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Molecular study of porcine circovirus type 2 circulating in Portugal

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ABSTRACT

Porcine circovirus type 2 (PCV2) belongs to the genus *Circovirus* of the *Circoviridae* family and has been associated with a disease named postweaning multisystemic wasting syndrome (PMWS). In Portugal the disease caused severe problems mainly between 1995 and 2003, but is nowadays less evident probably as the result of vaccination programmes initiated in 2007. This study reports the molecular analysis of 22 PCV2 strains obtained from domestic pigs originated from different regions of Portugal. One hundred and seventy-seven samples from PCV2 suspected cases received between 2003 and 2010 were analysed, 79 (44.6%) of which tested PCV2 PCR-positive. The entire genomes of 22 viruses were amplified and sequenced. Two major groups were observed, according to the genome length and in single nucleotide polymorphisms. The nucleotide similarity found among the PCV2 strains ranged between 94% and 99%. The Bayesian inference of phylogeny based on the genome of the strains studied revealed the existence of two distinct genetic groups consistent with the nucleotide sequence observations. The majority of the strains branched in genotype PCV2b, while six strains with a common geographical origin obtained between 2007 and 2009, were included in genotype PCV2a. The analysis performed with *cap* and *rep* genes confirmed that *cap* is a reliable alternative to full-length genome based phylogenetic studies of PCV2.

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1. Introduction

Porcine circoviruses (PCV) are spherical, small (16–18 nm of diameter) and non-enveloped viruses belonging to the genus *Circovirus* of the *Circoviridae* family that contain a single-stranded circular DNA genome (Finsterbusch and Mankertz, 2009).

Two types of PCV have been recognised. PCV type 1 (PCV1), non-pathogenic, was first detected in 1974 as a contaminant of the porcine kidney PK15 cell line (Dulac and Afshar, 1989; Tischer et al., 1982). PCV type 2 (PCV2) was initially identified in a Canadian swine herd in 1991 and has been implicated as the etiological agent of a disease named postweaning multisystemic wasting syndrome (PMWS) (Harding and Clark, 1997). This emerging disease in pigs is clinically characterised by growth retardation, paleness of skin, dyspnoea, enlargement of inguinal lymph nodes and occasionally jaundice and diarrhoea. The mortality rate may vary from 1% to up 40% (Muirhead, 2002). Recent studies have shown that PCV2 is crucial but by itself not a sufficient factor to the development of PMWS (Roca et al., 2004). Clearly the scope of possible PCV2 associated swine disease is expanding, as other diseases have been associated to PCV2, namely porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, proliferative and necrotizing pneumonia, congenital tremors and central nervous system diseases (Harding, 2004).

The genome of PCV2 with about 1768 nucleotides is composed by two intergenic regions flanked by two head-to-head arranged open reading frames (ORFs). ORF1 (*rep* gene) in the viral strand encodes two replicase proteins, Rep and Rep′. ORF2 (*cap* gene) occurs in the complementary strand of the double stranded replicative form and encodes the major structural capside protein, Cap. About ten other ORFs were detected in the genome of PCV2 (Hamel et al., 1998), but as far as we know, only ORF 3 located in the complementary strand has proven to be translated into protein which seems to play a role in virus-induced apoptosis (Liu et al., 2007).

Located in the nontranslated intergenic region of 83 nucleotides between the 5′-ends of *rep* and *cap* genes is a potential stem-loop structure containing a nonanucleotide motif (AAGTATTAC) in the apex, where rolling circle viral replication initiates (Mankertz et al., 2004; Phenix et al., 2001; Todd et al., 2001).

PCV2 infection have been reported worldwide and is nowadays considered to have a serious economic impact in swine industry (Kim et al., 2009). The disease has been also reported in Portugal, where severe problems were observed mainly between 1995 and 2003 (Segalés, 2007), but to date no molecular data was available for Portuguese strains of PCV2.

This study reports the nucleotide sequence analysis of 22 PCV2 strains obtained from naturally infected pigs (*Sus scrofa* ($n = 21$) and *Sus ibericus* ($n = 1$)) in Portugal between 2003 and 2010. The

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genetic relationship of these strains was examined and phylogenetically compared with those of porcine circoviruses obtained in different continents that were available in the database.

2. Materials and methods

2.1. Source of material

One hundred and seventy-seven samples from domestic pigs with PCV2-associated diseases originated from several regions of Portugal entered our laboratory for routine or differential diagnosis mainly with porcine reproductive and respiratory syndrome (PRRS). Lymph nodes, lung, kidney, intestine, skin, spleen and tonsil were collected and pooled for DNA extraction performed using a nucleic acid extraction workstation BioSprint 96 (Qiagen), according to manufacturer's instructions. Extracted DNA was used as template in a PCR reaction to investigate the presence of PCV2. From 2003 to the end of 2006, samples were assayed by the method described by Ouardani et al. (1999) for the amplification of a 493 bp fragment of the *cap* gene. Since 2007 onward, a real-time PCR developed in our laboratory has been used (unpublished). Primers and probe were designed on the *rep* gene allowing the amplification of a 144 bp conserved region. The reaction was performed with 500 ng of total DNA, 25 pmol of each primer and 5 pmol of probe, using FastStart PCR Master Kit (Roche), according to the manufacturer's protocol. The amplification program included an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 52 °C for 30 s and extension at 60 °C for 20 s.

2.2. Amplification and sequencing of the complete genome of PCV2

For molecular and phylogenetic analysis, 22 out of the 79 PCV2 PCR-positive samples obtained between 2003 and 2010 were selected randomly, but including samples from different farms and years. Twenty-one originating from 21 intensive pig farms (*S. scrofa*) and one from free-range pig production (*S. ibericus*). The full-length genome of PCV2 was amplified by PCR using overlapping primers described by Fenaux et al. (2002). The reaction was performed with 500 ng of total DNA and 25 pmol of each primer, using High Fidelity PCR Master (Roche), according to the manufacturer's protocol. The amplification program included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 3 min. A final extension was performed at 72 °C for 7 min and the expected fragments of about 1.8 kb were purified by agarose gel electrophoresis. DNA sequencing was performed with the amplifying and internal primers described in Table 1 using a Big-Dye Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The nucleotide sequences of the 22 PCV2s were determined on an automated 3130 Genetic

Table 1
Nucleotide sequences of primers used to determine the full-length genomes of PCV2.

Name	Sequence	Location (nt)	T_{hib} (°C)
RepF	ATGCCCAGCAAGAAGAATGG	51–70	60
S2[a]	AAGGTGGGTGTTCACGCT	95–112	52
AS2[a]	GTGTACATTGGTCTTCCAATCA	560–539	54
RepR	GTAATTTATTTCATATGGAAATT	992–970	60
CapR	AGGGTTAAGTGGGGGGT	1036–1052	60
S4[a]	CACGGATATTGTAGTCCTGGT	1093–1113	58
ORF2F	TAGGTTAGGGCTGTGGCCTT	1323–1342	57
EcoR	GAATTCTGGCCCTGCTCCCCATCAC	1426–1401	60
EcoF	GAATTCAACCTTAACCTTTCTTATTCT	1421–1447	60
AS4[a]	CCGCACCTTCGGATATACTGTC	1586–1565	60
CapF	ATGACGTATCCAAGGAGGCG	1735–1716	60

[a] Primers described by Ouardani et al. (1999).

Analyzer system (Applied Biosystems). In order to avoid possible errors introduced by the primers, the region encompassing the primers binding sequences was amplified with primers S2/AS2 (Ouardani et al., 1999) and sequenced. The complete genomic sequences of the PCV2 strains obtained were submitted to GenBank database and were given the accession numbers HQ831519 to HQ831540 (Table 2).

2.3. Bayesian analysis

Multiple alignments of the complete nucleotide sequences were generated by CLUSTAL W (Thompson et al., 1994) and the result was converted to the NEXUS format using Mesquite software (Maddison and Maddison, 2009). The phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software that uses a simulation technique called Markov chain Monte Carlo (or MCMC) to approximate the posterior probabilities of trees (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). MrBayes analysis was performed using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis was run for 10^6 generations (ngen = 10^6) with four chains of temperature (nchains = 4) and each chain was sampled every 10th generations (samplefreq = 10).

3. Results and discussion

3.1. Epidemiology

Between 2003 and 2010, 177 samples from domestic pigs exhibiting symptoms or lesions compatible with PCV2 infection, originated from several regions of Portugal mainland and from Azores islands were analysed. Seventy-nine samples (44.6%) were PCV2 PCR-positive.

The number of samples tested in each year and the results obtained can be observed in Fig. 1. Despite slight variations, the total number of samples analysed decreased gradually until 2008 and abruptly since then, which may be the result of an effective vaccination programme initiated in 2007 that lead to the control of more pathogenic forms of the disease. Vaccination against PCV2 is nowadays a common practice in Portugal and it is estimated that about 50% of the national swine population is vaccinated with complete or subunit PCV2 vaccines (Dr. J. Lopes Jorge, Merial Portuguesa, personal communication). On the other hand, the percentage of positive samples steadily increased after 2005 probably reflecting a higher accuracy of the veterinarians for the clinical recognition of PCV2 infections.

For molecular characterisation, 22 isolates were selected in order to include samples from different farms and years. Epidemiological data were obtained, such as the geographic location of the respective swine farms, the type of production system adopted and the country from which live animals or semen were imported. The information is summarised in Table 2.

3.2. Relevant features of the PCV2 genome

The full-length genomes of 22 PCV2 strains detected in Portugal were amplified with overlapping primers, sequenced and aligned revealing the characteristic genomic structure of circoviruses (Fig. 2). The nucleotide numbering was adopted from Todd et al. (2001), with position 1 corresponding to the "A" residue that occurs at the nucleotide position 8 in the nonanucleotide located at the apex of a potential stem-loop structure. The stem-loop structure, common in other circoviruses, appears in a putative intergenic region of 83 nucleotides (Table 3) and comprises the motif AAGTATTAC that constitutes an essential cis-acting element

Table 2
PCV2 strains used in this study. Year of collection, geography origin of the farms, type of production system used, importation from third countries, accession numbers and phylogenetic subgroups are indicated.

Strains	Year of collection	District of origin	Type of swine production system	History of importation from aboard	Origin of the animals' ancestors			Accession Nos.	Phylogenetic subgroup
					P[a]	GP[b]	GGP[c]		
14338-03	2003	S. Miguel, Azores	Close cycle	Rare	GB and DECA (board semen)	Unknown	Unknown	HQ831524	PCV2b
15152-03	2003	Leiria	Close cycle	Rare	ES	–	–	HQ831530	PCV2b
5742-04	2004	Setúbal	Close cycle	No	NA[d]	NA[d]	NA[d]	HQ831521	PCV2b
26216-04	2004	Lisboa	Close cycle	No	NA[d]	NA[d]	NA[d]	HQ831531	PCV2b
42034-05	2005	Évora	Unknown	Unknown	Unknown	Unknown	Unknown	HQ831529	PCV2b
55935-05	2005	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	HQ831532	PCV2b
34765-06	2006	Lisboa	Unknown	Unknown	Unknown	Unknown	Unknown	HQ831527	PCV2b
41060-06	2006	Lisboa	Close cycle	No	NA[d]	NA[d]	NA[d]	HQ831528	PCV2b
12722-07	2007	Évora	Fattening house	Unknown	Unknown	Unknown	Unknown	HQ831523	PCV2b
18359-07	2007	Beja	Free ranging	No	NA[d]	NA[d]	NA[d]	HQ831535	PCV2b
20750-07	2007	Evora	Fattening house	Yes	PT	PT	ES	HQ831525	PCV2b
32042-07	2007	Lisboa	Unknown	Unknown	Unknown	Unknown	Unknown	HQ831526	PCV2b
39360-07	2007	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831533	PCV2a
4221-08	2008	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831534	PCV2b
5693-08	2008	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831520	PCV2a
8296-08	2008	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831522	PCV2a
11001-08	2008	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831536	PCV2a
11751-08	2008	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831519	PCV2a
27168-08	2008	Leiria	Fattening house	Yes	PT	Unknown	NL	HQ831537	PCV2b
26633-09	2009	Lisboa	Unknown	Unknown	Unknown	Unknown	Unknown	HQ831538	PCV2b
20683-09	2009	Setúbal	Fattening house	Yes	PT	PT	ES	HQ831539	PCV2a
10810-10	2010	Leiria	Reproduction Centre	Yes	FR	FR	FR	HQ831540	PCV2b
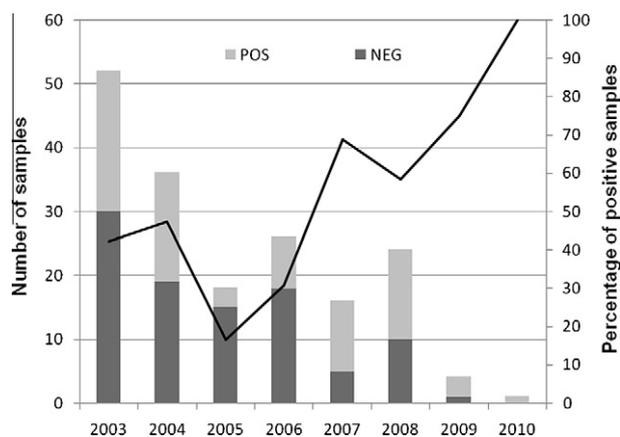
Fig. 1. Number of samples tested for PCV2 between 2003 and 2010 represented in columns. Positive results are represented in light grey on the top of each column. Line represents the percentage of positive samples.

required for DNA replication (Mankertz et al., 1997). A recent study demonstrated that only part of the conserved sequence (TATTAC) is essential for the cleavage of the viral strand (Steinfeldt et al., 2006). Indeed, although circoviruses infecting different species have different nonanucleotide motifs, the last six nucleotides of the sequence are conserved among them (Stewart et al., 2006).

As already reported (Mankertz et al., 2003), two pentamer (CACCT) and three hexamer (CGGCAG) repeats were found adjacent to the stem-loop (Table 3). These sequences are thought to play a role in virus replication, perhaps by acting as putative binding sites for the Rep protein. Indeed, the minimal binding site (MBS) for Rep was mapped to the right leg of the stem-loop and the two inner hexamer repeats, while binding of Rep′ required only the presence of two hexamer repeats (Steinfeldt et al., 2001).

Three polyadenylation signals, two direct (AATAAA) and one inverted (TTTATT) were found in all strains studied. Also five potential glycosylation sequences described by Hamel et al. (1998) as N-X-S or N-X-T were detected in the genome of the 22 strains circulating in Portugal that were analysed (Table 3).

Based on the nucleotide sequence and on the length of the circular genome the strains analysed were separated in two groups, corresponding to genotypes PCV2a and PCV2b (Cortey et al., 2011; Segales et al., 2008). Genotype PCV2b includes sixteen strains with 1767 nucleotides length, while genotype PCV2a includes the other six strains and is characterised by an insertion of a thymine in nucleotide position 1042, giving a total of 1768 nucleotides. Strain 11751-08 has a 1766 nucleotide-long genome due to the deletion of two nucleotides at positions 998 and 999 located in the nontranslated intergenic region between the 3′-ends of *rep* and *cap* genes. However it was included into genotype PCV2a since it has the characteristic insertion at position 1042 and shares 99% similarity with the other members of this genotype (Fig. 2).

The nucleotide variation observed between the two genotypes was calculated to range between 4% and 6%, suggesting different origins for these isolates. Within genotype PCV2a, 99% nucleotide similarity was observed while among the members of genotype PCV2b, 97–99% similarity was found. The higher variability observed in this genotype is mainly due to strains 41060/06 and 55935/05 that show, respectively, 97–98% and 98% similarity with all the strains analysed. The remaining fourteen viruses showed 99% similarity among them.

3.3. ORF 1

ORF 1, also named *rep* gene is present on the viral strand and encodes the major PCV2 proteins Rep and Rep′ that are responsible

for viral replication (Mankertz et al., 2004). The Rep protein is translated from the full-length transcript, while the Rep′ results from a spliced transcript (Finsterbusch and Mankertz, 2009). The positions of the first and last nucleotide of this ORF, as well as the estimated molecular weight of Rep protein of the strains circulating in Portugal are indicated in Table 4.

As already described for other strains, the Rep sequence of the strains analysed contains three conserved motifs typical for rolling circle replication (RCR), FTLNN (aa 19–23), GRTPHLQGF (aa 53–61) and YCSK (aa 96–99), and a P-loop for dNTP binding, GPPGCGKS (174–181). Also three additional conserved motifs, WWDGY (aa 202–206), DDFYGWLP (aa 215–222) and DRYP (aa 231–234), of no known function were detected in the genome of the strains analysed (Johne et al., 2006; Mankertz et al., 1998; Phenix et al., 2001; Stewart et al., 2006; Todd et al., 2007).

The nucleotide similarity observed among the *rep* gene of the 22 strains studied varied between 96% and 100%, while the amino acid similarity of the Rep protein ranged from 98% to 100%.

3.4. ORF 2

ORF 2 (*cap* gene) is present on the complementary strand and encodes the capsid protein Cap. Both the location of this ORF and the characterisation of Cap protein of the strains circulating in Portugal are indicated in Table 4.

This protein contains a nuclear localisation signal (NLS) consisting of 41 aminoacid residues at N-terminus (Shang et al., 2008), that directs viral DNA through the nuclear membrane to the nucleus for DNA synthesis (Heath et al., 2006). Also present in the Cap protein is a highly basic arginine-rich N-terminal region, probably necessary for packaging of the viral genome (Crowther et al., 2003).

Regarding ORF 2, the similarity observed among strains studied ranged from 90% to 99% and between 88% and 100%, respectively at nucleotide and amino acid levels, lower than that observed in the *rep* gene and Rep protein. As an outer protein, Cap is more subject to immunological pressure than Rep.

3.5. Other ORFs

The previously reported ORF 3 was detected in the complementary strand of all the stains analysed (Table 4). The protein encoded by this ORF seems to play a role in virus-induced apoptosis and in viral pathogenesis since ORF 3 protein has been found to interact with pPirh2, a protein responsible for maintaining the tumour suppressor p53 at low level. This interaction leads to an increase of p53 expression, resulting in apoptosis (Liu et al., 2007).
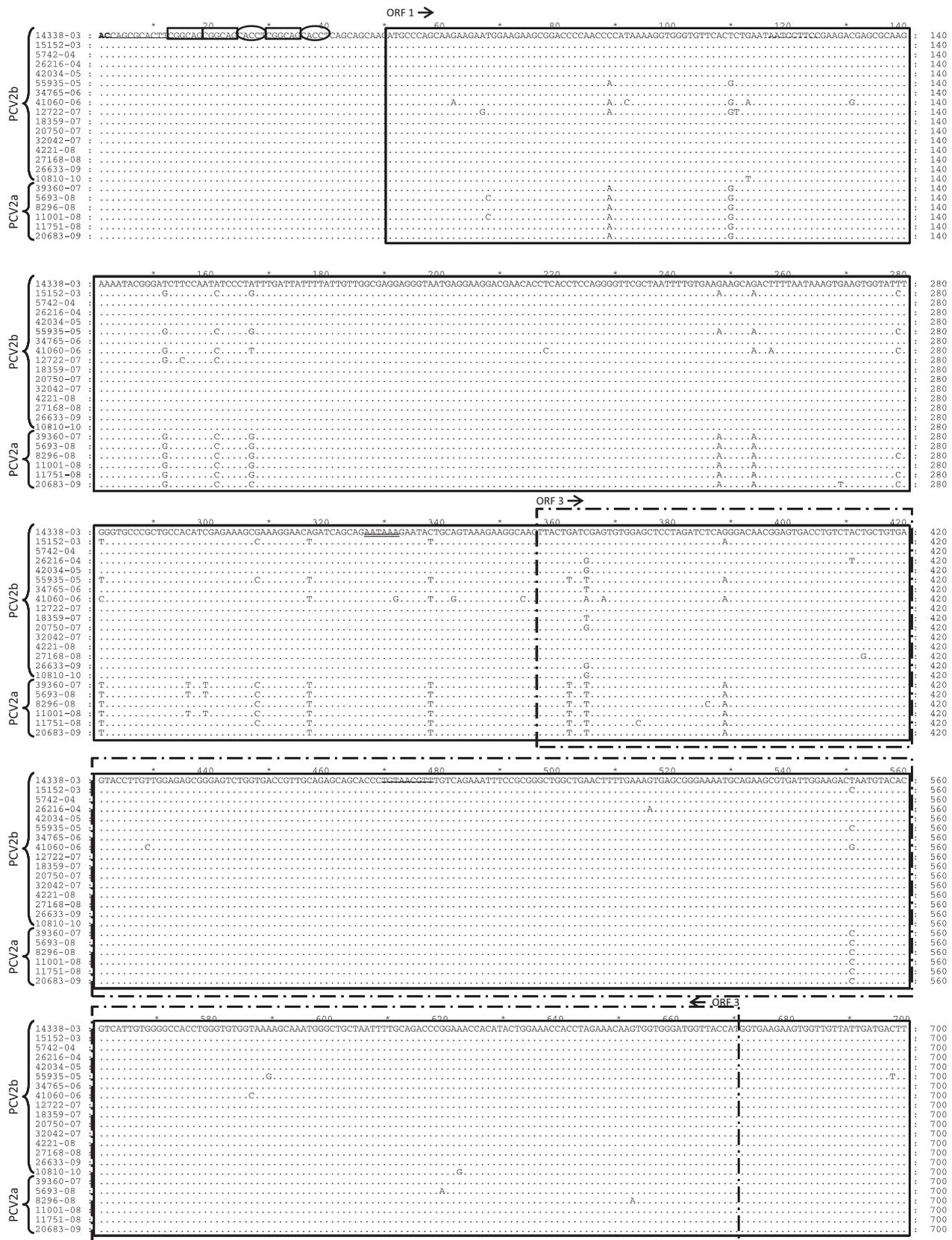
The strains circulating in Portugal showed between 97% and 100% nucleotide similarity in ORF 3, while in the encoded protein, 93–100% amino acid similarity was detected.

Eight shorter ORFs reported in the genome of PCV2, none of them proven to be expressed (Hamel et al., 1998), were also identified in the Portuguese strains.

3.6. Phylogenetic analysis

For PCV2 classification we adopted the previous proposed criteria in which PCV2 strains are divided in three major genotypic groups, PCV2a, PCV2b and PCV2c (Cortey et al., 2011; Segales et al., 2008). Two other genotypes, PCV2d and PCV2e, proposed by Wang et al. (2009) were not considered as genotype by Cortey et al. (2011) and were not included in this study. To date PCV2c genotype integrates only three Denmark strains from 1980, 1987 and 1990 (Segales et al., 2008).

The Bayesian analysis performed with the complete nucleotide sequences of the 22 strains analysed in this study and 28 strains

Fig. 2. Nucleotide alignment of PCV2 strains circulating in Portugal. The *rep* and *cap* genes are represented by filled and dashed boxes, respectively. ORF 3 is represented by a dot–dash box. The stem-loop structure and the nonanucleotide are underlined, this later in bold. Double underline indicates the location of polyadenylation signals while dashed underline points out the glycosylation sites. Hexamer and pentamer repeats are represented by rectangular and oval boxes, respectively. Grey boxes highlight signature motifs that distinguish genotype 1 from genotype 2.
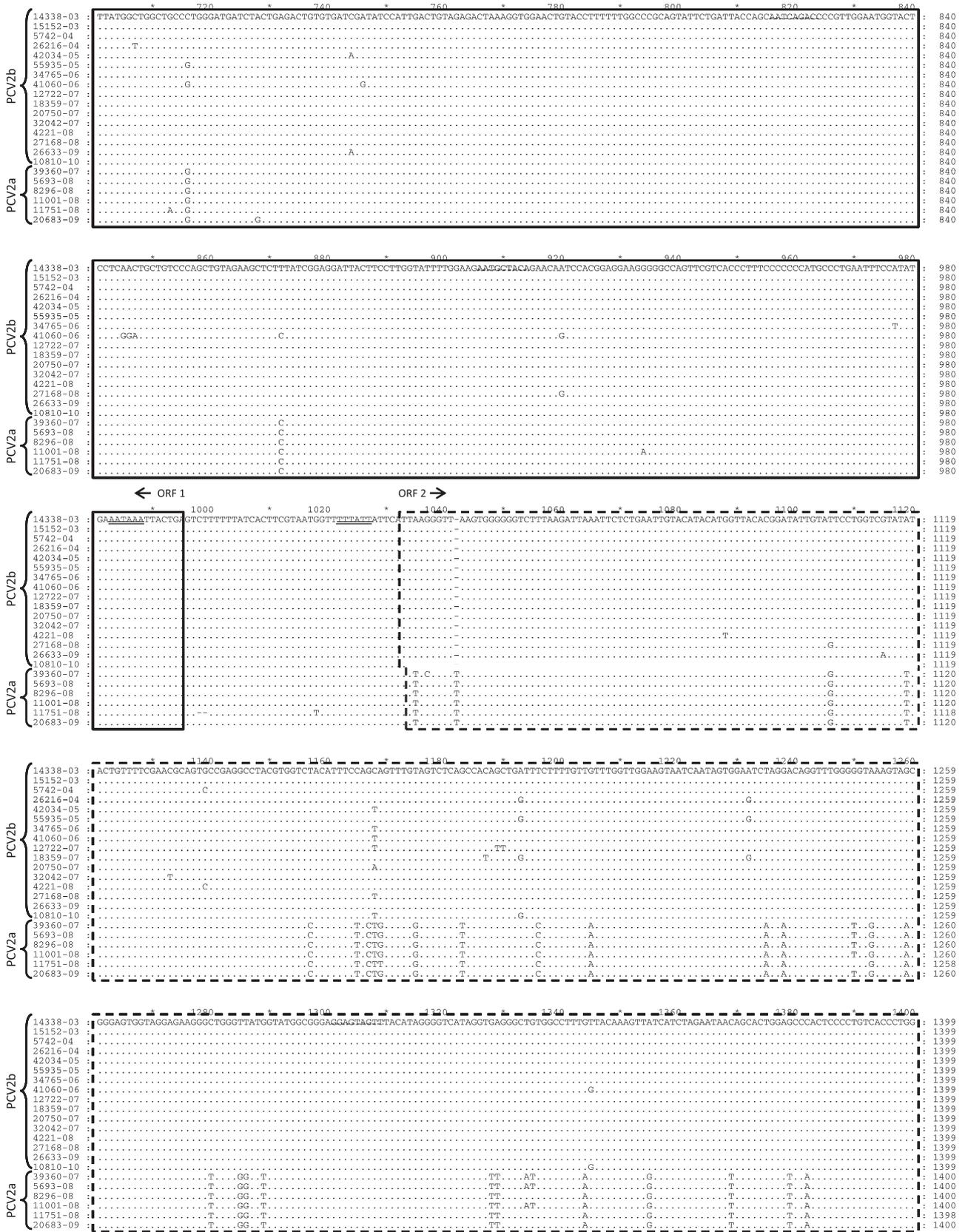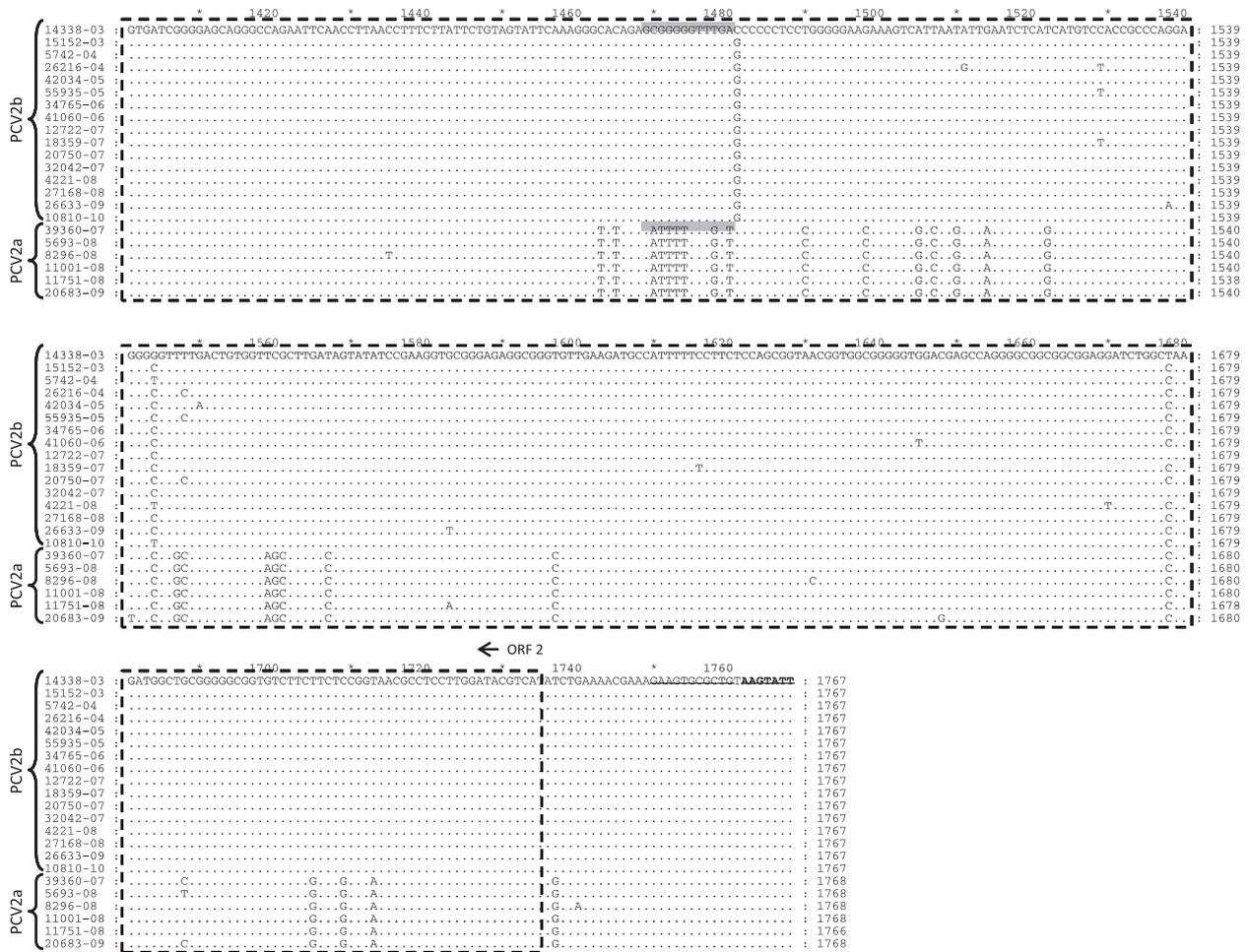
Fig. 2 (*continued*)

Fig. 2 (*continued*)

Table 3
Relevant genomic features of the genome of PCV2 strains circulating in Portugal.

	Group 1	Group 2
Genome length	1767 Nucleotides	1768 Nucleotides
Intergenic region	83 Nucleotides (nt 1735–1767 and 1–50)	83 nucleotides (nt 1736–1768 and 1–50)
Stem structure	CAGCGCACTTC (nt 3–13)	CAGCGCACTTC (nt 3–13)
	GAAGTGCGCTG (nt 1749–1759)	GAAGTGCGCTG (nt 1750–1760)
Nonanucleotide	AAGTATTAC (1761–1767 and 1–2)	AAGTATTAC (1762–1768 and 1–2)
Pentamer repeats	CACCT (nt 25–29 and nt 36–40)	CACCT (nt 25–29 and nt 36–40)
Hexamer repeats	CGGCAG (nt 13–18; nt 19–24 and nt 30–35)	CGGCAG (nt 13–18; nt 19–24 and nt 30–35)
Glycosylation sites (N-X-S or N-X-T)	AATCCTTCC (nt 117–125)	AATCCTTCC (nt 117–125)
	TGTAACGTT (nt 470–478 comp.)	TGTAACGTT (nt 470–478 comp.)
	AATCAGACC (nt 816–824)	AATCAGACC (nt 816–824)
	AATGCTACA (nt 906–914)	AATGCTACA (nt 906–914)
	GGAGTAGTT (nt 1300–1308 comp.)	GGAGTAGTT (nt 1301–1309 comp.)
Polyadenylation Signals	AATAAA (nt 327–332 and nt 983–988)	AATAAA (nt 327–332 and nt 983–988)
	TTTATT (nt 1022–1027)	TTTATT (nt 1022–1027)

Table 4
Characterisation of the major ORFs detected in the genome of PCV2 strains circulating in Portugal.

	ORF 1	ORF 2	ORF3
Position (strand)	nt 51–995 (viral)	Group 1: nt 1734–1033 (comp.) Group 2: nt 1735–1034 (comp.)	nt 671–357 (comp.)
Gene length	945 nt	702 nt	315 nucleotides
Protein encoded	314 aa (35.8 kDa)	233 aa (27.9 kDa)	104 aa (11.9 kDa)

previously reported found worldwide is presented in Fig. 3. The three genotypes are clearly observed in the resulting tree. Interestingly, two strains, one from Germany and the other from the Netherlands, previously classified as 1C (Olvera et al., 2007) are not included in any of the known genotypes. Thirteen strains from this study branched together in genotype PCV2b, representing 59.1% of the viruses analysed. Viruses of this cluster originated from different districts of Portugal mainland (Leiria, Lisboa, Setúbal, Évora and Beja) and from Azores (S. Miguel island), during 2003–2010. A retrospective inquiry allowed ascertaining that seven out of these thirteen PCV2b positive farms had intensive swine production systems, four of which received animals directly or indirectly from Spain, France, Netherland, UK and Germany or semen from Canada (Table 2). One sample was originated from a free ranging extensive swine production farm located in the south of the country (Alentejo).

Strains 15152-03, 55935-05 and 41060-06 were also classified into genotype PCV2b, but in separate branches, representing 13.6% of the isolates studied. Two of them were obtained from intensive swine production farms in the form of close cycles with no history of animal importations from third countries. Information concerning the origin of strain 55935-05 was not available.

Six strains obtained between 2007 and 2009, were included in genotype PCV2a, together with strains from USA (2000) and Austria (2003), but also with more recent isolates from USA (2006) and China (2008) (Fig. 3). It was suggested that genotype PCV2a would include mainly strains obtained from 2000 to 2003, while genotype PCV2b would harbour PCV2 strains detected after 2003. This temporal distribution is in accordance with Olvera et al. (2007) who proposed that genotype PCV2b strains could be more recent than those classified as genotype PCV2a. Additionally, two other studies carried out in Korea and Denmark suggested the occurrence of a global shift from genotype PCV2a to genotype PCV2b in year 2003 (Chae and Choi, 2010; Dupont et al., 2008). They also imply that strains from genotype PCV2b could be more pathogenic, since they were predominant in PMWS-affected herds, while strains from genotype PCV2a were more closely associated with apparently healthy herds.

The strains circulating in Portugal that belong to genotype PCV2b appear to reflect these findings as representatives of this genotype were found between 2003 and 2010. Genotype PCV2b may have been introduced in Portugal around 2003 through the importation of live animals from France, Netherlands or Spain, the main live swine exportation countries to Portugal. All strains
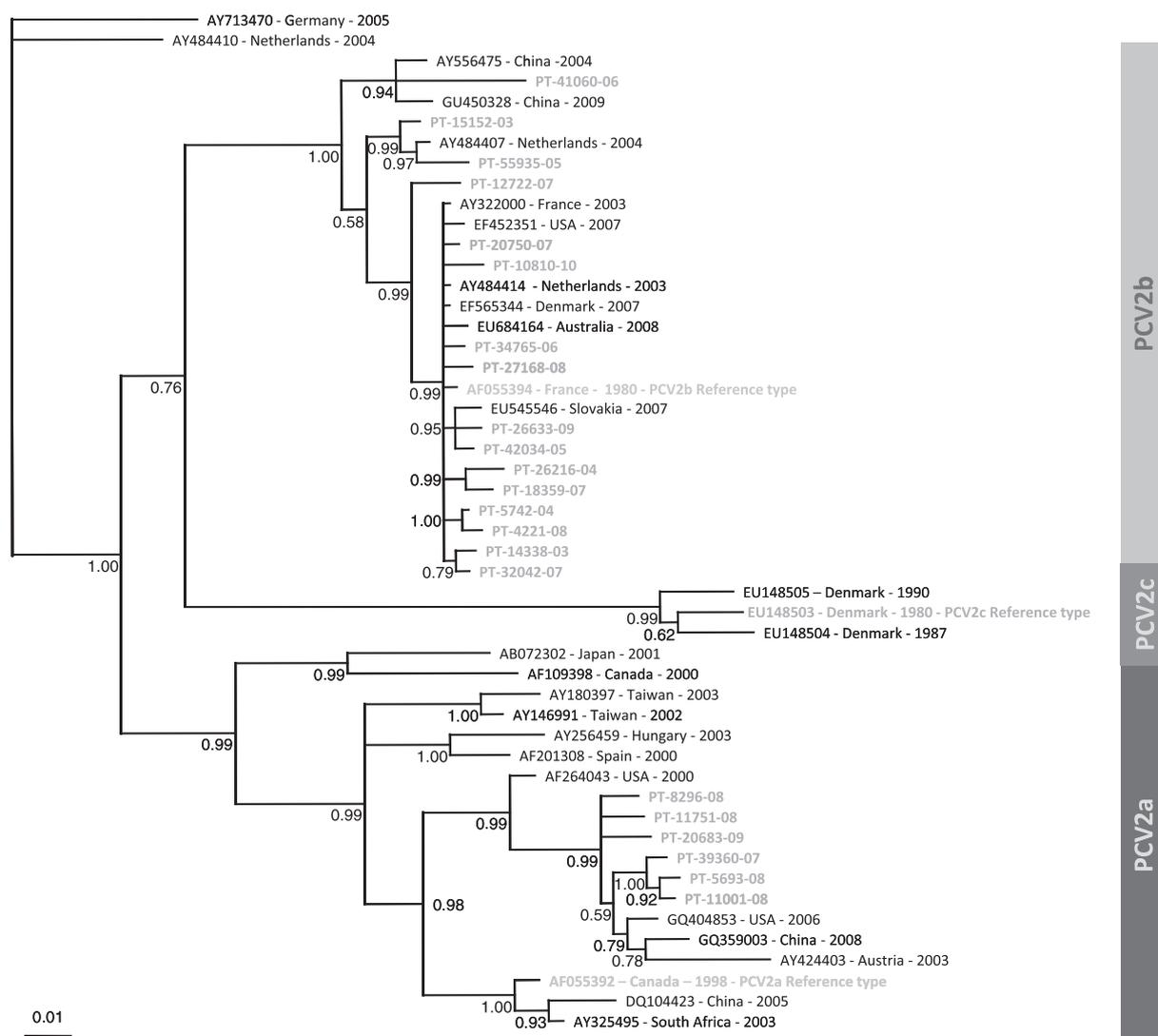
Fig. 3. Bayesian analysis of PCV2 full-length genome. A phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis was performed with ngen = 10^6, nchains = 4 and samplefreq = 10. The numbers included on each boot strap represent the Bayesian posterior probability.

included in genotype PCV2a were obtained after 2007 and therefore do not match the temporal pattern suggested for strains belonging to this genotype. One possible explanation is that viruses of genotype PCV2a may have also circulated in Portugal before 2003. As samples available for the present study report to 2003 onwards, this hypothesis could not be confirmed. Interestingly, all PCV2a genotype strains were obtained from fattening farms located in a circumscript area, all of them acquiring the F1 animals from a common national breeder farm. This farm received animals from another local company that in turn imported the grandparent breeders from Spain (Table 2). The phylogenetic and epidemiological data suggest that PCV2a strains were introduced in Portugal around 2007, through a common source of infection either national or from abroad. The biosecurity measures adopted by the swine industry in the area may have hampered the spread of these PCV2a circoviruses in the country.

In addition to the full-length genome, Bayesian analysis was done using rep and cap genes (Figs. 4 and 5). The overall topology of the tree with the cap gene was similar to that obtained with full-length genomes. Six strains detected in Portugal grouped in genotype PCV2a as for full-length genome phylogeny. The other sixteen strains grouped in genotype PCV2b, but all in the same branch (Fig. 4).

On the other hand, the overall topology of the tree obtained with the rep gene was distinct from those described above as none of the three major groups could be identified and several branches include strains of all genotypes (Fig. 5). Strong purifying selection acting on rep gene was referred as a possible cause for incongruent rep gene and complete genome based trees (Olvera et al., 2007).

As others, we found that the overall topology of the phylogenetic tree of cap gene is very similar to that obtained with the full-length genome, and that cap may be considered a reliable gene for PCV2 phylogenetic studies.

In conclusion, our results show that viruses of genotype PCV2b represent 72.7% of the 22 PCV2 characterised, suggesting that this genotype is dominant. Isolates from genotype PCV2b may have been introduced in Portugal around 2003 and are probably circulating since then, although possible additional introductions cannot be excluded. We also report the presence of genotype PCV2a strains from 2007 onward, in a confined area. The recent reappearance of genotype PCV2a strains have been described in other countries such as Korea (Kim et al., 2009), USA (Cheung, 2007) and China (GQ359003) and may represent a shift back of genotype PCV2b to genotype PCV2a.

The analysis of a higher number of PCV2 strains will allow investigating if less prevalent subgroups also circulate. Additionally,
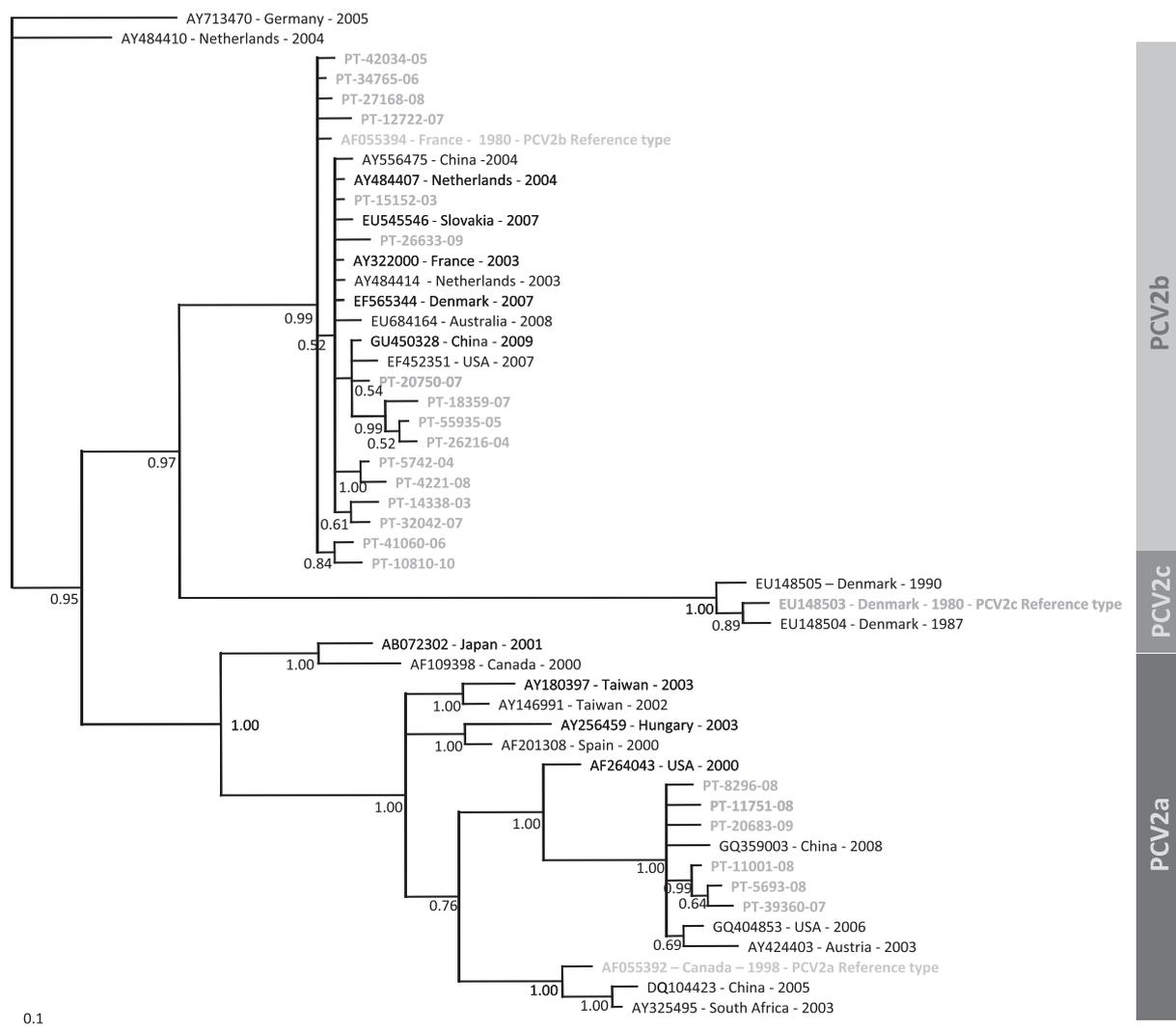
Fig. 4. Bayesian analysis of PCV2 cap gene nucleotide sequence. A phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis was performed with ngen = 106, nchains = 4 and samplefreq = 10. The numbers included on each boot strap represent the Bayesian posterior probability.
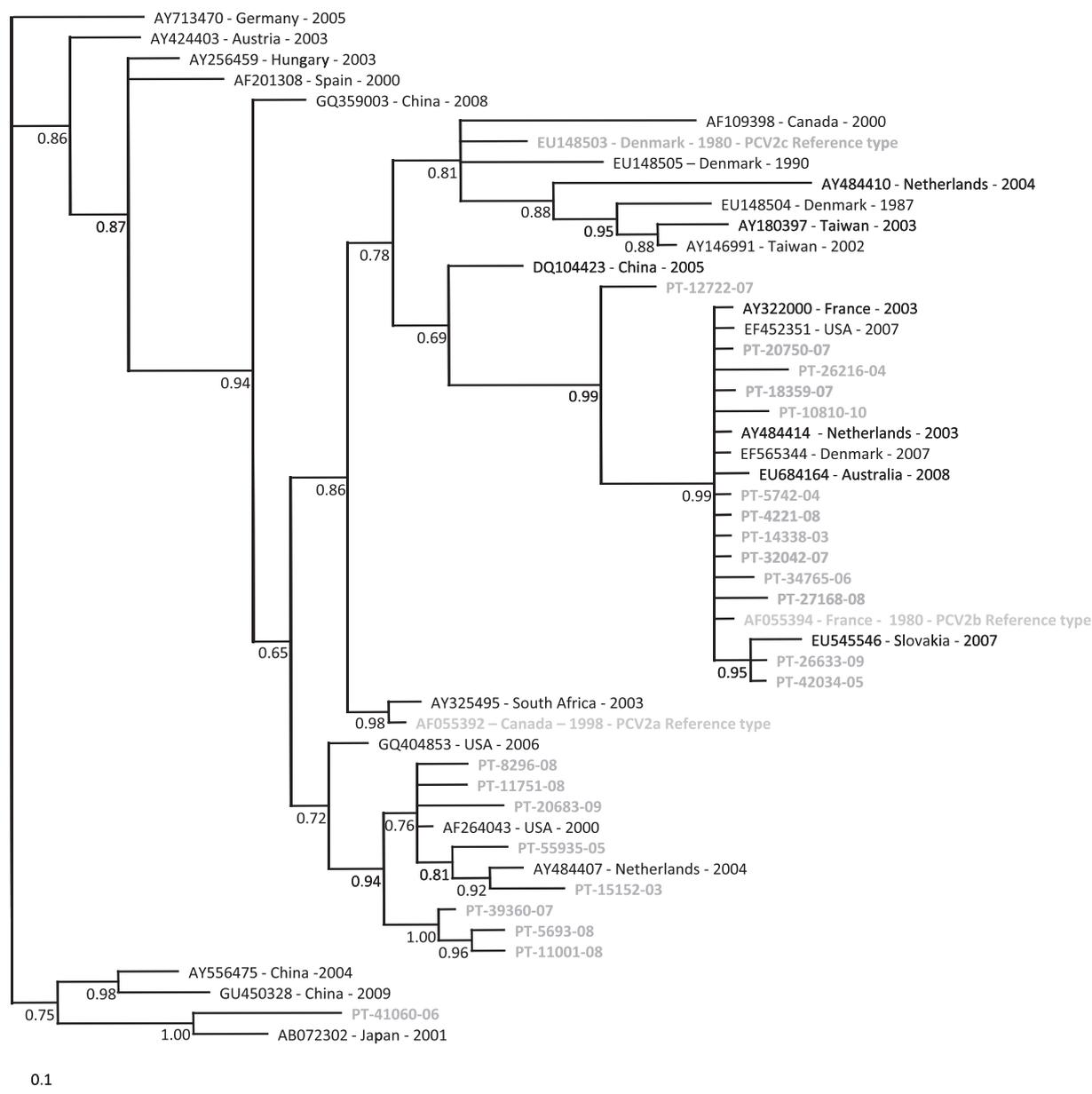
Fig. 5. Bayesian analysis of PCV2 *rep* gene nucleotide sequence. A phylogenetic tree was obtained with a Bayesian inference of phylogeny throughout the MrBayes v3.1.2 software, using the GTR model (nst = 6) with gamma-shaped rate variation with a proportion of invariable sites (rates = invgamma). The analysis was performed with ngen = 10^6, nchains = 4 and samplefreq = 10. The numbers included on each boot strap represent the Bayesian posterior probability.

retrospective analysis of samples collected before 2003 will clarify if strains of genotype PCV2a were present in Portugal in earlier years.

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References

Chae, J.S., Choi, K.S., 2010. Genetic diversity of porcine circovirus type 2 from pigs in Republic of Korea. Res. Vet. Sci. 88, 333–338.

Cheung, A.K., 2007. Homologous recombination plays minor role in excision of unit-length viral genomes from head-to-tail direct tandem repeats of porcine circovirus during DNA replication in *Escherichia coli*. Arch. Virol. 152, 1531–1539.

Cortey, M., Olvera, A., Grau-Roma, L., Segales, J., 2011. Further comments on porcine circovirus type 2 (PCV) genotype definition and nomenclature. Vet. Microbiol. 149, 522–523.

Crowther, R.A., Berriman, J.A., Curran, W.L., Allan, G.M., Todd, D., 2003. Comparison of the structures of three circoviruses: chicken anemia virus, porcine circovirus type 2, and beak and feather disease virus. J. Virol. 77, 13036–13041.

Dulac, G.C., Afshar, A., 1989. Porcine circovirus antigens in PK-15 cell line (ATCC CCL-33) and evidence of antibodies to circovirus in Canadian pigs. Can. J. Vet. Res. 53, 431–433.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case–control study supports a shift in genotypes with time. Vet. Microbiol. 128, 56–64.

Fenaux, M., Halbur, P.G., Haqshenas, G., Royer, R., Thomas, P., Nawagitgul, P., Gill, M., Toth, T.E., Meng, X.J., 2002. Cloned genomic DNA of type 2 porcine circovirus is infectious when injected directly into the liver and lymph nodes of pigs: characterization of clinical disease, virus distribution, and pathologic lesions. J. Virol. 76, 541–551.

Finsterbusch, T., Mankertz, A., 2009. Porcine circoviruses – small but powerful. Virus Res. 143, 177–183.

Hamel, A.L., Lin, L.L., Nayar, G.P., 1998. Nucleotide sequence of porcine circovirus associated with postweaning multisystemic wasting syndrome in pigs. J. Virol. 72, 5262–5267.

Harding, J.C.S., 2004. The clinical expression and emergence of porcine circovirus 2. Vet. Microbiol. 98, 131–135.

Harding, J.C.S., Clark, E.G., 1997. Recognizing and diagnosing postweaning multisystemic wasting syndrome (PMWS). Swine Health Prod. 5, 201–203.

Heath, L., Williamson, A.L., Rybicki, E.P., 2006. The capsid protein of beak and feather disease virus binds to the viral DNA and is responsible for transporting the replication-associated protein into the nucleus. J. Virol. 80, 7219–7225.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Johne, R., Fernandez-de-Luco, D., Hofle, U., Muller, H., 2006. Genome of a novel circovirus of starlings, amplified by multiply primed rolling-circle amplification. J. Gen. Virol. 87, 1189–1195.

Kim, H.H., Park, S.I., Hyun, B.H., Park, S.J., Jeong, Y.J., Shin, D.J., Chun, Y.H., Hosmillo, M., Lee, B.J., Kang, M.I., Cho, K.O., 2009. Genetic diversity of porcine circovirus type 2 in Korean pigs with postweaning multisystemic wasting syndrome during 2005–2007. J. Vet. Med. Sci. 71, 349–353.

Liu, J., Zhu, Y., Chen, I., Lau, J., He, F., Lau, A., Wang, Z., Karuppannan, A.K., Kwang, J., 2007. The ORF3 protein of porcine circovirus type 2 interacts with porcine ubiquitin E3 ligase Pirh2 and facilitates p53 expression in viral infection. J. Virol. 81, 9560–9567.

Maddison, W.P., Maddison, D.R., 2009. Mesquite: a modular system for evolutionary analysis, Version 2.75 http://mesquiteproject.org.

Mankertz, A., Persson, F., Mankertz, J., Blaess, G., Buhk, H.J., 1997. Mapping and characterization of the origin of DNA replication of porcine circovirus. J. Virol. 71, 2562–2566.

Mankertz, A., Mankertz, J., Wolf, K., Buhk, H.J., 1998. Identification of a protein essential for replication of porcine circovirus. J. Gen. Virol. 79 (Pt 2), 381–384.

Mankertz, A., Mueller, B., Steinfeldt, T., Schmitt, C., Finsterbusch, T., 2003. New reporter gene-based replication assay reveals exchangeability of replication factors of porcine circovirus types 1 and 2. J. Virol. 77, 9885–9893.

Mankertz, A., Caliskan, R., Hattermann, K., Hillenbrand, B., Kurzendoerfer, P., Mueller, B., Schmitt, C., Steinfeldt, T., Finsterbusch, T., 2004. Molecular biology of porcine circovirus: analyses of gene expression and viral replication. Vet. Microbiol. 98, 81–88.

Muirhead, M., 2002. Sources of information on PMWS/PDNS. Vet. Rec. 150, 456.

Olvera, A., Cortey, M., Segales, J., 2007. Molecular evolution of porcine circovirus type 2 genomes: phylogeny and clonality. Virology 357, 175–185.

Ouardani, M., Wilson, L., Jette, R., Montpetit, C., Dea, S., 1999. Multiplex PCR for detection and typing of porcine circoviruses. J. Clin. Microbiol. 37, 3917–3924.

Phenix, K.V., Weston, J.H., Ypelaar, I., Lavazza, A., Smyth, J.A., Todd, D., Wilcox, G.E., Raidal, S.R., 2001. Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus Circovirus of the family Circoviridae. J. Gen. Virol. 82, 2805–2809.

Roca, M., Balasch, M., Segales, J., Calsamiglia, M., Viaplana, E., Urniza, A., Hattermann, K., Mankertz, A., Plana-Duran, J., Domingo, M., 2004. In vitro and in vivo characterization of an infectious clone of a European strain of porcine circovirus type 2. J. Gen. Virol. 85, 1259–1266.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Segalés, J., 2007. Porcine circovirus disease (PCVD) – have we won the war in Europe? Adv. Pork Prod. 18, 49–56.

Segales, J., Olvera, A., Grau-Roma, L., Charreyre, C., Nauwynck, H., Larsen, L., Dupont, K., McCullough, K., Ellis, J., Krakowka, S., Mankertz, A., Fredholm, M., Fossum, C., Timmusk, S., Stockhofe-Zurwieden, N., Beattie, V., Armstrong, D., Grassland, B., Baekbo, P., Allan, G., 2008. PCV-2 genotype definition and nomenclature. Vet. Rec. 162, 867–868.

Shang, S.B., Li, Y.F., Guo, J.Q., Wang, Z.T., Chen, Q.X., Shen, H.G., Zhou, J.Y., 2008. Development and validation of a recombinant capsid protein-based ELISA for detection of antibody to porcine circovirus type 2. Res. Vet. Sci. 84, 150–157.

Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2001. Rep and Rep′ protein of porcine circovirus type 1 bind to the origin of replication in vitro. Virology 291, 152–160.

Steinfeldt, T., Finsterbusch, T., Mankertz, A., 2006. Demonstration of nicking/joining activity at the origin of DNA replication associated with the rep and rep′ proteins of porcine circovirus type 1. J. Virol. 80, 6225–6234.

Stewart, M.E., Perry, R., Raidal, S.R., 2006. Identification of a novel circovirus in Australian ravens (*Corvus coronoides*) with feather disease. Avian Pathol. 35, 86–92.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal-W – improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature 295, 64–66.

Todd, D., Weston, J.H., Soike, D., Smyth, J.A., 2001. Genome sequence determinations and analyses of novel circoviruses from goose and pigeon. Virology 286, 354–362.

Todd, D., Scott, A.N., Fringuelli, E., Shivraprasad, H.L., Gavier-Widen, D., Smyth, J.A., 2007. Molecular characterization of novel circoviruses from finch and gull. Avian Pathol. 36, 75–81.

Wang, F., Guo, X., Ge, X., Wang, Z., Chen, Y., Cha, Z., Yang, H., 2009. Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res. 145, 151–156.