Short Communication

Emerging novel porcine parvoviruses in Europe: origin, evolution, phylodynamics and phylogeography

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To elucidate the spatiotemporal phylodynamics, dispersion and evolutionary processes underlying the emergence of novel porcine parvovirus 2 (PPV2), PPV3 and PPV4 species, we analysed all available complete capsid genes, together with ours, obtained in Europe. Bayesian phylogeography indicates that Romania (PPV2 and PPV4) and Croatia (PPV3) are the most likely ancestral areas from which PPVs have subsequently spread to other European countries and regions. The timescale of our reconstruction supported a relatively recent history of the currently circulating novel PPV species (1920s to 1980s) in the domestic or sylvatic host. While PPV2 strains exhibited a large genetic exchange characterized by significant recombination and gene flow between distinct regions and hosts, PPV3 and PPV4 showed a diversification reflected by the accumulation of geographically structured polymorphisms. The RNA-like evolutionary rates detected inter- and intrahost recombination and the positive selection sites provided evidence that the PPV2–4 capsid gene plays a prominent role in host adaptation.

Recently, several new members of the subfamily *Parvovirinae* have been discovered in animals, particularly in domestic pigs. The first such novel parvovirus, provisionally designated porcine parvovirus 2 (PPV2), has been described worldwide (Hijikata *et al.*, 2001; Wang *et al.*, 2010; Xiao *et al.*, 2013a). PPV3 (Cheung *et al.*, 2010), initially designated porcine hokovirus (Lau *et al.*, 2008) and also known as porcine partetravirus (Tse *et al.*, 2011), porcine PARV4-like virus (Szelei *et al.*, 2010) and porcine PARV4 (Xiao *et al.*, 2012), shows a global presence in domestic pigs. Among newly described parvoviruses of swine, PPV4, a novel genetically divergent member of the subfamily *Parvovirinae* was discovered initially in the USA in porcine circovirus-associated disease (PCVAD)-affected pigs (Cheung *et al.*,

2010) and subsequently reported in Asia, Europe and Africa (Huang *et al.*, 2010; Zhang *et al.*, 2011; Cságola *et al.*, 2012; Cadar *et al.*, 2013; Ndze *et al.*, 2013).

With little knowledge on their pathogenicity, these emerging parvoviruses are currently receiving more attention due to the tentative association with diseases like PCVAD (Xiao *et al.*, 2012; Li *et al.*, 2013; Opriessnig *et al.*, 2013) or 'high fever disease' (Wang *et al.*, 2010), which highlights the need to investigate the molecular evolution, epidemiology and genetic diversity of these novel PPVs in Suidae hosts. The aim of this study was to reconstruct the phylogeny and evolution of PPV2–4 on a spatiotemporal scale in order to detect the driving forces shaping their evolution, and estimate the time of origin and patterns of geographical dispersal of the different strains in general and in Europe in particular. These data will help to fill several gaps in the understanding of dispersion, evolution and phylodynamics of these emerging novel porcine parvoviruses.

To detect the presence of PPV2–4 in different regions of Europe, tissues and serum samples collected (2006–2011)
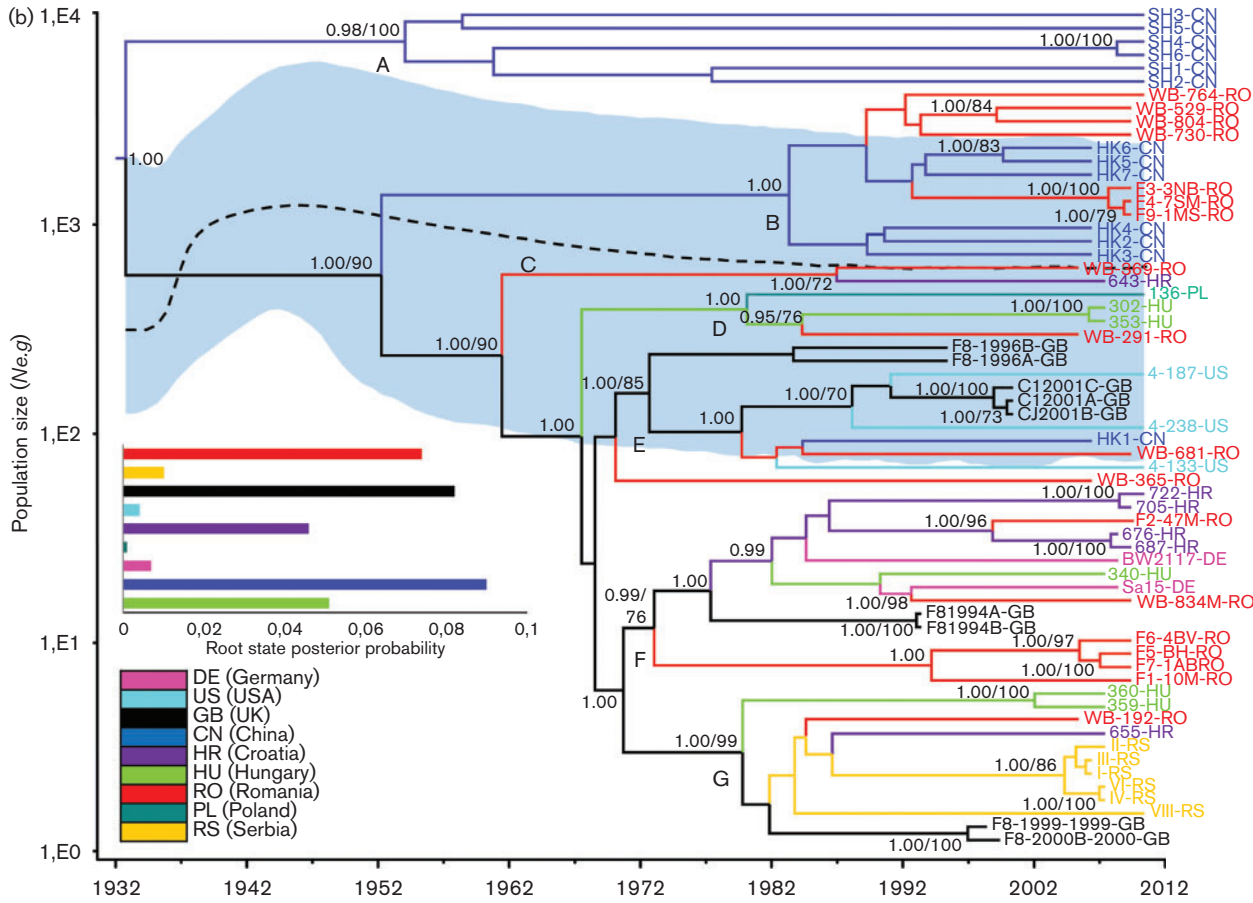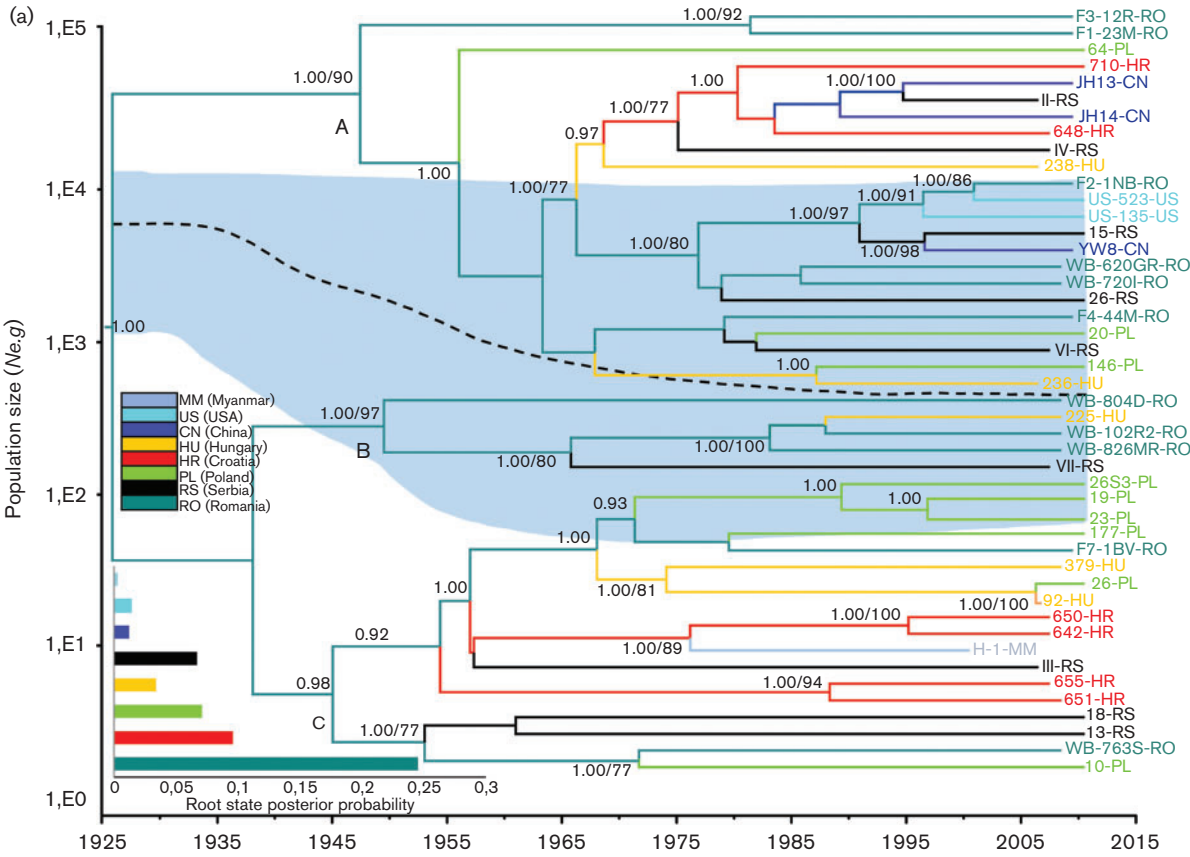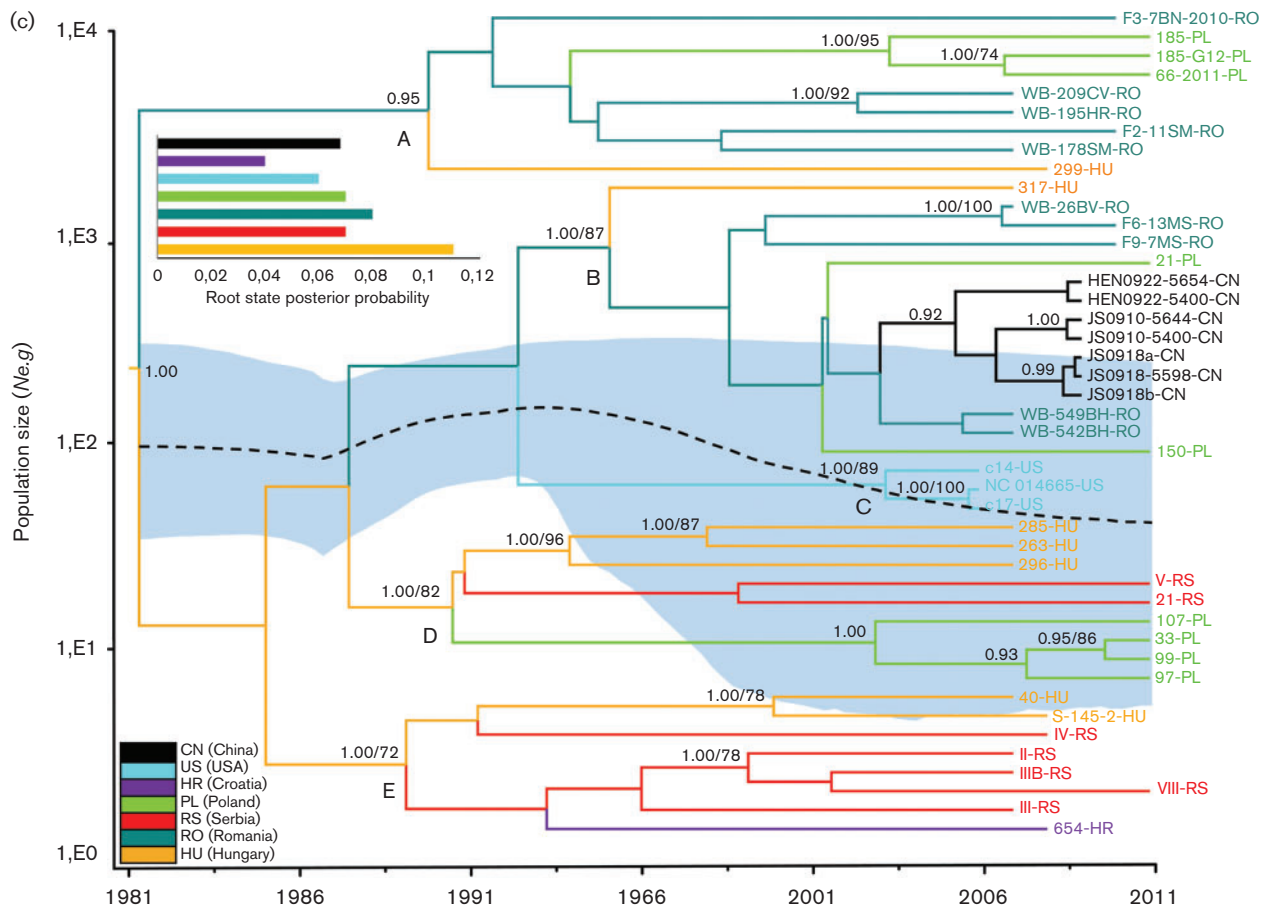
Fig. 1. Bayesian MCC trees that summarized global PPV2 (a), PPV3 (b) and PPV4 (c) complete *VP* gene datasets were generated using geospatial Bayesian analysis. We coloured branches according to the most probable location state of their descendent nodes. The location s.p.p. distributions of ancestral nodes corresponding to PPV2–4 are on the left. ML bootstrap scores (>70 %) and BPP (>90 %) are shown above the branches. Time is reported in the axis below the tree. The BSPs including ten coalescent interval groups obtained by analysing the PPV2–4 sequences sampled at different times are shown in the background. The thick dashed line indicates the median estimates, and the light blue area shows the 95 % highest posterior density (HPD). The *x*-axis is the timescale in years, and the *y*-axis is a logarithmic scale of *Ne.g* (where *Ne* is the effective population size and *g* is the generation time).

from domestic pigs from Croatia ($n=89$), Poland ($n=185$) and Serbia ($n=78$) were used in this study. In addition, Hungarian and Romanian samples from our previous studies (Cságola *et al.*, 2012; Cadar *et al.*, 2013) were also used. The detection and amplification of the complete PPV2–4 *VP* gene sequences were performed using previously described specific PCR protocols (Cadar *et al.*, 2011, 2013; Cságola *et al.*, 2012). We retrieved from GenBank all available PPV2–4 complete *VP* gene sequences. Their accession numbers and other additional information including PPV2–4 sequences of this study are listed in Table S1 (available in JGV Online).

To reconstruct the evolutionary history of each dataset (global and European), maximum likelihoods (MLs) using Treefinder (Jobb *et al.*, 2004) and the Bayesian Markov chain Monte Carlo (MCMC) method implemented in the BEAST v1.6.2 package (Drummond & Rambaut, 2007) were

applied. Moreover, we estimated the rate of evolutionary change (substitutions per site per year), the time to most recent common ancestor (tMRCA) and *Ne.g* (i.e. the effective number of transmission events, *Ne*, times the generation time, *g*, of the pathogen) under a Bayesian skyline coalescent tree prior (Drummond *et al.*, 2005). The extent and pattern of geographical structure in PPV2–4 *VP* gene phylogenies were assessed using the program BaTS (Parker *et al.*, 2008).

We used a timescaled phylogeny to reconstruct the PPV2–4 spread patterns using a standard continuous-time Markov chain process over discrete sampling locations with the Bayesian stochastic search variable selection (BSSVS) model, which allows the diffusion rates to be zero with some positive prior probability (Lemey *et al.*, 2009). The putative spatiotemporal pattern of PPV2–4 spread in

Table 1. Bayesian estimates of evolutionary rates (substutions per site per year) and the times of the most recent common ancestor (tMRCA) inferred from complete *VP* genes of global and European novel porcine parvovirus PPV2–4 datasets

Virus datasets	Number of sequences	Date range of sequences	Clock/demographic model	Rate	Rate 95 % HPD	Node age	95 % HPD (node age)	tMRCA (year)
PPV2 global	46	2001–2011	UCLN/BSP	3.38×10^{-4}	1.97×10^{-5}– 6.60×10^{-4}	86	37-224	1925
PPV2 Europe	40	2006–2011	UCLN/BSP	4.59×10^{-4}	3.14×10^{-5}– 9.79×10^{-4}	71	40-335	1940
PPV3 global	63	1994–2011	UCLN/BSP	2.57×10^{-4}	1.61×10^{-4}– 3.55×10^{-4}	81	49-122	1930
PPV3 Europe	36	2006–2011	UCLN/BSP	2.48×10^{-4}	1.17×10^{-4}– 4.08×10^{-4}	45	16-119	1967
PPV4 global	44	2006–2011	UCLN/BSP	3.34×10^{-4}	7.47×10^{-5}– 5.97×10^{-4}	30	13-79	1981
PPV4 Europe	34	2006–2011	UCLN/BSP	2.98×10^{-4}	4.61×10^{-5}– 5.27×10^{-4}	32	13-146	1979

UCLN, uncorrelated lognormal distribution of rates; BSP, Bayesian skyline plot.

Europe was analysed using the SPREAD (Bielejec *et al.*, 2011) program.

The selection pressures analyses were performed using the SLAC, FEL, REL (Kosakovsky Pond & Frost, 2005) and FUBAR (Murrell *et al.*, 2013) methods of the HyPhy package. In order to detect both episodic and pervasive positive selection at the level of individual sites, the mixed effects model of evolution (Murrell *et al.*, 2012) method was implemented. We used the automated algorithms contained within the RDP3 program (Martin *et al.*, 2010) to screen for putative recombination events. The mosaic structures of any recombinants were also inferred by means of bootscanning using GARD (Kosakovsky Pond *et al.*, 2006). The SplitsTree program v4.12.3 (Huson & Bryant, 2006) was also employed to confirm the phylogenetic relationship of the recombinant samples of each dataset.

Fig. 1 shows the Bayesian MCMC trees, the estimation of viral population dynamics and the tMRCA of global PPV2–4 datasets. The root of the PPV2 tree had a tMRCA of 86 years before present (y.b.p.). From this ancestor, three main clades (A–C) were identified, having tMRCAs of 63, 61 and 65 y.b.p., respectively. The phylogeographic reconstruction clearly showed that all clades and subclades had a MRCA location in Romania [state posterior probability (s.p.p.)=0.24] (Fig. 1a). While the root of the PPV3 tree had a tMRCA of 81 y.b.p., the seven major clades identified had a mean ranging between 23 and 56 y.b.p. The phylogeographic reconstruction was unable to identify a single location for the root of the tree of the PPV3 global dataset, due to almost identical s.p.p. for two localities (China and UK) (Fig. 1b). The PPV4 phylogeny showed a more recent tMRCA (30 y.b.p.) in comparison with the other two PPV species studied (Fig. 1c and Table 1). Five distinct clades were identified, which diverged at

about 8–21 y.b.p. The AI and PS statistics using the program BaTS indicated that there was very strong geographical clustering of PPV3 (except in Germany and Poland) and PPV4 (except in Croatia) strains by country of origin ($P<0.05$), reflecting a significant population subdivision, except for the PPV2 dataset, for which there was evidence of significant gene flow between distinct regions ($P>0.05$) (Table S3).

Fig. 2 illustrates the Bayesian MCC tree for PPV2–4 in Europe, and the colour of each lineage and internal node represents its most probable geographical locality. The spatial reconstruction suggested that PPV2 and PPV4 in Europe most likely originated in Romania (highest s.p.p. values), but we could not accurately pinpoint the geographical origin of PPV3 due to almost identical s.p.p. for two localities (Romania and Croatia) (Fig. 2b). Thus, investigation of the diffusion patterns showed a well-supported connection [Bayes factor (BF) >3] for Croatia using the BF test under BSSVS analysis (Fig. S3b). Our analysis recognized six, seven and four main clades corresponding to PPV2, PPV3 and PPV4 European datasets having the root of the tree tMRCA of 71, 45 and 32 y.b.p. (Fig. 2). Bayesian skyline plot (BSP) analysis of the PPV2 European dataset showed similar *Ne.g* values and dynamics as observed for the global dataset (Figs 1a and 2a). PPV3 dynamics showed an increased growth phase that started around the mid-1960s, with a plateau from the 1970s to 1990s associated with a decreased phase, until the mid-2000s followed by a phase when *Ne.g* values remained constant and still persist (Fig. 2b). PPV4 population dynamics are characterized by an initial constant phase from the 1980s to 1990s, followed by a decreasing level of *Ne.g* values towards the 2000s, continued with a stabilized value persisting until today (Fig. 2c). The histories of dispersal of PPV2–4 among European countries reconstructed using the equal rates model
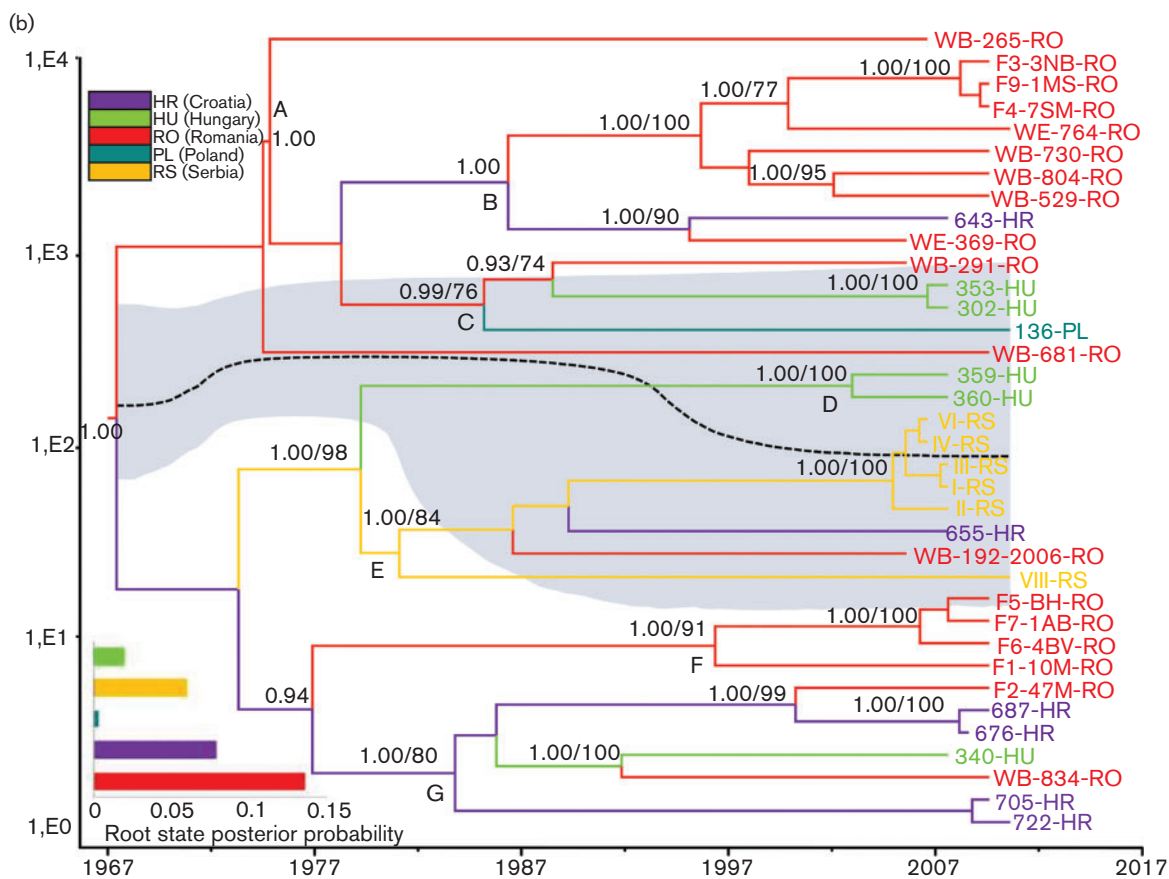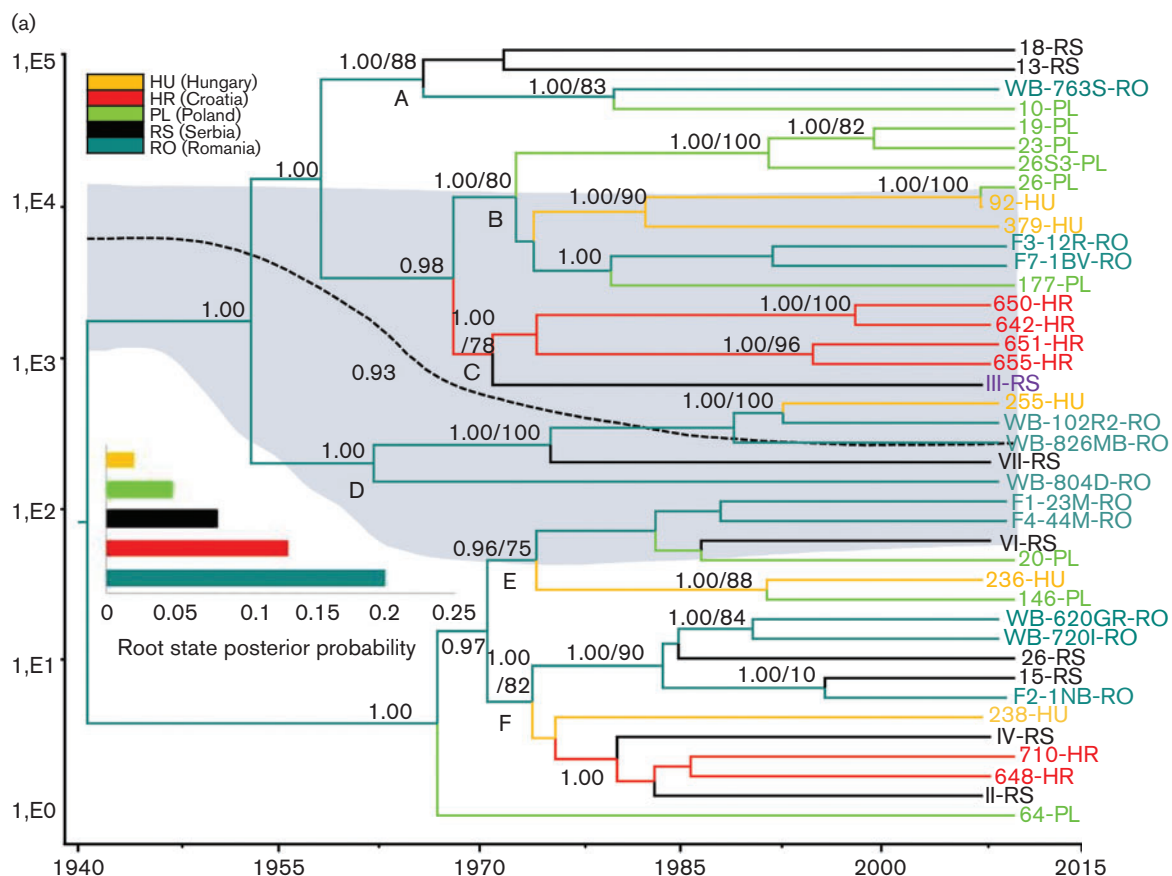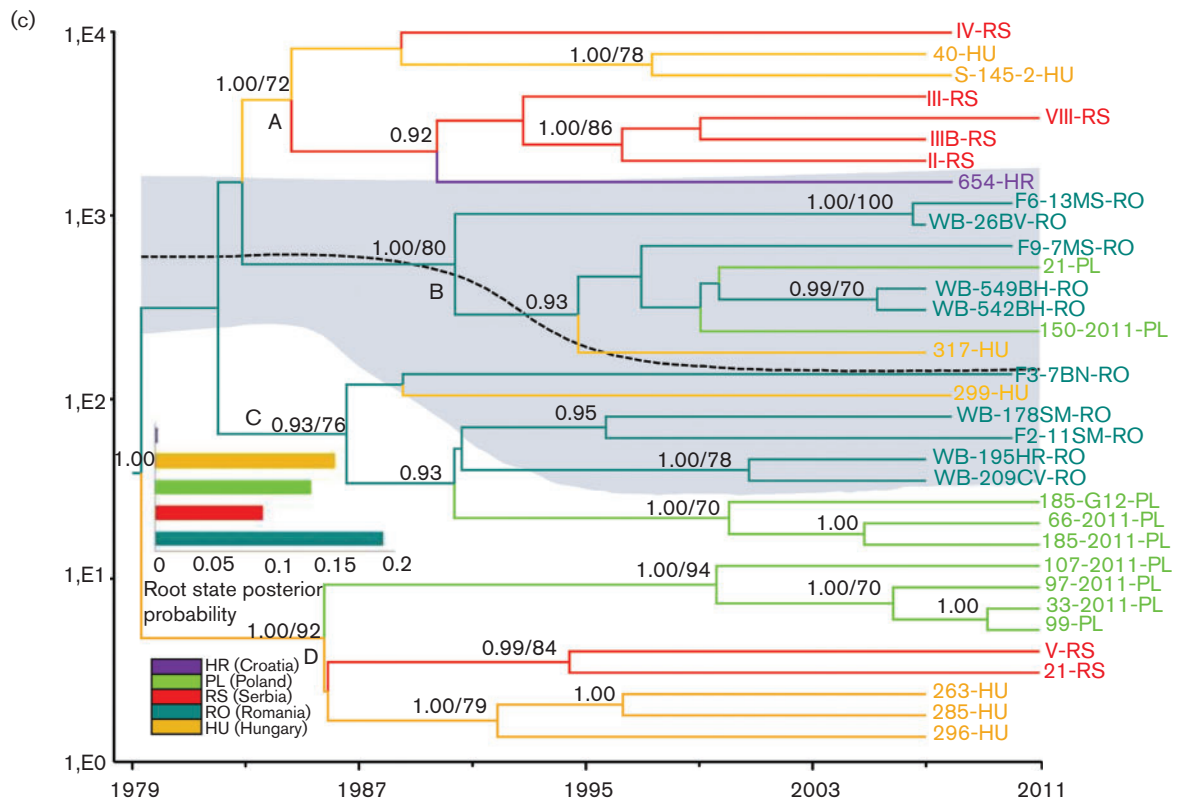
Fig. 2. Bayesian MCC trees summarized for European PPV2 (a), PPV3 (b) and PPV4 (c) complete *VP* gene datasets were generated using geospatial Bayesian analysis. Branches are coloured according to the most probable location state of their descendent nodes. The location s.p.p. distributions of ancestral nodes corresponding to PPV2–4 are on the left. ML bootstrap scores (>70 %) and BPP (>90 %) are shown above the branches. Time is reported in the axis below the tree. The BSPs, including ten coalescent interval groups obtained by analysing the PPV2–4 sequences sampled at different times, are shown in the background. The thick dashed line indicates the median estimates, and the light blue area shows the 95 % HPD. The *x*-axis is the timescale in years, and the *y*-axis is a logarithmic scale of *Ne.g* (where *Ne* is the effective population size and *g* is the generation time).

are shown in Fig. S2. Fig. S3 shows only those linkages that are statistically well supported (i.e. non-zero rates supported by a BF >3). Our reconstruction of discrete ancestral states (Fig. 2) had the higher BPP values for Romania (PPV2, PPV4) and Croatia (PPV3).

Table S2 shows the prevalence of PPV2–4 infections detected in this study and previously reported worldwide. Multiple nucleotide alignments of PPV3 datasets revealed one codon deletion (position 714) in two strains from Serbia (13-RS, 18-RS). The overall d_N/d_S ratios in *VP*s were 0.134 (PPV2), 0.176 (PPV3) and 0.234 (PPV4), indicating that most sites are subject to strong purifying selection. However, positive selection sites were detected in each parvovirus species under the five implemented algorithms (Table S5). Evolutionary fingerprints of PPV2–4 *VP* gene alignments clearly reflected and supported the presence of positively selected individual sites and purifying selection (Fig. S4). The paraphyletic $Pro_{990}Ala$ and $Ala_{1009}Val/His$ mutations located in the C terminus of the PPV2 *VP* gene

appeared only in European domestic and sylvatic strains, while the paraphyletic $Ser_{1010}Thr$ mutation was detected worldwide. The geographical structure of the $Pro_{233}Ser$ mutation indicated the presence of this amino acid replacement only in wild boar origin PPV3 *VP* gene sequences, while the PPV4 $Gln_{722}Pro$ mutation appeared only in domestic and sylvatic strains from Romania and domestic strains from China. Strong recombination signals were detected only in the PPV2 dataset, and confirmed by GARD and SplitTree network analysis (Table S4, Fig. S1). Almost all PPV2 strains from the European dataset exhibited potential recombination events. These events were detected both within and between clades and within and between countries, and also between domestic and sylvatic (wild boars) strains (Table S4).

In this study, we sought to elucidate the possible origin, spatiotemporal dynamics and factors that shape the evolution of novel PPV2–4 species in general, and in Europe especially. Although several distinct clades in the phylogenetic trees of PPV2–4 were detected, there is so far

no available classification methodology for PPV2–4. Very recently, Xiao *et al.* (2013b) proposed three new tentatively designated genera within the subfamily *Parvovirinae*, from which the tentative genus *PARV4-like* included PPV2 and PPV3, and a currently unnamed novel clade 2 for PPV4.

Our study is the first attempt to reconstruct the phylodynamics and phylogeography history of PPV2–4 species. Although PPV2–4 have received more attention only recently, our estimates suggest that they must have been around at least since the 1920s (PPV2), 1930s (PPV3) and 1980s (PPV4), respectively, in the domestic or sylvatic host. Recently, it has been shown that PPV1 originated approximately 120 years ago, with the main divergence occurring in the past 20–60 years (Cadar *et al.*, 2012). This suggests that the novel PPV2–4 and PPV1 have a relatively recent and similar evolutionary history.

These results suggest the occurrence of interspecies transmissions of PPV2–4 between domestic and sylvatic hosts and are supported by our previous study (Cadar *et al.*, 2013), but they do not allow us to pinpoint the host of origin for each PPV species, and by which the viruses were introduced into specific geographical regions. An extended surveillance, especially in wild boars among these regions, will be necessary to answer this question. The spread of PPV2 and PPV4 in Europe indicates Romania as the initial source of their diffusion. For PPV3, we were unable to detect the most probable ancestral location due to the almost identical state probability for two basal localities, but based on BSSVS analysis, the diffusion patterns showed well-supported connections (BF >3) for Croatia as the likely source of PPV3 diffusion in Europe. Although there is clear evidence of gene flow of PPV2 among countries within Europe, and worldwide as well, BaTS analysis also detected strong structuring of the PPV3 and PPV4 phylogeny by country, suggesting that *in situ* evolution also plays an important role in the maintenance of these viruses worldwide. Indeed, few genetic clusters included viruses isolated from single geographical areas, suggesting the existence of epidemiological and commercial connections among different countries.

Evolutionary rates have been calculated for the *VP* gene of several ssDNA viruses and range from more than 1.2×10^{-3} to as low as 9.4×10^{-5} substitutions per site per year (Shackleton *et al.*, 2005; Duffy & Holmes, 2008; Hoelzer *et al.*, 2008; Firth *et al.*, 2009; Streck *et al.*, 2011; Cadar *et al.*, 2012, 2013). The evolutionary rates of the *VP* genes of PPV2–4 (2.57–3.38×10^{-4} substitutions per site per year) fall in the middle of this spectrum and could maintain evolutionary dynamics of these viruses closer to those of ssRNA viruses than to those of dsDNA viruses. A larger sample of viruses from both hosts is needed to confirm whether the immune escape and tropism shifts are responsible for the high substitution rates. The overall low d_N/d_S ratio in these novel PPVs indicates that most amino acid residues are subject to purifying selection with adaptive evolution restricted to specific residues within *VP* genes. It is unknown if these mutations are a consequence of the mechanisms of antigenic escape or further adaptation to the host, but they may affect the antigenic profile and confer an evolutionary advantage to the viruses.

It has been shown that parvoviruses are able to emerge in new hosts (Parrish & Kawaoka, 2005; Shackleton *et al.*, 2005). Recombination events of PPV2 *VP* genes, including the likely intra- and interspecies and within and between country strain recombinations, are reported in this study. All lineages included closely related PPV2 variants from distant locations and different host species together with the radiation pattern observed for diversification of PPV2 strains, suggesting that genetic flow has occurred. Our analysis provides novel data about the spatiotemporal phylodynamics, dispersion and evolutionary scenario shaping these emerging PPVs in general and in Europe in particular, and supports the notion that Romania (PPV2 and PPV4) and Croatia (PPV3) are possible sources of PPVs that have subsequently spread to other countries of the continent. While circulating PPV2 strains exhibited a large genetic exchange characterized by significant gene flow between distinct regions, PPV3 and PPV4 showed a diversification of viral lineages reflected by the accumulation of geographically structured polymorphisms. The present study also provides data on the evolutionary dynamics of PPVs, serving as a useful substrate for further studies regarding the effect of specific mutations, geographical genetic diversity and the possible implication or association as a co-factor in the development of swine diseases such as PCVAD.

Acknowledgements

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