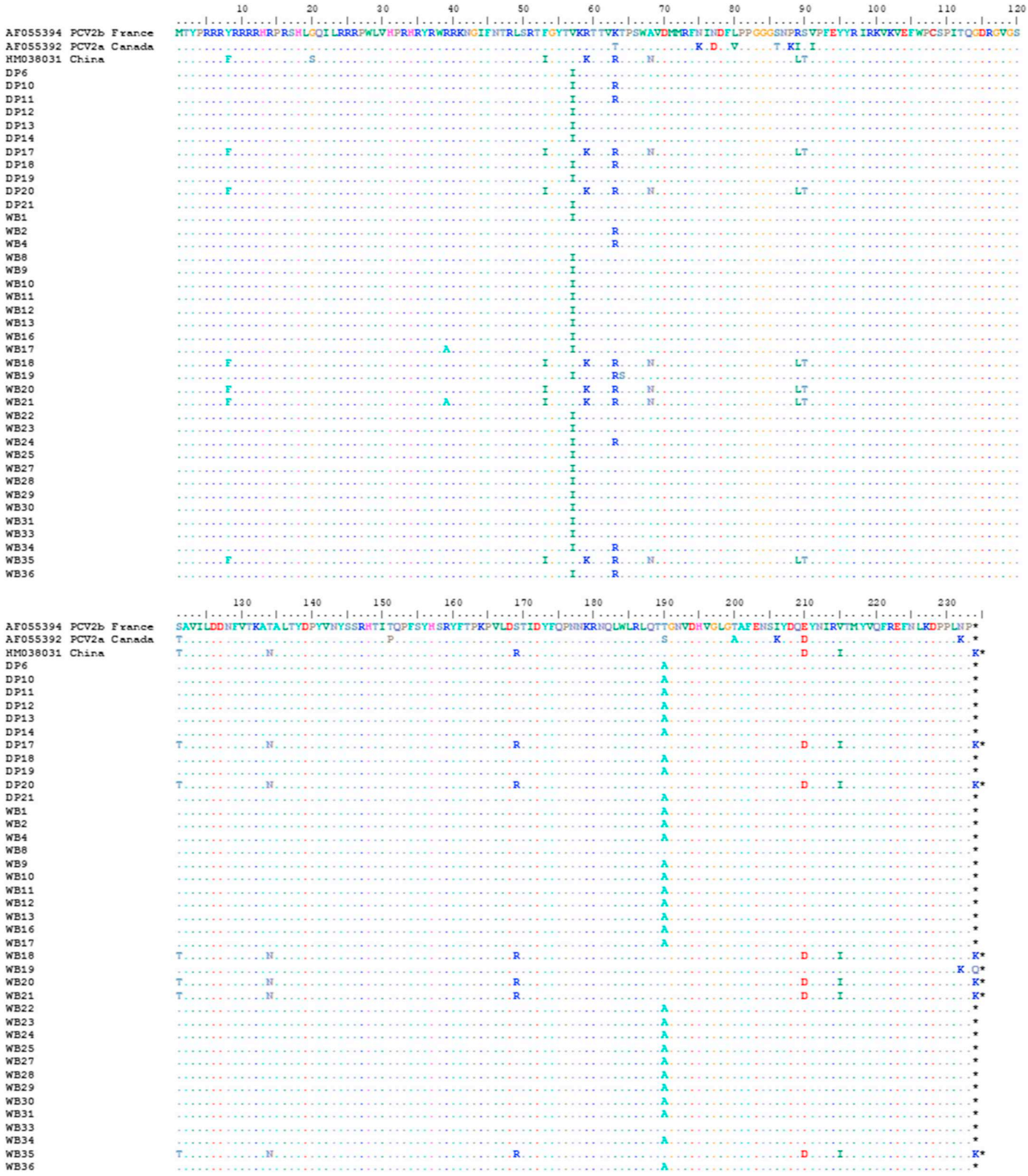

Fig. 1. A cap protein comparison between Sardinian strains and reference strains PCV2a, −2b, and -2c. DP: domestic pig; WB: wild boar.

scaled phylogenetic reconstruction and for population dynamics analysis. All the data sets were checked for recombination by RDP, GENECONV, MaxChi, and 3Seq methods in the RDP4 software (Martin et al., 2015). All the sequences that contained recombinant events detected by more than two methods with statistical significance (p value $< 10^{-5}$ with Bonferroni's correction) were removed from the data sets.

The evolutionary model that best fitted the data for all the data sets was selected by means of JmodelTest v.2.1.7 (Posada, 2008) (freely available at http://darwin.uvigo.es/software/jmodeltest.html). The phylogenetic signal of all the data sets was subjected to the likelihood mapping analysis with 10,000 random quartets in the TreePuzzle software as already described (Zehender et al., 2011). Groups of four

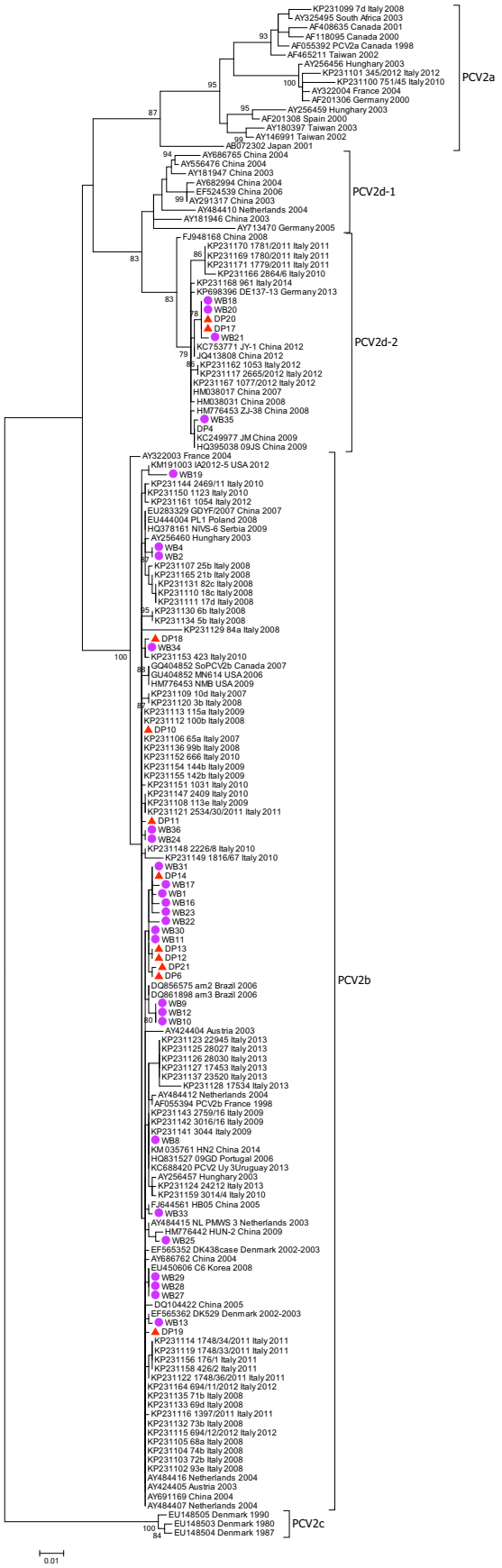

Fig. 2. A maximum likelihood phylogenetic tree inferred from the first data set by the GTR + G + I model of nucleotide substitution. Isolates under study are indicated with different symbols. Red triangles: domestic pigs, purple circles: wild boars. Significant statistical support for the clade in the tree is presented (bootstrap values > 75%). The scale bar indicates 1% of nucleotide divergence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

randomly chosen sequences (quartets) were evaluated. For each quartet, the three possible unrooted trees were reconstructed by the maximum likelihood method via the selected substitution model. If > 30% of the dots fell into the centre of the triangle, then the data were considered unreliable for the purposes of phylogenetic inference.

Phylogeny of the first data set was estimated in MEGA 7 (Tamura et al., 2011) via the maximum likelihood and GTR + G + I model of nucleotide substitution pre-estimated. Statistical support for specific clades was obtained via 1000 bootstrap replicates.

The Bayesian phylogenetic trees of the second and third data sets were reconstructed by means of Beast software v.1.8.2 (http://beast.bio.ed.ac.uk) using the GTR + G + I and TrN + I models of nucleotide substitution, pre-estimated, respectively for the second and third data set. The mean evolutionary rate of the second data set was estimated by the Bayesian Markov Chain Monte Carlo (MCMC) approach in Beast v.1.8.2 (Drummond and Rambaut, 2007). To investigate demographic history, independent MCMC runs were carried out enforcing both a strict and relaxed clock with an uncorrelated log normal rate distribution and one of the following coalescent priors: constant population size, exponential growth, expansion growth, non-parametric smooth skyride plot, Gaussian Markov random field, and non-parametric Bayesian skyline plot. Chains were carried out for at least 200×10^6 generations and sampled every 20,000 steps. The best-fitting models were selected via a Bayes factor (BF; using marginal likelihoods). In accordance with the study by Kass and Raftey (1995), the strength of evidence against the null hypothesis (H0) was evaluated as follows: 2lnBF < 2, no evidence; 2–6, weak evidence; 6–10, strong evidence; > 10, very strong evidence. A negative value indicated evidence in favour of H0. Only values ≥6 were considered significant. The Bayesian maximum clade credibility tree was obtained for the third data set, by means of the mean evolutionary rate, the clock, and demographic models, pre-estimated on the second data set. Chains were carried out for 100×10^6 generations and were sampled every 10,000 steps. For both data sets, convergence of the MCMC method was assessed by calculating the Effective Sample Size for each parameter in Tracer software v.1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Only parameter estimates with ESSs > 200 were accepted. Uncertainty in the estimates was indicated by 95% highest posterior density (95% HPD) intervals. Maximum clade credibility trees were constructed from the tree posterior distributions in the Tree-Annotator software, v.1.5.

Population dynamics were analysed on the third data set by applying the Bayesian skyline plot in the Beast software to estimate changes in effective population size with time.

3. Results

The determined sequences of the 40 strains were deposited in GenBank under the following accession numbers: KR559686–KR559725, as presented in Table 1.

The *cap* gene sequences from domestic pigs showed 93.3–100% similarity among themselves and 92.9–100% similarity with sequences from wild boars. Some sequences from the animals belonging to the same farm or municipality had the same haplotype (data not shown).

The *cap* gene sequences were 702 or 705 bp long. The amino acid (aa) analysis revealed that some strains had a T → A transition at the aa position 700, thus modifying the stop codon and inserting a lysine (K) or glutamine (Q) residue (WB19, sequence accession number

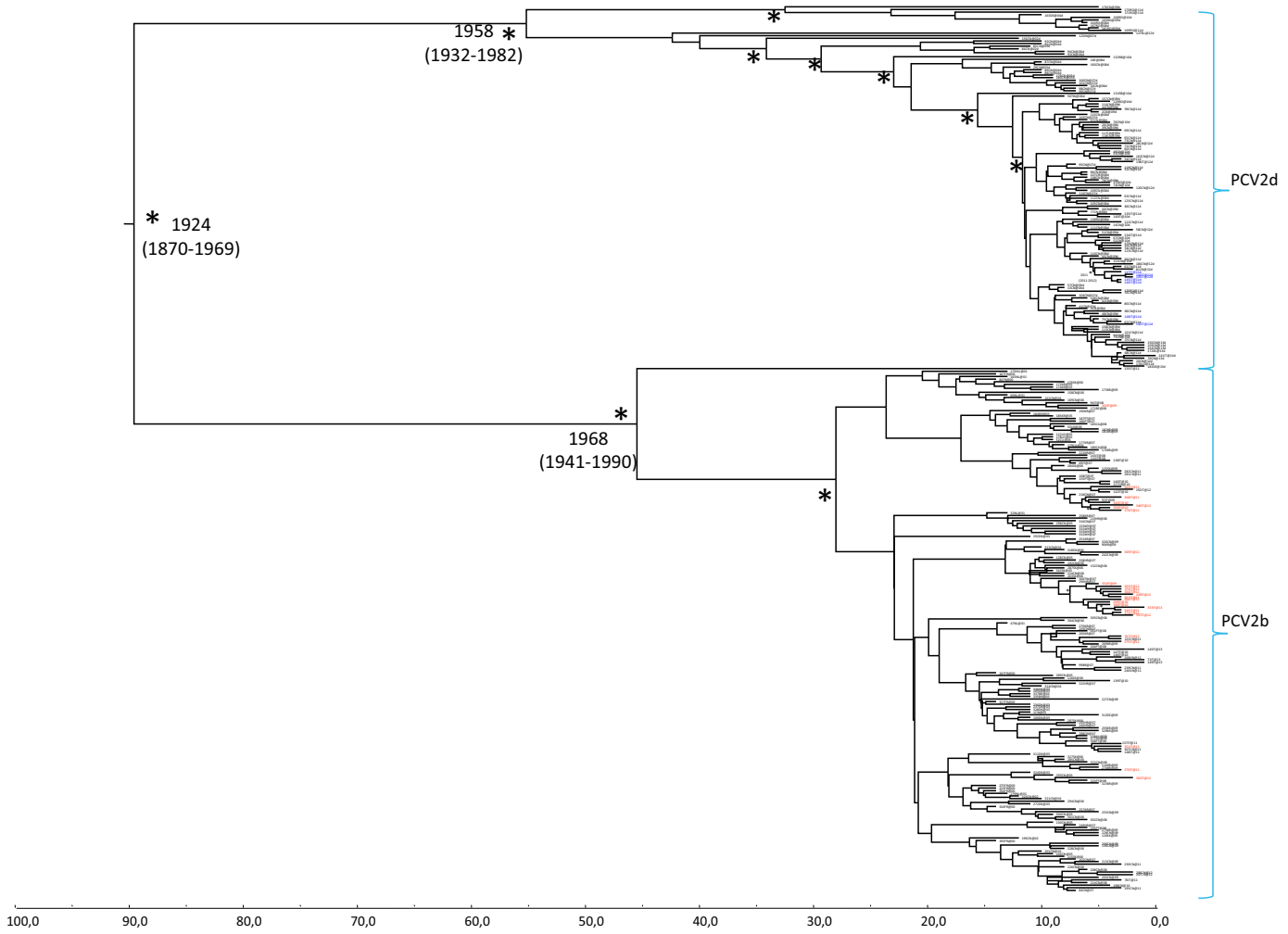

Fig. 3. The Bayesian maximum clade credibility tree of the second data set including 138 and 200 sequences belonging respectively to subtypes PCV2d and PCV2b. (*)Significant statistical support for the clade subtending the branch (PP > 0.90). The scale at the bottom of the tree represents time in years. tMRCA and 95% HPD of tMRCA for the root and the internal nodes are indicated near the nodes. Sardinian strains belonging to the PCV2d genotype are highlighted in blue; Sardinian strains belonging to genotype PCV2b are highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

KR559710). Overall, 17 aa substitutions relative to the reference CPV2b strain (AF055392) were detected, along with other silent nucleotide substitutions. The aa alignment of the sequences analysed in this study with reference strains CPV2a (AF055394), CPV2b (AF055392), and CPV2d (HM038017) is depicted in Fig. 1. The recombination analysis performed in RDP4 produced no evidence of recombination between the sequences included in the data sets being analysed. The phylogenetic signals of the three data sets are reported in Supplementary material 1, showing that the data sets contained sufficient phylogenetic information. The phylogenetic tree obtained from the first data set is illustrated in Fig. 2. Nine strains from domestic pigs and 24 from wild boars turned out to belong to genotype PCV2b, whereas three strains from domestic pigs and four from wild boars belonged to genotype PCV2d-2 (Table 1 and Fig. 2), as confirmed by statistical support of bootstrap values ≥75%. No strains belonging to genotype PCV2a or 2c were found.

The BF analysis of the second data set showed that the relaxed clock fitted significantly better than the strict clock (2 lnBF = 228.3 for the relaxed clock). With the relaxed clock, the BF analysis indicated that the constant-size model was better than the other models (2 lnBF > 1800). The estimated mean value of the evolutionary rate of the ORF2 sequences analysed (second data set) was 8.07×10^{-4} (95% HPD: $6.27–9.90 \times 10^{-4}$). Fig. 3 presents the time-scaled tree calculated from the second data set. The root of the tree dates back to the year 1924 (95% HPD: 1870–1969). Time to the most common ancestor (tMRCA) was predicted to be 1958 for PCV2d with posterior probability (PP) = 1 (95% HPD: 1932–1982) and to be 1968 for PCV2b (95% HPD: 1941–1990, PP = 1). For both genotypes PCV2b and PCV2d, Sardinian sequences clustered in different branches of the tree, suggesting that different introduction events of the virus from other countries could have occurred.

For PCV genotype 2d, two introduction events could have occurred. In both cases, Sardinian viruses clustered mostly with Chinese strains, confirming the results yielded by the maximum likelihood phylogenetic analysis. One of these clusters comprised five out of seven PCV2d Sardinian strains from domestic pigs and wild boars collected in two municipalities ~30 km apart, and its tMRCA was predicted to be 2011 (95% HPD: 2011–2012, PP = 0.99).

Concerning Sardinian PCV2b strains, at least five different clusters were noted. Strain WB2 (355IT@09) clustered with Italian, Belgian, and Chinese strains. Strains DP10, DP11, DP18, WB19, WB34 (345IT@10, 346IT@12, 350IT@10, DP365IT@11, and 376IT@11, respectively) clustered in two close groups along with other Italian sequences. They were isolated from domestic pigs and wild boars and came from three Sardinian provinces. Another cluster consisted of 13 Sardinian strains and was divided into two groups well sustained by high PP (0.99). They

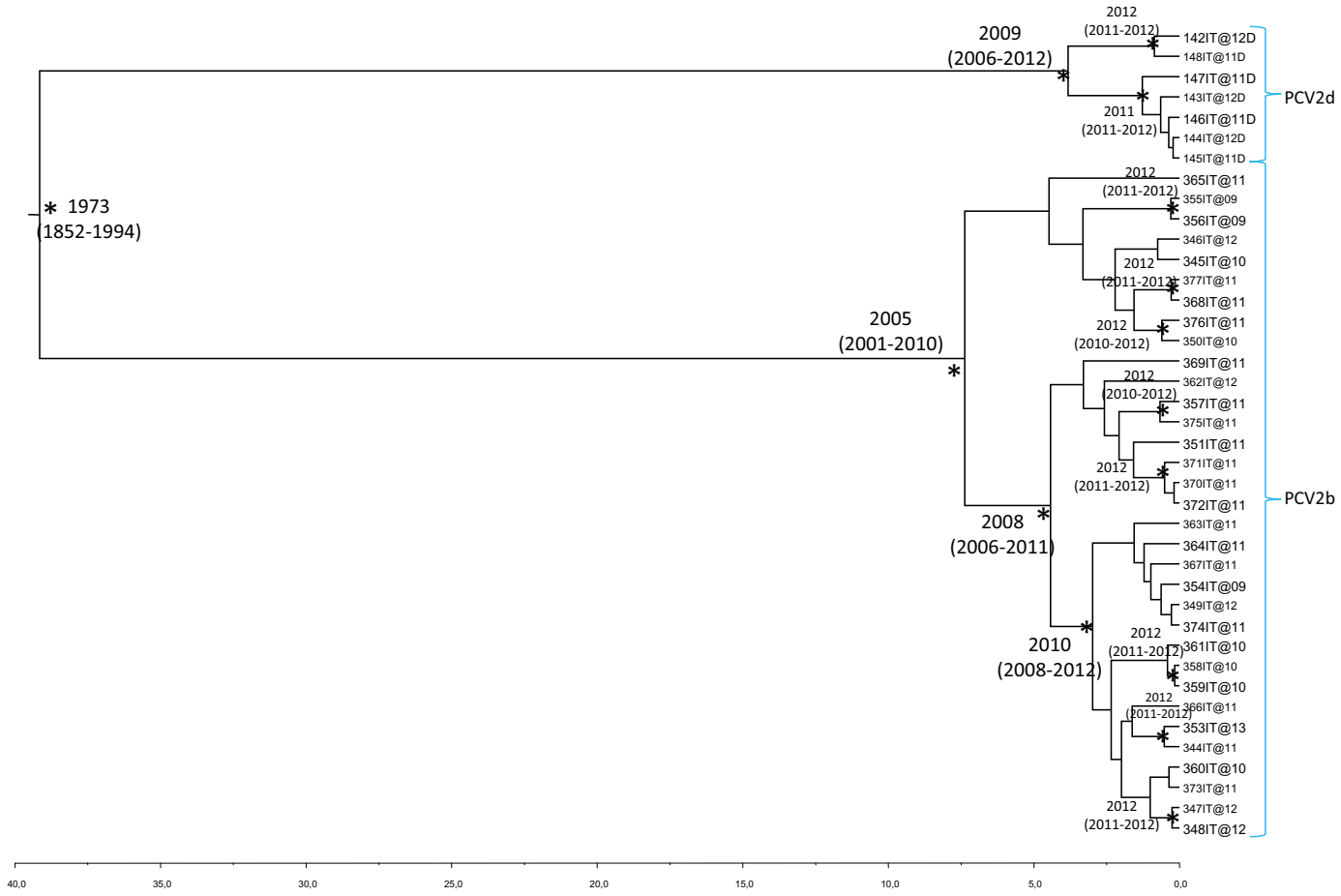

Fig. 4. The Bayesian maximum clade credibility tree of the third data set including 40 PCV2 strains from Sardinia. (*)Significant statistical support for the clade subtending the branch (PP > 0.90). The scale at the bottom of the tree represents time in years. tMRCA and 95% HPD of tMRCA for the root and the internal nodes are presented near the nodes.

were found to be closely related to isolates from Croatia and Taiwan, were isolated from domestic pigs and wild boars, and came from three Sardinian provinces. Strain DP19 (351IT@11) clustered with Chinese and Italian sequences. Other, more disperse sequences from wild boars clustered near isolates from China, Korea, and Portugal, thereby confirming the results obtained in the previous phylogenetic analysis above.

Fig. 4 shows the Bayesian phylogenetic tree calculated from the third data set. The root of the tree dates back to the year 1973 (95% HPD: 1852–1994, PP = 1). tMRCA of the Sardinian sequences was predicted to be 2009 (95% HPD: 2006–2012, PP = 1) for those belonging to the PCV2d-2 genotype and to be 2005 (95% HPD: 2001–2010, PP = 1) for those belonging to the PCV2b genotype. Inside PCV2d, two sub-clades were identified: the first one dates back to the year 2012 (95% HPD: 2011–2012, PP = 0.99), and the second dates back to the year 2011 (95% HPD: 2011–2012, PP = 0.99). Inside PCV2b, a major sub-clade that dates back to 2008 (95% HPD: 2006–2011) was found. This sub-clade was characterised by different epidemic entries, registered in different time intervals. One epidemic entry corresponded to the year 2010 (95% HPD: 2008–2012), and the other epidemic entries corresponded to 2012 (95% HPD: 2010–2012). Outside this sub-clade, three clusters that dated back to 2012 were also identified (95% HPD: 2010–2012).

Fig. 5 presents the results of the Bayesian skyline plot. The analysis revealed a relatively constant phase from the middle of the 1970s until the year 2005. A decrease occurred between 2005 and 2010; after that, a growth phase took place.

4. Discussion

The aim of this study was to analyse the PCV2 strains circulating on the island of Sardinia (Italy) from 2009 to 2013 in domestic pigs and wild boars. There are few studies on the molecular epidemiology and time-scaled phylogeny of this virus in Italy, and this is the first report describing the presence of PCV2 in wild boars. As expected, no strains belonging to PCV2c were found in this study. Indeed, PCV2c, which was detected only in Danish archive samples (Dupont et al., 2008), had been considered extinct (Xiao et al., 2015) until a recent report about Brazilian feral pigs (Franzo et al., 2015c). The PCV2b genotype was found to be dominant in Sardinia, whereas only one PCV2a strain was detected (GenBank accession number KR559726) and subsequently excluded from the phylogenetic analysis because of the incomplete ORF2 sequencing. Despite the limited time interval of our study, these findings appear to be in agreement with several reports indicating that a genotype shift from PCV2a to PCV2b occurred approximately in 2003 (Franzo et al., 2016; Segalés et al., 2013; Grau-Roma et al., 2011). In line with other findings (Franzo et al., 2016), our results confirmed the presence of the PCV2d-2 genotype in Italy, infecting both domestic pigs and wild boars. It has been suggested that a second major genotype shift from PCV2b to PCV2d is currently in progress (Franzo et al., 2016); likewise, PCV2d could be expected to become dominant in Italy in the near future as already seen in China (Xiao et al., 2015).

The results of Beast analysis of the second data set (Fig. 3), composed of sequences belonging to subtypes PCV2d and PCV2b, showed an estimated mean evolutionary rate of ORF2 of 8.07×10^{-4} (95%

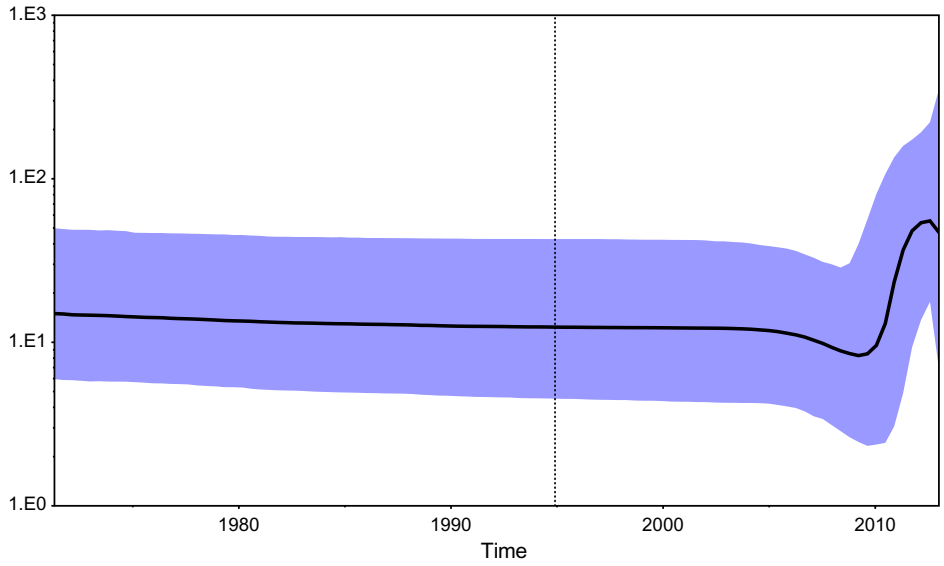

Fig. 5. The Bayesian skyline plot of the third data set including 40 PCV2 strains from Sardinia. On the Y-axis, the effective number of infections is presented. Time is represented by the X-axis. The coloured area corresponds to the credibility interval based on 95% HPD.

HPD: 6.27–9.90 × 10^{-4}). These findings are consistent with the results of other authors (Franzo et al., 2016; Xiao et al., 2015) that were obtained by analysing each subtype independently. tMRCA was predicted to be 1958 (95% HPD: 1932–1982) for PCV2d and 1968 (95% HPD: 1941–1990) for PCV2b, thus confirming the results of some authors (Franzo et al., 2016), but in contrast to another study (Xiao et al., 2015), which dated PCV2d tMRCA back to the beginning of the 1990s. A lower number of sequences used by Xiao et al. (2015) could have possibly yielded the earlier tMRCA estimate for PCV2d.

The first Italian and Sardinian PCV2d sequences were respectively found in 2010 (Franzo et al., 2015a) and 2011. All the Italian strains appeared to be closely related to the main PCV2d-2 haplotypes (Fig. 3) that were first identified in China in 2007 (Guo et al., 2010). The results of the Beast analysis of the third data set, including only sequences from Sardinia (Fig. 4), revealed that tMRCA for PCV2d is 2009 (95% HPD: 2006–2012, PP = 0.99) indicating a recent introduction. As evidenced in Figs. 1 and 4, two introduction events could have occurred for PCV2d in Sardinia. The strains clustered with Chinese isolates, as already seen for viral strains from other Italian areas, albeit clustering in different clades (Franzo et al., 2016). The PCV2d genotype was first identified in China, but further studies have highlighted its presence in Switzerland in 1998 (Franzo et al., 2016). The hypothesis of the major role played by China in the spread of PCV2d proposed by Xiao et al. (2015) is controversial because China is mainly a swine importer (Vidigal et al., 2012), whereas importation of livestock and meat products from China is forbidden in many countries (Wang, 2006). Some authors point out the peculiar resistance features of the virus to different environmental conditions; these data open up multiple possibilities involving passive vectors, including human beings (Li et al., 2010). In a recent study on macro-areas with a more balanced data set, researchers proposed a new scenario where PCV2a and PCV2d most likely originated in Europe and PCV2b in China (Franzo et al., 2016).

Concerning PCV2b Sardinian strains (Figs. 1 and 4), at least eight introduction events could have occurred. Our sequences clustered mostly with those from Italian isolates and with strains from China, Belgium, Croatia, Taiwan, Korea, and Portugal. The presence of a large cluster exclusively composed of Sardinian strains (Fig. 1) suggests that the variability of PCV2 in Sardinia is due to local viral evolution, not only to different epidemic entries. Recent introduction events were also identified for PCV2b Sardinian strains.

Pig farming in Sardinia is not a major industry and consists of small and family-operated farms producing mostly piglets (5–8 kg) for human consumption (Mur et al., 2016). Live animals are imported from other Italian regions and from European countries (National data base - BDN, 2019). Moreover, a large number of pigs are bred illegally in a free-ranging system, posing a substantial risk of the spread and persistence of infectious diseases.

The presence of African swine fever (Mur et al., 2016; Cappai et al., 2018), which limits the trade to imports only, and the Sardinian unique geographical features generated new PCV2 variants (not detectable in other countries) possibly with the help of high selective pressure caused by frequent culling. The presence of the same PCV2d and PCV2b genetic variants in domestic pigs and wild boars, found in different areas separated by up to 30 km, raises issues regarding to what extent wild boars may be involved in PCV2 transmission. Nevertheless, the epidemiological role of wild boars is still controversial, and one could hypothesise, as already suggested for African swine fever in Sardinia (Jurado et al., 2018), that PCV2 spreads from domestic pigs to wild boars. Indeed, several authors agree on the secondary role of wild boars in African swine fever transmission and on the importance of the interaction between free-ranging pigs and wild boars for the persistence of this disease (Mur et al., 2016; Laddomada et al., 2019).

Although the available vaccines based on genotypes PCV2a and -2b seem to cross-protect against all PCV2 genotypes (as reviewed by Karuppannan and Opriessing, 2017), including PCV2d, they may not confer protection under field conditions and in the presence of other cofactors, thus possibly being able to minimise PCV2d replication but not abrogate it. The spread of new genotypes and variants could be responsible for immunity failure, thereby underscoring the need for constant evaluation of genetic variability through molecular studies to prevent and control PCV2 infection.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.03.013.

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