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Tripping over emerging pathogens around the world: A phylogeographical approach for determining the epidemiology of Porcine circovirus-2 (PCV-2), considering global trading

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ABSTRACT

Porcine circovirus-2 (PCV-2) is an emerging virus associated with a number of different syndromes in pigs known as Porcine Circovirus Associated Diseases (PCVAD). Since its identification and characterization in the early 1990s, PCV-2 has achieved a worldwide distribution, becoming endemic in most pig-producing countries, and is currently considered as the main cause of losses on pig farms. In this study, we analyzed the main routes of the spread of PCV-2 between pig-producing countries using phylogenetic and phylogeographical approaches. A search for PCV-2 genome sequences in GenBank was performed, and the 420 PCV-2 sequences obtained were grouped into haplotypes (group of sequences that showed 100% identity), based on the infinite sites model of genome evolution. A phylogenetic hypothesis was inferred by Bayesian Inference for the classification of viral strains and a haplotype network was constructed by Median Joining to predict the geographical distribution of and genealogical relationships between haplotypes. In order to establish an epidemiological and economic context in these analyses, we considered all information about PCV-2 sequences available in GenBank, including papers published on viral isolation, and live pig trading statistics available on the UN Comtrade database (http://comtrade.un.org/). In these analyses, we identified a strong correlation between the means of PCV-2 dispersal predicted by the haplotype network and the statistics on the international trading of live pigs. This correlation provides a new perspective on the epidemiology of PCV-2, highlighting the importance of the movement of animals around the world in the emergence of new pathogens, and showing the need for effective sanitary barriers when trading live animals.

1. Introduction

The emergence of infectious agents has followed the evolutionary history of man and the domestication of animals. Over the past 50 years, the world's livestock population has increased significantly with the development of intensive animal production, resulting in a 60% increase in livestock between 1960 and 2000 (Tilman et al., 2002). In addition, the economic integration of world markets has enhanced the movement of animals and animal products between countries. Together, these two factors have contributed to the spread and emergence of infectious agents from animals in different regions of the world.

Swine infectious agents have received a great deal of attention since the early 1990s when many pig-producing countries suffered significant economic losses due to emerging pathogens such as Porcine circovirus-2 (PCV-2). Porcine circovirus-2 belongs to the family *Circoviridae*, and was initially identified as an agent of Post-weaning Multisystemic Wasting Syndrome (PMWS) (Madec et al., 2008; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). It is now associated with a number of different syndromes in pigs known as Porcine Circovirus Associated Diseases (PCVAD) (Madec et al., 2008; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). Porcine circovirus-2 is a non-enveloped virus with an ambisense genome composed of single-stranded circular DNA of ~1.76 kb, which is converted into double-stranded DNA during replication, with three open reading frames (ORFs). In the positive strand, ORF1 encodes two proteins (rep and rep′) involved in viral replication

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(Mankertz et al., 2004). In the negative strand, ORF2 encodes a structural protein (cap) of the viral capsid and ORF3 encodes a protein involved in the induction of apoptosis (Liu et al., 2005, 2006).

Since its identification and characterization, PCV-2 has become distributed worldwide, and PMWS, which was first described in western Canada in 1991 (Clark, 1997; Harding, 1997), has become an endemic syndrome in most pig-producing countries, and is currently considered as the main cause of losses on pig farms (Madec et al., 2008; Ramamoorthy and Meng, 2009).

In this context, the economic impact of PCVAD on pig farms has stimulated studies on the evolution and epidemiology of PCV-2 (Chiarelli-Neto et al., 2009; Firth et al., 2009; Larochelle et al., 2002; Olvera et al., 2007; Timmusk et al., 2008). Based on phylogenetic studies, a classification model for PCV-2 was proposed that divides the viral strains into three major groups: genotypes PCV-2a, PCV-2b and PCV-2c (Segales et al., 2008).

Phylogenetic and phylogeographical approaches have become important strategies for studying the epidemiology of infectious agents from animals for which molecular sequences are available, since these approaches can elucidate the evolution of pathogens and also establish the pattern of their dispersal in a historical and geographical context (Avise, 2000; Felsenstein, 2003).

Thus, the objective of this study was to analyze the main routes of spread of Porcine circovirus-2 (PCV-2) between pig-producing countries, using phylogenetic and phylogeographical approaches.

2. Materials and methods

2.1. Preparing the dataset for analyses

Complete genome sequences of 420 PCV-2 isolates (Table S1) were downloaded from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank), and all countries of origin were sampled. These sequences represent ≈56.2% of PCV-2 genome sequences available in GenBank and were submitted to database between 1997 and 2011 (Table S1). The sequences were aligned using ClustalW (Thompson et al., 1994) and the number of segregating sites was calculated using DnaSP v5 (Librado and Rozas, 2009). The 1761 nt multiple alignment (excluding sites with gaps) of 420 genome sequences contained 737 (41.9%) polymorphic sites.

In order to avoid the interference of too many polymorphisms in analyses of the PCV-2 genealogy, only the coding sequences of genomes were selected. The ORF1 and ORF2 sequences were concatenated and placed in tandem (ORF1-ORF2). The ORF3 sequences were not selected because they are superposed by ORF1 in the PCV-2 genome. The number of segregating sites in the three codon positions of ORF1-ORF2 sequences was calculated using DnaSP v5 (Librado and Rozas, 2009). The sites in the first codon position were the least polymorphic and were thus selected. Via these approaches, the number of polymorphic sites was reduced to 156 in 547 nt (28.5%).

Following this, these sequences were grouped into haplotypes (groups of sequences with 100% identity) using SNAP Workbench v2.0 (Aylor et al., 2006; Price and Carbone, 2005). The infinite sites model of genome evolution (Kimura, 1969) was considered, and the violations to this model were removed (30 sites). The sequences of 420 PCV-2 isolates were grouped into 112 haplotypes (Table S1) containing 126 segregating sites in 517 nt (24.4%).

2.2. Genotyping of PCV-2 isolates

The 420 PCV-2 isolates were classified according to the methodology and nomenclature proposed by Segales et al. (2008). The ORF2 sequences in the PCV-2 genome were selected and aligned using ClustalW (Thompson et al., 1994). A pairwise distance (p-distance) matrix was calculated and the isolates were grouped by the Unweighted Pair Group Method plus Arithmetic Mean (UPGMA) method (Fig. S1) using MEGA version 4 (Tamura et al., 2007). The PCV-2 isolates were assigned to different genotypes (PCV-2a, PCV-2b, or PCV-2c) when the p-distance between them was greater than 0.035 (Grau-Roma et al., 2008; Segales et al., 2008).

2.3. Recombination tests

The presence of recombinant viral isolates may confuse the interpretation of haplotype networks. Recombinant isolates represent breakpoints in the PCV-2 genealogies. In order to detect possible recombinants in the dataset, the 420 genomes of PCV-2 were analyzed using the RDP (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), Chimaera (Posada and Crandall, 2001), MaxChi (Smith, 1992), 3Seq (Boni et al., 2007), and Bootscan/Recscan (Martin et al., 2005) methods (P-value < 0.01) implemented in the Recombination Detection Program v.3.44 (Martin et al., 2010).

2.4. Phylogenetic and phylogeographical methods

Reconstructing phylogenies from intraspecific data (such as PCV-2 viral isolates) is often a challenging task due to the large sample sizes and small genetic distances between individuals (Bandelt et al., 1999). To group the PCV-2 haplotypes in an evolutionary way, a phylogenetic hypothesis was inferred by Bayesian Inference (BI) using the program MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). To expedite the construction of phylogenetic trees, a model of nucleotide substitution was estimated using the program MrModeltest (Nylander et al., 2004). This approach provided the parameters to be estimated by the program MrBayes, avoiding excess parameters in the models for estimating the topologies and sizes of branches. The phylogenetic trees were calculated using the Bayesian Markov Chain Monte Carlo (MCMC) method in four runs with 50,000,000 generations and a sample frequency of 1000. The sequence EU148503 (PCV-2c) was selected as the outgroup and 25% of the trees generated were burned to produce the consensus tree. Once the phylogenetic hypothesis had been developed, the haplotypes were grouped according to the genotyping of PCV-2 isolates.

In order to represent the geographical distribution and provide an alternative view of the genealogical relationships between haplotypes of PCV-2 represented in the phylogenetic tree, a haplotype network was constructed using the program Network 4.5.1.0 (http://www.fluxus-technology.com) and the Median Joining algorithm (MJ) (Bandelt et al., 1999). The algorithms for reconstruction of networks are more obvious generalizations of the representations of evolution than trees, which impose a strictly bifurcated structure (Morrison, 2005).

2.5. Economic statistics and epidemiological history

In order to establish an economic context in the phylogeographical analysis, we considered the statistics of live pig trading (commodity code 0103), available from the United Nations Commodity Trade Statistics Database DESA/UNSD, UN Comtrade (http://comtrade.un.org/; accessed on June 1, 2011). This database contains annual statistics on the international trade in live pigs from the early 1990s, at which time PCV-2 had achieved worldwide distribution. A summary of these economic statistics is presented in Table S2.

Table 1
Summary of the genotype classification of PCV-2 isolates.

Geographic region	Country	Genotype classification of PCV-2		
		PCV-2a	PCV-2b	PCV-2c
Africa	South Africa	1	–	–
Asia	China	15	114	–
Asia	Japan	4	–	–
Asia	India	–	1	–
Asia	Indonesia	–	2	–
Asia	Malaysia	–	11	–
Asia	South Korea	4	12	–
Asia	Taiwan	9	–	–
Caribbean	Cuba	–	19	–
Europe	Austria	3	2	–
Europe	Belgium	–	3	–
Europe	Croatia	2	10	–
Europe	Denmark	2	35	1
Europe	France	1	24	–
Europe	Germany	3	–	–
Europe	Greece	1	2	–
Europe	Hungary	4	2	–
Europe	Netherlands	–	9	–
Europe	Portugal	4	12	–
Europe	Romania	–	1	–
Europe	Serbia	–	4	–
Europe	Slovakia	–	10	–
Europe	Spain	3	2	–
Europe	Sweden	1	1	–
North America	Canada	17	21	–
North America	USA	15	13	–
Oceania	Australia	8	2	–
South America	Argentina	–	1	–
South America	Brazil	5	4	–
Total		102 (24.3%)	317 (75.5%)	1 (0.2%)

The epidemiological correlation between viral isolates was estimated using all the information available in GenBank regarding the 420 genome sequences of PCV-2, including papers published on viral isolation.

3. Results

3.1. Genotyping of PCV-2 isolates

Of the 420 isolates of PCV-2, 102 (24.3%) were classified as the PCV-2a genotype, 317 (75.5%) as PCV-2b, and 1 (0.2%) as PCV-2c. The genotyping of PCV-2 isolates is detailed in Table S1 and summarized in Table 1.

The viral isolates of genotypes PCV-2a and PCV-2b were distributed throughout the countries of Asia, Europe, North America, Oceania, and South America. Only one isolate of PCV-2a was identified in Africa, and only isolates of PCV-2b were identified in the Caribbean. China was the country of origin of the highest number of viral isolates ($n = 129$; 30.7%). Germany, Japan, South Africa, and Taiwan only contained isolates of PCV-2a. Argentina, Belgium, Cuba, India, Indonesia, Malaysia, the Netherlands, Romania, Serbia, and Slovakia only contained isolates of PCV-2b. The only representative of the PCV-2c genotype was isolated in Denmark.

3.2. Recombinant analysis

No recombination events were detected using the GENECONV method. Thus, we considered only the recombinants that were detected by the other methods. Three possible recombination events were predicted in the analysis of PCV-2 genomes: event 1: isolate AY146992 (Taiwan, PCV-2a) as a recombinant of AY146991 (Taiwan, PCV-2a) and EU148507 (Denmark, PCV-2a) (P-value = 1.539×10^{-13}); event 2: isolate FJ218002 (USA, PCV-2a) as a recombinant of AF085695 (Canada, PCV-2a) and AY321984 (France, PCV-2b) (P-value = 1.211×10^{-7}); event 3: isolate EU057187 (Brazil, PCV-2a) as a recombinant of AF086834 (Canada, PCV-2a) and EU450586 (South Korea, PCV-2b) (P-value = 5.013×10^{-13}). These viral isolates were not considered in the haplotype network reconstruction, following the criteria proposed for MJ networks (Bandelt et al., 1999).

3.3. Phylogenetic and phylogeographical analysis

The isolates of PCV-2 were grouped into 112 haplotypes in the phylogenetic and phylogeographical analysis (Table S1). These haplotypes represent groups of viral isolates that are closely related in evolutionary terms. The phylogenetic hypothesis of the haplotypes was in accordance with the genotyping of viral isolates. In the phylogenetic tree (Fig. 1), the clusters of genotypes PCV-2a and PCV-2b were separated by high values of posterior probability, and the representative of the PCV-2c genotype was selected as the outgroup.

In the haplotype network (Fig. 2), the PCV-2 haplotypes were represented according to the geographic origin of the viral isolates: Africa, Asia, Caribbean, Europe, North America, Oceania, and South America. The haplotype network is an alternative representation of the genealogy of viral isolates (Morrison, 2005) and enables a more feasible discussion of epidemiology. In this network, the central haplotypes are the possible ancestors and the peripheral haplotypes are the descendants. Among the haplotypes of PCV-2a (Fig. 2A), it was not possible to define a single ancestral line that gave rise to the other viral isolates. On other hand, haplotype H38 was identified as the ancestor of all isolates of PCV-2b (Fig. 2B).

Of the 112 haplotypes, only 12 (10.7%) haplotypes that grouped viral isolates of PCV-2 originated from different countries (Fig. 4; Table S3). These 12 haplotypes corresponded to 273 (65.0%) viral isolates, and were positioned as ancestors in the haplotype network (Fig. 2). Haplotype H38 was the most common ($n = 163$, 38.8%) with a worldwide distribution (Figs. 2 and 4). The viral isolates included in this haplotype were identified in 18 (62.1%) countries of Asia, Europe, North America, Oceania, and South America (Table S3).

Based on the relationships between ancestors and descendants established in the phylogenetic tree (Fig. 1) and in the haplotype network (Fig. 2), the dispersal routes of PCV-2 between the countries were predicted. From an epidemiological point of view, the 12 haplotypes that grouped the viral isolates from two or more countries (Fig. 4; Table S3) may be related to the main events of introduction and the spread of PCV-2 between pig-producing countries.

The meaning of these dispersal routes was considered in an epidemiological and economic context by analyzing the history of the PCV-2 isolates of these 12 haplotypes and the statistics on the world trade in live pigs (commodity code 0103), available from the United Nations Commodity Trade Statistics Database DESA/UNSD, UN Comtrade (Table S2). In this context, we correlated the relationships between ancestors and descendants of the haplotype network (Fig. 2) with a timeline of reports of viral isolation (origin, year, associated diseases, and outbreaks) (Fig. 4; Table S3), and the import and export history of live pigs (flow and economic value, year, and number of animals) (Table S2). These routes are illustrated in Fig. 3, and mainly originate from Europe and North America.

According to UN Comtrade (Table S2), the Netherlands ($ 20.4 billion), Canada ($ 13.4 billion), China ($ 10.3 billion), and Denmark ($ 9.2 billion) were the major exporters of live pigs between 1989 and 2010. On the other hand, Germany ($ 21.6 billion) and the USA ($ 13 billion) were the major importers. The analysis of trading between the countries, the timeline of viral reports, and the predicted routes are described in detail in the Supplemental Material.

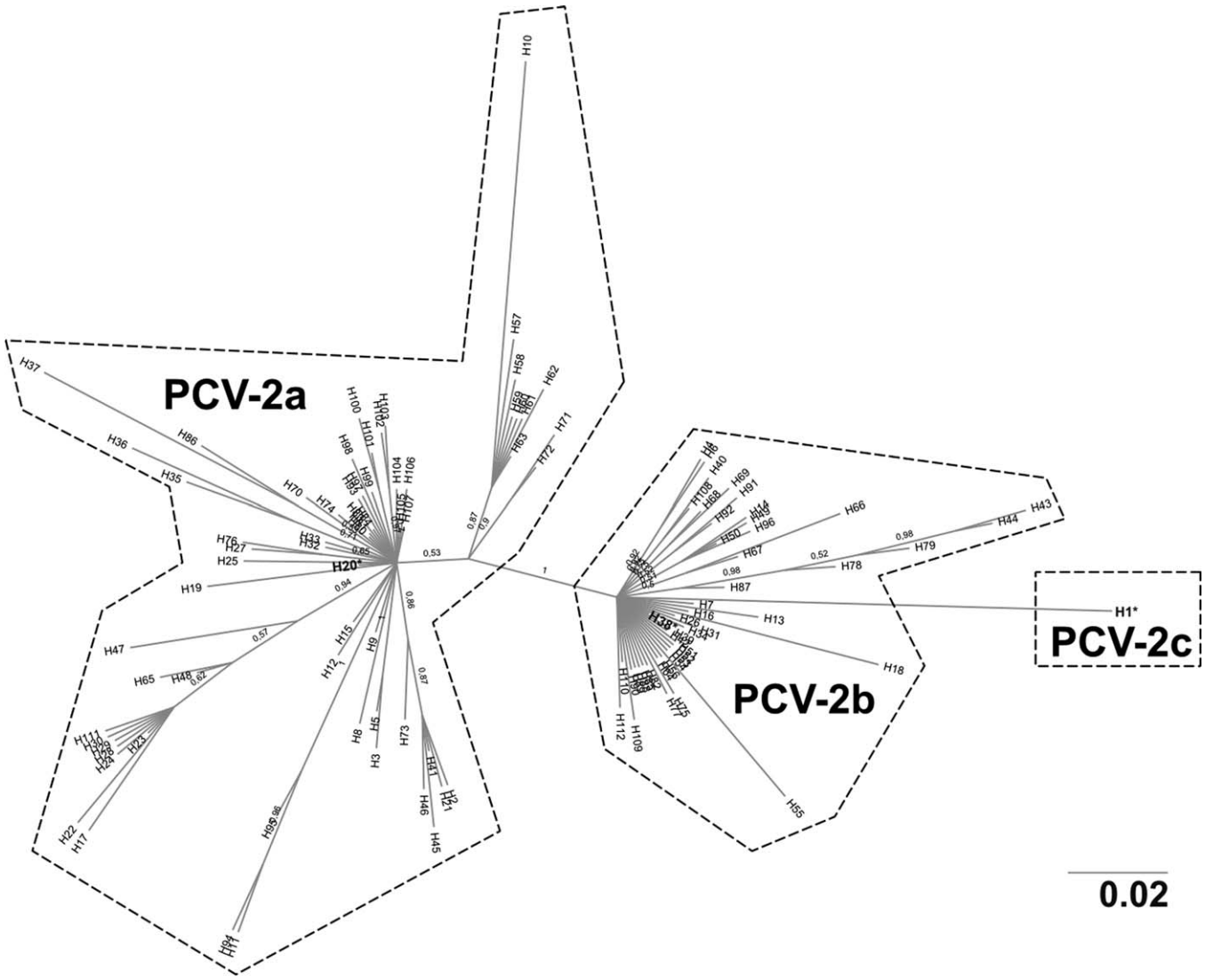

Fig. 1. Genotyping of PCV-2 haplotypes. The majority rule consensus tree was obtained from a Bayesian analysis of the 112 haplotypes of PCV-2. The posterior probability values calculated using the best trees found by MrBayes are shown beside each node. The branches of the phylogenetic tree were grouped according to the classification model proposed by Segales et al. (2008). Haplotype H1 (genotype PCV-2c) was selected as the outgroup.

4. Discussion

These results provide a new perspective on the epidemiology of Porcine circovirus-2, predicting the main routes of the spread of PCV-2 in pig-producing countries within the context of change and economic development.

Most of the isolates in this study were classified as genotype PCV-2b (Table 1). Although all of the PCV-2 genotypes were found in pigs with PMWS, several studies found that isolates from animals that had PMWS often contained PCV-2b, while isolates from animals that did not have the disease contained PCV-2a (An et al., 2007; Grau-Roma et al., 2008). However, it is known that not all pigs infected with PCV-2 will develop PMWS (Silva et al., 2011). More pathogenic viral variants are sometimes capable of causing clinical disease in a large number of animals and it is possible that they were isolated and their genomes sequenced at a greater frequency, which may have contributed to this increased frequency of PCV-2b sequences in the GenBank database.

It has been suggested that a switch from PCV-2a to PCV-2b occurred around 2003 (Dupont et al., 2008; Firth et al., 2009). This is not obvious when analyzing the GenBank submission dates of genome sequences (Table S1). Although the submission of genomes of PCV-2b became more frequent after 2003, the submission of PCV-2a genomes remained relatively constant between 1997 and 2011. On the other hand, the timeline of viral reports shows that viral isolates of PCV-2a were identified more frequently before 2003, and isolates of PCV-2b after 2003 (Fig. 4). These data suggest that PCV-2a could be older than PCV2-b.

The only representative of PCV-2c was isolated in Denmark (Table 1). To date, only three viral isolates recovered from samples collected in Denmark during the 1980s contained the PCV-2c genotype (Segales et al., 2008). The length of branches in the phylogenetic tree (Fig. 1) and the number of mutations represented in the haplotype network (Fig. 2B) suggest that the viral isolate of PCV-2c is highly divergent from the isolates of the other genotypes. As the isolates of PCV-2c were identified in the 1980s, there is no other evidence that would allow us to speculate why the PCV-2c genotype is so rare.

Three possible recombinant isolates of genotypes PCV-2a and PCV-2b were predicted in the analysis of PCV-2 genomes. The occurrence of recombination events among PCV-2 genotypes has been reported previously (Hesse et al., 2008), and the possible recombination among isolates from different countries supports

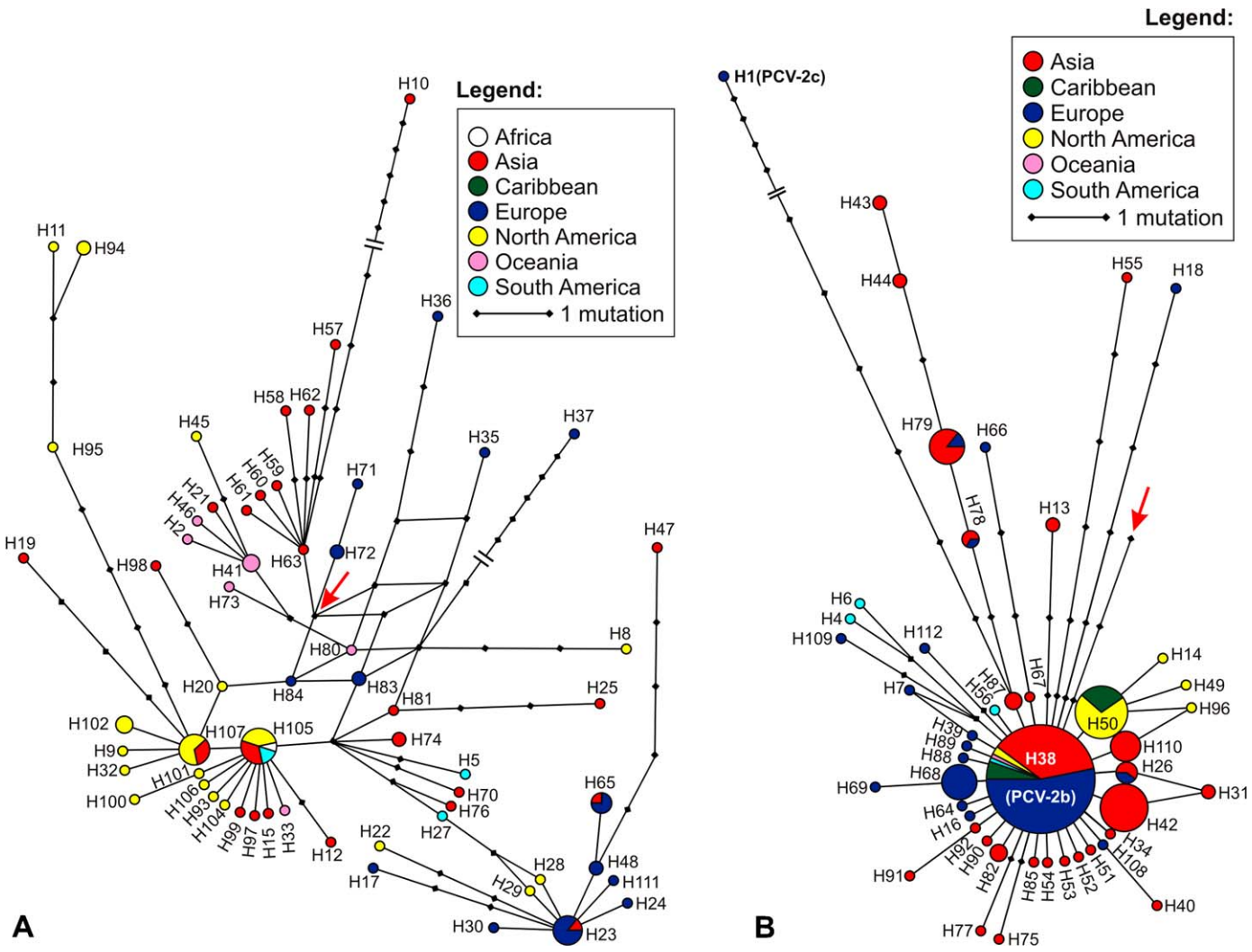

Fig. 2. Haplotype network of PCV-2. Median-joining (MJ) network of all PCV-2 haplotypes built on the Network 4.5.1.0 program. The haplotypes were grouped according to genotype classification. The size of the circumferences is proportional to the haplotype frequencies and the size of the branches is proportional to the number of mutations between haplotypes. A. PCV-2 haplotypes of PCV-2a. B. PCV-2 haplotypes of PCV-2b and PCV-2c. The red arrow marks the point of connection between the networks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the hypothesis that animal movements could contribute to the diversity of isolates of PCV-2.

According to Firth et al. (2009), the isolates of PCV-2 found in South America are restricted to PCV-2b and those in Australia (Oceania) to PCV-2a. However, the results showed that the isolates of PCV-2 are widely distributed throughout the pig-producing countries with representatives of PCV-2a and PCV-2b in Asia, Europe, North America, Oceania, and South America (Table 1). These observations are consistent with those described by Chiarelli-Neto et al. (2009).

The prediction of the spread of PCV-2 among the countries in the haplotype network (Fig. 2) showed a strong correlation with the statistics on the international trade of live pigs, available from the United Nations Commodity Trade Statistics Database DESA/UNSD (UN Comtrade), and with the timeline of reports of viral isolation (Fig. 4; Table S3), which suggests overlap in these reports between countries. Twelve haplotypes grouped isolates of PCV-2 that originated from different countries (Fig. 2). The main routes of the spread of PCV-2 in pig-producing countries were predicted from these haplotypes (Fig. 3). These routes mainly originated from Europe and North America, and are detailed in the Supplemental Material.

European countries showed intensive trading of live pigs (Supplemental Material), with a high level of animal movement between countries. The analysis suggests that Denmark, France, and the Netherlands may have been the major sources of the dissemination of PCV-2 throughout European pig herds (Fig. 3). The analysis also showed that the high level of dispersion of viral isolates of haplotype H38 throughout Europe has even reached wild boars, which may act as natural reservoirs.

Analysis of trading in Asia (Supplemental Material) showed that the role of the Chinese dispersal of PCV-2 in swine may be restricted to regional viral distribution among Asian countries (Fig. 3).

In North America, it is likely that the spread of PCV-2 throughout the country originated from exports from Canada to the USA (Supplemental Material). The introduction of PCV-2 to the Caribbean may have occurred via North America (Canada) and may be related to the emergence of PCVAD in Cuba. The viral isolates of PCV-2a found in South America may have originated from North America (Canada), and the isolates of PCV-2b may have originated from North America (Canada) and Europe (France) (Supplemental Material).

The introduction of PCV-2 into Africa (South Africa) may be linked with viral isolates from North America (USA) (Fig. 2A),

Fig. 3. Prediction of the main routes of dispersal of PCV-2 in the swine industry. These routes were predicted from the haplotype network and considered the groups of viral isolates that were identified in more than one country and the statistics on the international trading of live pigs.

as suggested by Drew et al. (2004). In Oceania (Supplemental Material), most of the viral isolates were introduced to Australia from haplotype H80, and the origin of this haplotype was difficult to establish due to reticulations in the haplotype network (Fig. 2A). The PCV-2b viral isolates were introduced from haplotype H38 (Fig. 2B). The viral isolates of PCV-2a and PCV-2b may have originated from USA (Fig. 4).

Many factors related to the transmission and pathogenesis of PCV-2 have contributed to its rapid spread within the swine industry worldwide. Porcine circovirus-2 is stable in the farm environment and has a great capacity for dispersal in pig production systems through direct contact with infected animals (via oronasal, fecal, and urinary routes), vertical transmission from sows to piglets, and via contaminated semen (Madec et al., 2008; Opriessnig et al., 2007; Schmoll et al., 2008).

Thus, the marketing of subclinically infected animals and the selection of these animals for breeding programs (PCV-2 is apparently transmitted through semen (Schmoll et al., 2008) and from sows to piglets) have played a major role in the spread of PCV-2 around the world.

Given that the majority of pig sounders, if not all, are infected with PCV-2, the selection of PCV-2-free animals would be impossible. Thus, parameters such as determining the viral load of samples and the diagnosis of co-infectious agents related to PCVAD could be used to control the spread of PCV-2 in the course of commercial transactions between pig-producing countries. Another alternative would be the development of a diagnostic method to detect viral isolates of haplotype H38, which were shown to be the most dispersed isolates among the countries included in this study.

Our data showed correlations between the PCV-2 dispersal routes in pigs, as predicted by the phylogeographical analysis, and the statistics on the international trade of live pigs. This correlation shows the importance of animal movements around the world in the emergence of new pathogens and the need to establish sanitary barriers that are effective in the trading of live animals.

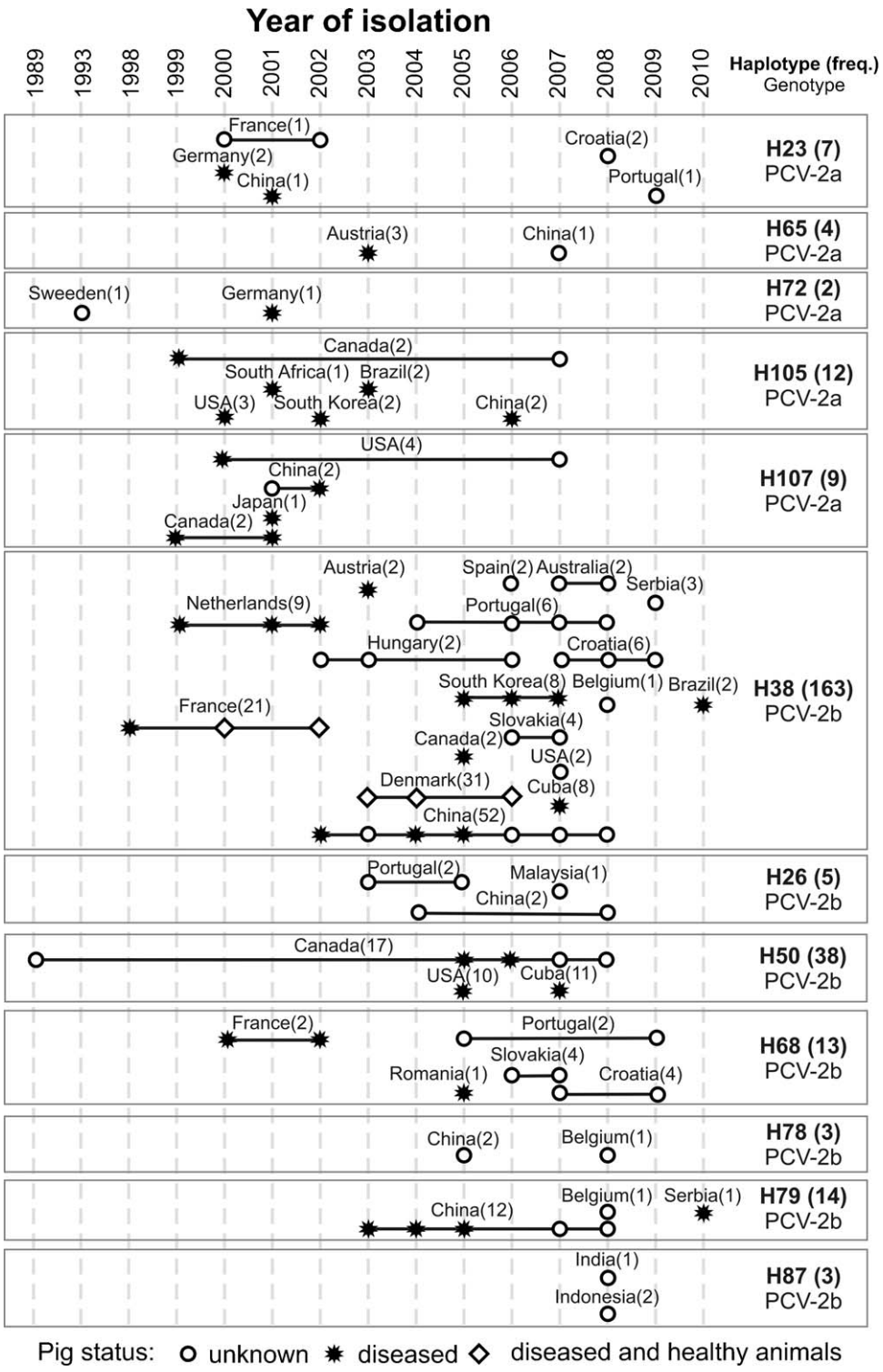

Fig. 4. Timeline of reports of isolation of PCV-2. The 12 haplotypes represented in the timeline were those that grouped viral isolates of PCV-2 originating from different countries. The references concerning viral isolation are detailed in Table S3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.10.019.

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