

Porcine reproductive and respiratory syndrome virus strains of exceptional diversity in eastern Europe support the definition of new genetic subtypes

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Supplementary figures and a table showing details of the Belarusian and Polish herds from which sequences were obtained are available.

Porcine reproductive and respiratory syndrome virus (PRRSV) ORF5 and ORF7 sequences from Belarus were found to be of the European (EU) genotype, but grouped separately from all other EU genotype sequences described so far, including live-attenuated EU genotype PRRSV vaccines and Italian EU genotype sequences, some of which have been associated with reduced vaccine efficacy. Also, the Belarusian EU-PRRSV exhibited extreme ORF7 size polymorphism, ranging from 375 nt (the smallest EU genotype ORF7 yet described) to 393 nt (the largest ORF7 yet described for any arterivirus). With the Belarusian sequences, the diversity of EU genotype PRRSV now exceeds that of the North American (US) genotype PRRSV, suggesting a European origin of PRRSV. Finally, a very sharp geographical demarcation of highly diverse EU genotype PRRSV was observed along the eastern Polish border. The new Belarusian sequences have relevance for vaccine and diagnostic-antigen design and show that sequence analysis of PRRSV from more eastern parts of Europe may offer further insights into the emergence and evolution of PRRSV.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a recently emerged pathogen. Based on reporting of disease symptoms and retrospective serological surveys, the virus emerged in Canada in 1979 (Carman *et al.*, 1995), in the USA in 1985 (Zimmerman *et al.*, 1997), in South Korea in 1985 (Shin *et al.*, 1993), in the Asian part of the former Soviet Union in 1986 (Grebennikova *et al.*, 2004), in Japan in 1987 (Yoshii *et al.*, 2005), in the former German Democratic Republic in 1987 (Ohlinger *et al.*, 2000), in the Philippines in 1987 (Thanawongnuwech *et al.*, 2003) and in Thailand in 1989 (Thanawongnuwech *et al.*, 2004). In western Europe, the first clinical outbreaks were reported in November 1990 in Germany, with outbreaks in the Netherlands, Spain, UK, France, Belgium and Denmark occurring through 1991–1992 (OIE, 1992).

Thus, PRRSV emerged globally through a brief time window. Surprisingly, the viruses that appeared in Europe and North America were related only distantly (55–70 % nucleotide identity). PRRSV was first isolated in the Netherlands (Wensvoort *et al.*, 1991) and that isolate (Lelystad virus), together with the first North American isolate (VR2332), now define the two recognized genotypes of PRRSV: European (EU genotype, type I) and North American (US genotype, type II) (Snijder *et al.*, 2004).

The cotemporal emergence of genetically very different viruses could be due to two independent species jumps in Europe and North America (triggered by as-yet-unknown factors) or a single species-jump event at an unknown location followed by very quick global spread, coupled with unusually quick evolution of the virus (Forsberg, 2005; Hanada *et al.*, 2005). However, phylogenetic analysis places the most recent common ancestor (MRCA) for the EU and US genotypes at least 100 years back in time (Forsberg, 2005; Hanada *et al.*, 2005), providing strong support for the hypothesis that EU and US viruses evolved in parallel in North America and Europe prior to their cotemporal species jump into pigs and emergence as clinical entities in the latter 1980s.

Originally, EU genotype viruses were thought to form a very homogeneous, 'Lelystad-like' group (Wensvoort *et al.*, 1991; Suarez *et al.*, 1996; Drew *et al.*, 1997; Le Gall *et al.*, 1998). More recently, the view that EU genotype viruses are genetically homogeneous and Lelystad-like was challenged by the reporting of unusually diverse EU genotype PRRSV strains, first in Denmark (Oleksiewicz *et al.*, 2000) and later in Italy (Forsberg *et al.*, 2002), the Czech Republic (Indik *et al.*, 2000), Poland (Stadejek *et al.*, 2002), Spain (Mateu *et al.*, 2003), Germany and the Netherlands (Pesch *et al.*, 2005) and even Thailand (Thanawongnuwech *et al.*, 2004). In a recent, groundbreaking study, by using a Lelystad-like, live-attenuated vaccine strain and an Italian isolate as challenge, it was demonstrated that the genetic diversity within EU genotype viruses is sufficient to impact vaccine efficacy (Labarque *et al.*, 2004). The Italian challenge virus represented one of the most diverse EU genotype field isolates known at the time (Forsberg *et al.*, 2002). Since then, new studies have demonstrated that eastern European countries such as Lithuania harbour EU genotype strains of much higher diversity than does Italy (Stadejek *et al.*, 2002).

Because of the great importance of PRRSV diversity for vaccine development and because we wished to further explore the diversity of EU genotype PRRSV in eastern Europe,

we sequenced EU genotype field strains from 11 Belarusian herds. The herds were large, ranging from 2500 to 9000 sows, and included farrow to finish and nucleus herds. All herds were infected persistently with PRRSV and presented the full range of disease conditions that could be ascribed to PRRSV. For comparison with the Belarusian sequences, we obtained sequences from four herds located in north-eastern Poland, close to the Lithuanian, Russian and Belarusian borders, and from one herd from western Poland (Fig. 1; Table 1; Supplementary Table S1). RNA purification from pig serum, RT-PCR amplification of open reading frames (ORFs) 5 and 7 and sequencing of bulk (not cloned) PCR product were done essentially as described previously (Stadejek *et al.*, 2002). Briefly, for RT-PCR of ORF5, previously described primers were used (Stadejek *et al.*, 2002). RT-nested PCR of ORF7 was performed by using the following primers: external, 5'-GCCCCTGCCCAICACG-3' and 5'-TCGCCCTAATTGAATAGGTGA-3' (Oleksiewicz *et al.*, 1998), and internal, 5'-TCGCCCTAATTGAATAGGTGACTC-3' and 5'-CGAGCTGTAAACGAGGAGTG-3' (Drew *et al.*, 1997). The ORF5 RT-PCR was specific for EU genotype PRRSV (i.e. the primer-binding sites are not conserved in US genotype PRRSV), whereas the ORF7 primer-binding sites are conserved between EU and US genotype viruses. The three currently available EU genotype live-attenuated vaccines, Porcilis PRRS (Intervet), Amervac-PRRS (HIPRA) and Pysrvac-183 (SYVA), were also sequenced (Table 1).

All of the new Belarusian and Polish sequences were of the EU genotype. For phylogenetic analysis, we used a set of reference sequences representing the maximal global diversity of EU and US genotype PRRSV (Table 1). When analysed together with the new Belarusian EU genotype sequences, the highly diverse reference EU type sequences coalesced to form a single group, termed EU subtype 1 (Fig. 2). In contrast, the Belarusian and Lithuanian sequences were separated from EU subtype 1. Thus, the new Belarusian sequences demonstrated that EU genotype PRRSV consists of several genetic subtypes that can be separated with high bootstrap support (Fig. 2a). An identical picture was seen after phylogenetic analysis of complete ORF7 sequences (see Supplementary Fig. S1). Only one sequence, Bel-42, exhibited different positioning in the ORF5 and ORF7 trees. This could be due to recombination, which has been described previously in EU as well as US genotype field strains (van Vugt *et al.*, 2001) and for which conserved sequence stretches between ORFs 5 and 7 are known to serve as hot spots (Forsberg *et al.*, 2002).

There was a sharp geographical demarcation along the eastern Polish border between the highly diverse EU subtypes (Fig. 1 and 2). Thus, our data show that, in most of Europe (west of the eastern Polish border), relatively closely related EU genotype strains circulate, whereas east of the eastern Polish border, a great diversity of EU genotype strains can be observed (Fig. 1). We believe that this EU genotype PRRSV distribution pattern is explained by trade: whilst Belarus imports breeding animals from the west, there is essentially no livestock trade from Belarus to Poland. Because ancestral populations are generally held to be more diverse than descendant populations, we suggest that this lack of livestock movement from east to west may have serendipitously preserved phylogenetic evidence of PRRSV emergence in eastern Europe. Other reasons also make a 'Eurasian PRRSV origin' hypothesis attractive: the

currently available sequences place the MRCA of EU genotype PRRSV strains between 1946 and 1967, i.e. during the post-World War II development of Europe (R. Forsberg, personal communication). It seems plausible that the post-war expansion of the former Soviet Union might have created an environment that allowed a new virus to emerge or an already emerged virus to spread. Also, because Poland and other east and central European countries were influenced much less by the Soviet Union animal-breeding policies than, for example, Belarus and the Baltic states (e.g. Lithuania), it seems plausible that, if new PRRSV subtypes arose during the post-war upheavals, they would be dominant in Lithuania, Belarus and probably other countries of the former Soviet Union, but would not necessarily be present west of the Polish border (Fig. 1).

The nucleocapsid protein is one of the most conserved PRRSV proteins. Accordingly, without exception, all previous studies covering the time period 1989–2005 in western, northern, central and southern Europe, North America and Asia, yielding approximately 270 sequences deposited in GenBank of both EU and US genotypes, have so far failed to reveal size polymorphisms in the PRRSV nucleocapsid protein (Meng *et al.*, 1995; Suarez *et al.*, 1996; Drew *et al.*, 1997; Le Gall *et al.*, 1998; Forsberg *et al.*, 2002). Accordingly, based on deduced amino acid sequence, all of the new Polish strains sequenced in the present study had ORF7 sizes prototypical for EU genotype PRRSV (128 aa). In contrast, the three Belarusian PRRSV strains had ORF7 protein sizes from 124 aa, the lowest ORF7 size reported so far for EU genotype PRRSV, to 130 aa, the largest ORF7 size yet reported for any arterivirus (Snijder *et al.*, 2004) (see Supplementary Fig. S2).

In addition to the extreme ORF7 polymorphism, the Belarusian PRRSV strains also provided a rare example of variability in the otherwise highly conserved N-46 glycosylation site of GP₅ (Chen *et al.*, 2000; Wissink *et al.*, 2004; Mateu *et al.*, 2005). N-46 was found to be important for infectious virion production in the context of an infectious cDNA clone of Lelystad virus (Wissink *et al.*, 2004). In contrast, we found that viruses without N-46 were relatively common in the field in Belarus. Also, we found that, in the same virus derived from different age groups, N-46 was present in some age groups and not in others (see Bor and Zad farm sequences in Supplementary Fig. S3). In LDV, it was found that N-46 and N-53 were always present in non-neuropathogenic strains, whereas in neuropathogenic strains, N-46 was always lacking and this correlated with a higher susceptibility to antibody neutralization (Chen *et al.*, 2000). Similarly for PRRSV, GP₅ glycosylation has recently been shown to be important for antibody neutralization (Ansari *et al.*, 2006). Thus, based on the observation of N-46 variability in pigs of different age groups (see Bor and Zad farm sequences in Supplementary Fig. S3), it could be hypothesized that PRRSV uses variability of the N-46 glycosylation site as a genetic switch to adjust immune-system interactions to the age of its host.

In an ORF5-based phylogeny between EU and US genotype viruses, including the new Belarusian sequences, the whole EU genotype cluster exhibited clearly higher diversity than the US genotype cluster (Fig. 2b). As mentioned in the introduction, PRRSV appears to have emerged independently in Europe and North America in the 1980s (Forsberg, 2005; Hanada *et al.*, 2005). Thus, a pre-PRRS virus must be postulated to have existed in reservoir species,

making species jumps into pigs triggered by global factors that acted in Europe and North America almost simultaneously. Global candidate triggering factors can easily be conceived. For example, the global emergence in the early 1980s of porcine respiratory coronavirus, which shares cell tropism with PRRSV in the pulmonary tract (Laude *et al.*, 1993), could be hypothesized to have helped PRRSV emergence. However, there remains the question of the origin of pre-PRRSV. Most likely, pre-PRRSV existed in either Europe or North America and spread to the other continent by means of export or migration of the unknown original host, well before the species jump into pigs the 1980s. By using arguments similar to those used to track human immunodeficiency virus emergence (Mokili & Korber, 2005), it seems plausible that the ancestral population of pre-PRRSV should exhibit a larger genetic diversity than the colonist population and that this situation would be reflected in PRRSV diversity post-emergence. Thus, because of the large diversity of EU genotype viruses revealed in the present study (Fig. 2b), a European or Eurasian origin of pre-PRRSV currently seems most likely. Further indirect support for a European origin of pre-PRRSV comes from the fact that house mice have been suggested as the most likely reservoir species for pre-PRRSV (Plagemann, 2003) and these rodents colonized North America from Europe (Tichy *et al.*, 1994). An alternative hypothesis suggested that PRRSV was spread to the USA by wild-boar imports from Europe (Plagemann, 2003).

In summary, a quite large number of recent studies have examined the genetic diversity of EU genotype PRRSV in Europe (Suarez *et al.*, 1996; Drew *et al.*, 1997; Le Gall *et al.*, 1998; Indik *et al.*, 2000, 2005; Forsberg *et al.*, 2002; Stadejek *et al.*, 2002; Mateu *et al.*, 2003, 2005; Pesch *et al.*, 2005). On this basis, the value of performing yet more molecular-phylogeny work should be questioned. However, the current study illustrates that sampling new geographical regions in Europe remains a highly worthwhile undertaking, revealing a hitherto-unsuspected diversity of EU genotype PRRSV east of Poland (Figs 1 and 2). In contrast, all known EU genotype sequences from west of the Poland/Belarus border formed a single phylogenetic cluster, operationally termed subtype 1 (Figs 1 and 2). Whilst subtype 1 appears microheterogeneous by comparison with the new Belarusian sequences (Fig. 2), the diversity within the European subtype 1 is in fact at least as large as within the total body of US genotype PRRSV (Forsberg *et al.*, 2002) (Fig. 2b) and sufficiently large to impact vaccine efficacy (Labarque *et al.*, 2004). Because all current EU-PRRSV vaccines, as well as all current EU genotype PRRSV ELISA antigens, belong to subtype 1 (Fig. 2 and Table 1), the new subtypes of EU genotype PRRSV described in this study are relevant for vaccine and diagnostic-assay development. In short, further molecular-epidemiology studies in the far-eastern parts of Europe and in Asia would combine applied and basic scientific gains, namely knowledge for design of second-generation diagnostic assays and vaccines, as well as unravelling the emergence of PRRSV in Europe.

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References

- Ansari, I. H., Kwon, B., Osorio, F. A. & Pattnaik, A. K. (2006).** Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. *J Virol* **80**, 3994–4004.
- Carman, S., Sanford, S. E. & Dea, S. (1995).** Assessment of seropositivity to porcine reproductive and respiratory syndrome (PRRS) virus in swine herds in Ontario – 1978 to 1982. *Can Vet J* **36**, 776–777.
- Chen, Z., Li, K. & Plagemann, P. G. W. (2000).** Neuropathogenicity and sensitivity to antibody neutralization of lactate dehydrogenase-elevating virus are determined by polylectosaminoglycan chains on the primary envelope glycoprotein. *Virology* **266**, 88–98.
- Drew, T. W., Lowings, J. P. & Yapp, F. (1997).** Variation in open reading frames 3, 4 and 7 among porcine reproductive and respiratory syndrome virus isolates in the UK. *Vet Microbiol* **55**, 209–221.
- Forsberg, R. (2005).** Divergence time of porcine reproductive and respiratory syndrome virus subtypes. *Mol Biol Evol* **22**, 2131–2134.
- Forsberg, R., Storgaard, T., Nielsen, H. S., Oleksiewicz, M. B., Cordioli, P., Sala, G., Hein, J. & Bøtner, A. (2002).** The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. *Virology* **299**, 38–47.
- Grebennikova, T. V., Zaberezhny, A. D., Vlasova, A. N. & 8 other authors (2004).** Genetic variability of the nucleocapsid protein of the virus of the porcine reproductive and respiratory syndrome. *Mol Gen Mikrobiol Virusol* **2**, 37–40 (in Russian).
- Hanada, K., Suzuki, Y., Nakane, T., Hirose, O. & Gojobori, T. (2005).** The origin and evolution of porcine reproductive and respiratory syndrome viruses. *Mol Biol Evol* **22**, 1024–1031.
- Indik, S., Valíček, L., Klein, D. & Klánová, J. (2000).** Variations in the major envelope glycoprotein GP5 of Czech strains of porcine reproductive and respiratory syndrome virus. *J Gen Virol* **81**, 2497–2502.
- Indik, S., Schmoll, F., Sipos, W. & Klein, D. (2005).** Genetic variability of PRRS virus in Austria: consequences for molecular diagnostics and viral quantification. *Vet Microbiol* **107**, 171–178.
- Labarque, G., Van Reeth, K., Nauwynck, H., Drexler, C., Van Gucht, S. & Pensaert, M. (2004).** Impact of genetic diversity of European-type porcine reproductive and respiratory syndrome virus strains on vaccine efficacy. *Vaccine* **22**, 4183–4190.
- Laude, H., Van Reeth, K. & Pensaert, M. (1993).** Porcine respiratory coronavirus: molecular features and virus-host interactions. *Vet Res* **24**, 125–150.

- Le Gall, A., Legeay, O., Bourhy, H., Arnauld, C., Albina, E. & Jestin, A. (1998).** Molecular variation in the nucleoprotein gene (ORF7) of the porcine reproductive and respiratory syndrome virus (PRRSV). *Virus Res* **54**, 9–21.
- Mateu, E., Martín, M. & Vidal, D. (2003).** Genetic diversity and phylogenetic analysis of glycoprotein 5 of European-type porcine reproductive and respiratory virus strains in Spain. *J Gen Virol* **84**, 529–534.
- Mateu, E., Díaz, I., Darwich, L., Casal, J., Martín, M. & Pujols, J. (2005).** Evolution of ORF5 of Spanish porcine reproductive and respiratory syndrome virus strains from 1991 to 2005. *Virus Res* **115**, 198–206.
- Meng, X.-J., Paul, P. S., Halbur, P. G. & Lum, M. A. (1995).** Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. *Arch Virol* **140**, 745–755.
- Mokili, J. & Korber, B. (2005).** The spread of HIV in Africa. *J Neurovirol* **11** (Suppl. 1), 66–75.
- Ohlinger, V. F., Pesch, S. & Bischoff, C. (2000).** History, occurrence, dynamics, and current status of PRRS in Europe. *Vet Res* **31**, 86–87.
- OIE (1992).** Animal health status and disease control methods (part one: reports). In *World Animal Health 1991*, vol. VII, no. 2., p. 126. Paris: Office International Épizooties.
- Oleksiewicz, M. B., Bøtner, A., Madsen, K. G. & Storgaard, T. (1998).** Sensitive detection and typing of porcine reproductive and respiratory syndrome virus by RT-PCR amplification of whole viral genes. *Vet Microbiol* **64**, 7–22.
- Oleksiewicz, M. B., Bøtner, A., Toft, P., Grubbe, T., Nielsen, J., Kamstrup, S. & Storgaard, T. (2000).** Emergence of porcine reproductive and respiratory syndrome virus deletion mutants: correlation with the porcine antibody response to a hypervariable site in the ORF 3 structural glycoprotein. *Virology* **267**, 135–140.
- Pesch, S., Meyer, C. & Ohlinger, V. F. (2005).** New insights into the genetic diversity of European porcine reproductive and respiratory syndrome virus (PRRSV). *Vet Microbiol* **107**, 31–48.
- Plagemann, P. G. W. (2003).** Porcine reproductive and respiratory syndrome virus: origin hypothesis. *Emerg Infect Dis* **9**, 903–908.
- Shin, J.-H., Kang, Y.-B., Kim, Y.-J. & 12 other authors (1993).** Sero-epidemiological studies on porcine reproductive and respiratory syndrome in Korea. I. Detection of indirect fluorescent antibodies. *J Agric Sci* **35**, 572–576.
- Snijder, E. J., Brinton, M. A., Faaborg, K. S., Godeny, E. K., Gorbalenya, A. E., MacLachlan, N. J., Mengeling, W. L. & Plagemann, P. G. W. (2004).** Family *Arteriviridae*. In *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses*. Edited by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger & L. A. Ball. London: Elsevier/Academic Press.
- Stadejek, T., Stankevicius, A., Storgaard, T., Oleksiewicz, M. B., Belák, S., Drew, T. W. & Pejsak, Z. (2002).** Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. *J Gen Virol* **83**, 1861–1873.

- Suarez, P., Zardoya, R., Martin, M. J., Prieto, C., Dopazo, J., Solana, A. & Castro, J. M. (1996).** Phylogenetic relationships of European strains of porcine reproductive and respiratory syndrome virus (PRRSV) inferred from DNA sequences of putative ORF-5 and ORF-7 genes. *Virus Res* **42**, 159–165.
- Thanawongnuwech, R., Damrongwatanapokin, S. & Horcharoen, A. (2003).** PRRS virus in Southeast Asia. In *2003 PRRS Compendium*, pp. 269–273. Edited by J. Zimmerman & K.-J. Yoon. Des Moines: National Pork Board.
- Thanawongnuwech, R., Amonsin, A., Tatsanakit, A. & Damrongwatanapokin, S. (2004).** Genetics and geographical variation of porcine reproductive and respiratory syndrome virus (PRRSV) in Thailand. *Vet Microbiol* **101**, 9–21.
- Tichy, H., Zaleska-Rutczynska, Z., O’Huigin, C., Figueroa, F. & Klein, J. (1994).** Origin of the North American house mouse. *Folia Biol (Praha)* **40**, 483–496.
- van Vugt, J. J. F. A., Storgaard, T., Oleksiewicz, M. B. & Bøtner, A. (2001).** High frequency RNA recombination in porcine reproductive and respiratory syndrome virus occurs preferentially between parental sequences with high similarity. *J Gen Virol* **82**, 2615–2620.
- Wensvoort, G., Terpstra, C., Pol, J. M. A & 19 other authors (1991).** Mystery swine disease in the Netherlands: the isolation of Lelystad virus. *Vet Q* **13**, 121–130.
- Wissink, E. H. J., Kroese, M. V., Maneschijn-Bonsing, J. G., Meulenbergh, J. J. M., van Rijn, P. A., Rijsewijk, F. A. M. & Rottier, P. J. M. (2004).** Significance of the oligosaccharides of the porcine reproductive and respiratory syndrome virus glycoproteins GP_{2a} and GP₅ for infectious virus production. *J Gen Virol* **85**, 3715–3723.
- Yoshii, M., Kaku, Y., Murakami, Y., Shimizu, M., Kato, K. & Ikeda, H. (2005).** Genetic variation and geographic distribution of porcine reproductive and respiratory syndrome virus in Japan. *Arch Virol* **150**, 2313–2324.
- Zimmerman, J. J., Yoon, K.-J., Wills, R. W. & Swenson, S. L. (1997).** General overview of PRRSV: a perspective from the United States. *Vet Microbiol* **55**, 187–196.

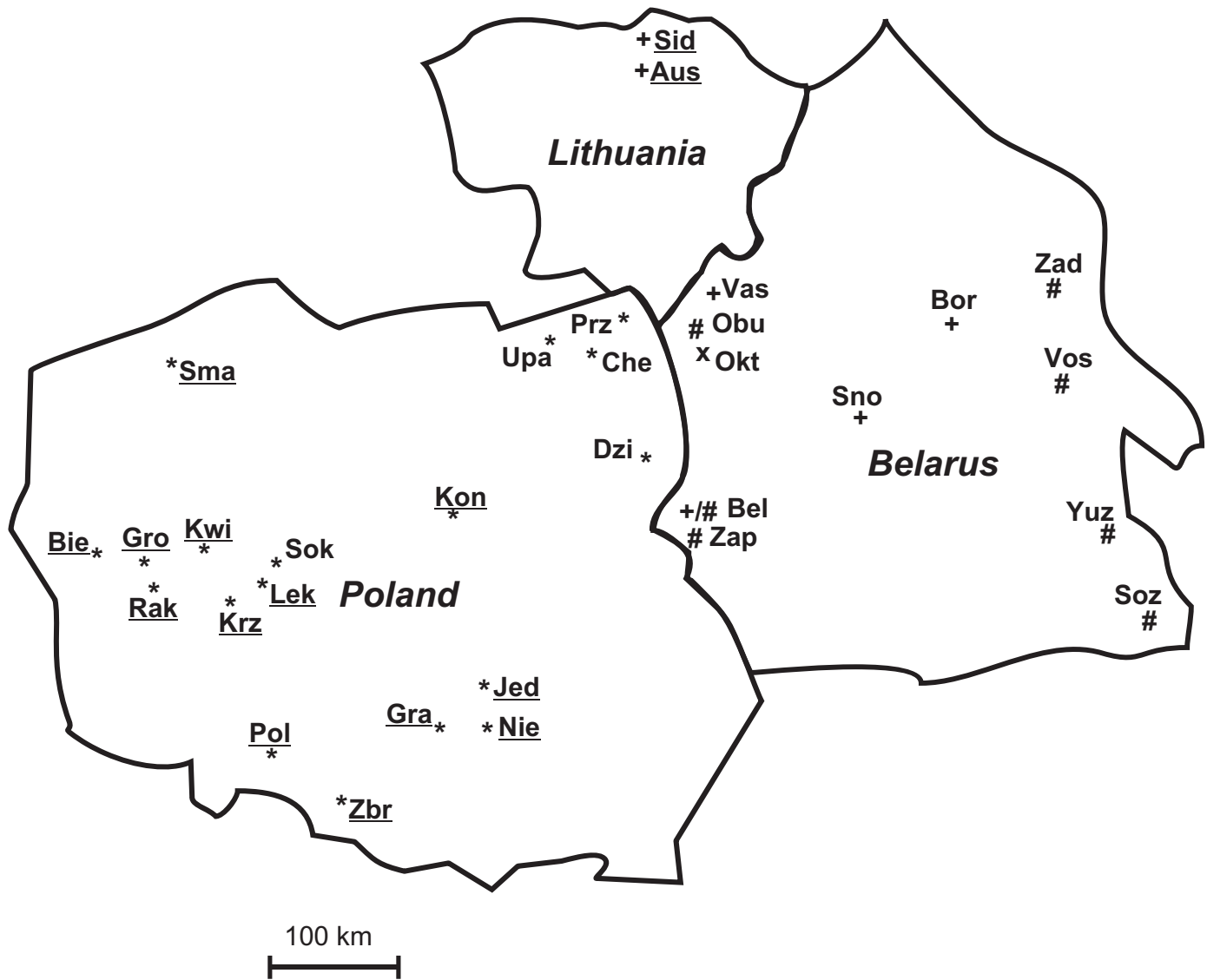


Fig. 1. The eastern Polish border separates radically diverse EU genotype PRRSVs. The locations of the five Polish and 11 Belarusian herds from which new sequences were derived in the present study are labelled by a three-letter designation. The locations of the herds from which sequences were derived in a previous study (Stadejek *et al.*, 2002) are indicated by underlined names. Herds with EU subtype 1 viruses are marked '*', EU subtype 2 '+', EU subtype 3 '#' and EU subtype 4 'x'. Herd Bel, where sequence analysis indicated recombinant virus composed of subtype 2 and subtype 3 material, is marked '+/#'.

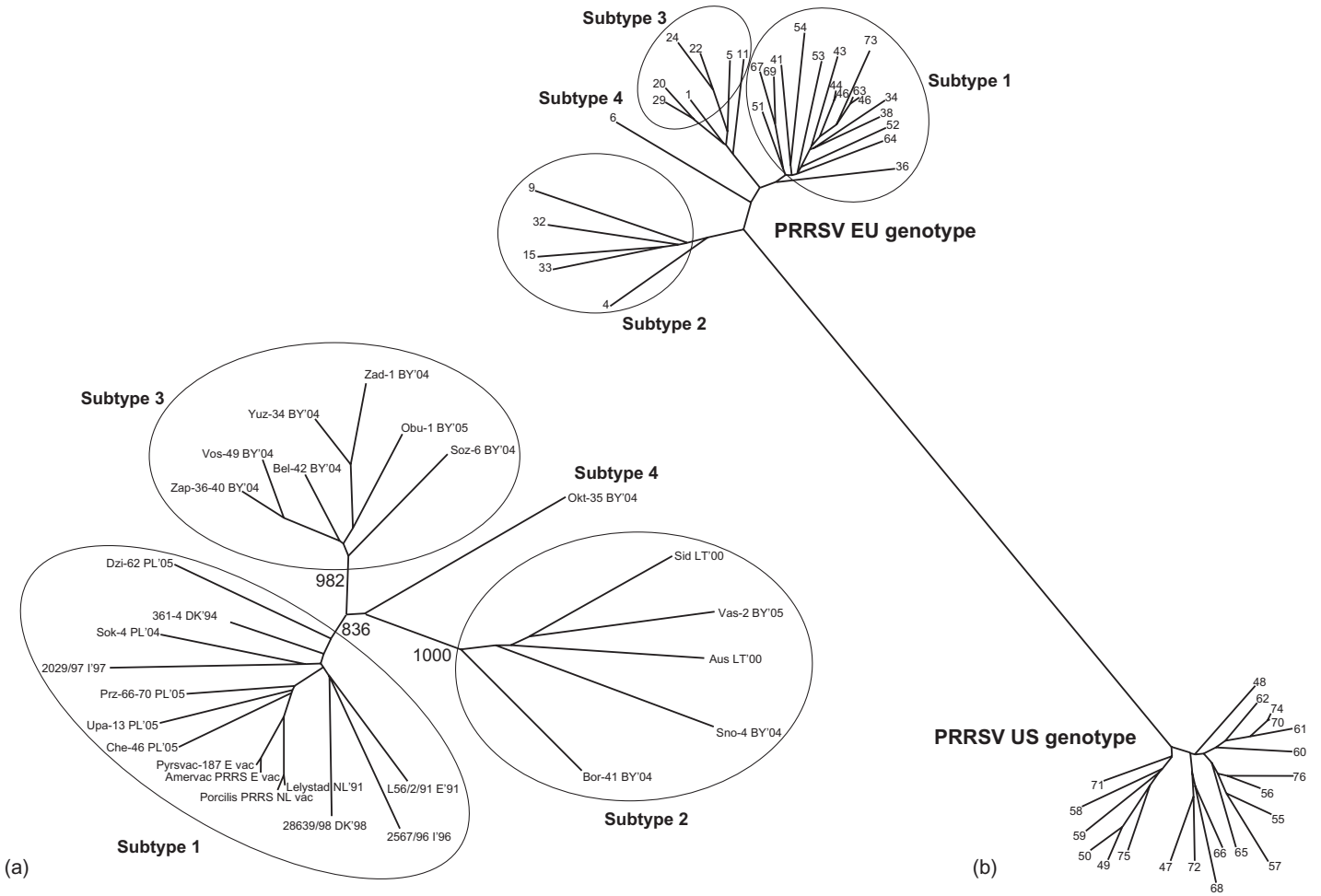


Fig. 2. Belarusian strains expand the diversity of EU genotype PRRSV beyond that of US genotype PRRSV. (a) EU genotype ORF5 tree. The bootstrap values adjacent to the main nodes represent the number of 1000 trees that supported the clustering. The Sid, Aus, Bor and Sno herds had trade links (replacement animals and semen). (b) Pan-PRRSV (EU+US genotype) ORF5 tree. Branch numbers correspond to the first column of Table 1.

Table 1. PRRSV sequence summary

Sequences 1–46 were obtained as part of this study (all EU genotype). The two Lithuanian sequences (Aus and Sid) have been described previously, but were resequenced from original material for the present study (Stadejek *et al.*, 2002). Sequences 47–76 are reference sequences, representing the most diverse EU and US genotype sequences available in GenBank (Forsberg *et al.*, 2002; Thanawongnuwech *et al.*, 2004; Yoshii *et al.*, 2005). The consecutive numbers 1–76 are also used in branch labelling in the phylogenetic trees in Fig. 2(b). ORF7 size includes the stop codon.

No.	Sequence name (herd ID-serum no.)	Pig age (days)	Country	Year	GenBank accession no.		ORF7 size (nt)	Genotype-subtype
					ORF5	ORF7		
1	Bel-42	65	Belarus	2004	DQ324669	DQ324699	378	EU-3/2
2	Bel-43	65	Belarus	2004	DQ324670	DQ324700	378	EU-3/2
3	Bor-41	65	Belarus	2004	DQ324671	DQ324701	393	EU-2
4	Bor-54	120	Belarus	2004	DQ324672	DQ324702	393	EU-2
5	Obu-1	30	Belarus	2005	DQ324676	DQ324707	375	EU-3
6	Okt-35	65	Belarus	2004	DQ324677			EU-4
7	Okt-46	65	Belarus	2004		DQ324708	375	EU-4
8	Okt-47	65	Belarus	2004		DQ324709	375	EU-4
9	Sno-4		Belarus	2004	DQ324683	DQ324713	378	EU-2
10	Sno-6		Belarus	2004		DQ324714	378	EU-2
11	Soz-6	80	Belarus	2004	DQ324686	DQ324719	375	EU-3
12	Soz-8	80	Belarus	2004	DQ324687	DQ324720	375	EU-3
13	Soz-42	65	Belarus	2004		DQ324717	375	EU-3
14	Soz-43	65	Belarus	2004		DQ324718	375	EU-3
15	Vas-2	55	Belarus	2005	DQ324689	DQ324722	378	EU-2
16	Vas-3	55	Belarus	2005		DQ324723	378	EU-2
17	Vas-4	55	Belarus	2005		DQ324724	378	EU-2

No.	Sequence name (herd ID-serum no.)	Pig age (days)	Country	Year	GenBank accession no.		ORF7 size (nt)	Genotype-subtype
					ORF5	ORF7		
18	Vos-29	220	Belarus	2004		DQ324725	375	EU-2
19	Vos-41	220	Belarus	2004		DQ324726	375	EU-3
20	Vos-49	220	Belarus	2004	DQ324690			EU-3
21	Vos-50	220	Belarus	2004	DQ324691			EU-3
22	Yuz-34	65	Belarus	2004	DQ324692	DQ324727	375	EU-3
23	Yuz-48	65	Belarus	2004	DQ324693	DQ324728	375	EU-3
24	Zad-1	30	Belarus	2004	DQ324694	DQ324729	375	EU-3
25	Zad-13	60	Belarus	2004		DQ324730	375	EU-3
26	Zad-14	60	Belarus	2004	DQ324695	DQ324731	375	EU-3
27	Zad-37	120	Belarus	2004		DQ324732	375	EU-3
28	Zad-39	120	Belarus	2004		DQ324733	375	EU-3
29	Zap-36-40	65	Belarus	2004	DQ324696			EU-3
30	Zap-41	65	Belarus	2004		DQ324734	375	EU-3
31	Zap-46-50	65	Belarus	2004	DQ324697			EU-3
32	Aus		Lithuania	2000	DQ324667	AF438362	378	EU-2
33	Sid		Lithuania	2000	DQ324682	AF438363	378	EU-2
34	Che-46	63	Poland	2005	DQ324673	DQ324703	387	EU-1
35	Che-50	63	Poland	2005	DQ324674	DQ324704	387	EU-1
36	Dzi-62	91	Poland	2005	DQ324675	DQ324705	387	EU-1
37	Dzi-64	91	Poland	2005		DQ324706	387	EU-1
38	Prz-66-70	49	Poland	2005	DQ324679			EU-1
39	Prz-71-75	63	Poland	2005	DQ324680			EU-1
40	Prz-87	120	Poland	2005		DQ324711	387	EU-1
41	Sok-4	42	Poland	2004	DQ324684	DQ324715	387	EU-1
42	Sok-9	56	Poland	2004	DQ324685	DQ324716	387	EU-1

No.	Sequence name (herd ID-serum no.)	Pig age (days)	Country	Year	GenBank accession no.		ORF7 size (nt)	Genotype-subtype
					ORF5	ORF7		
43	Upa-13	101	Poland	2005	DQ324688	DQ324721	387	EU-1
44	Amervac PRRS		Spain	Vaccine	DQ324668	DQ324698	387	EU-1
45	Pyrsvac-183		Spain	Vaccine	DQ324681	DQ324712	387	EU-1
46	Porcilis PRRS		The Netherlands	Vaccine	DQ324678	DQ324710	387	EU-1
47	Quebec 807/94		Canada	1994	Z82995			US
48	FJ-1		China		AY881994			US
49	GDCZ1		China		AY857635			US
50	GDCZ2		China		AY857636			US
51	361-4		Denmark	1994	AY035915	AY035960	387	EU-1
52	28639/98		Denmark	1998	AY035912	AY035957	387	EU-1
53	2567/96		Italy	1996	AY035932	AY035976	387	EU-1
54	2029/97		Italy	1997	AY035930	AY035973	387	EU-1
55	Gu922M		Japan	1992	AB175721			US
56	Nagasaki 93		Japan	1993				US
57	Jeh1		Japan	2000	AB175691			US
58	Jis1		Japan	2000	AB175694			US
59	Jis2		Japan	2000	AB175695			US
60	Jos1		Japan	2000	AB175699			US
61	Jyt2		Japan	2000	AB175713			US
62	Jam2		Japan	2000	AB175690			US
63	Lelystad		The Netherlands	1991	M96262	M96262	387	EU-1
64	L56/2/91		Spain	1991	AY035935	AY035979	387	EU-1
65	MD-001		Taiwan		AF121131			US
66	01UD6		Thailand	2001	AY297113			US
67	01CB1		Thailand	2001	AY297119			EU-1

No.	Sequence name (herd ID-serum no.)	Pig age (days)	Country	Year	GenBank accession no.		ORF7 size (nt)	Genotype-subtype
					ORF5	ORF7		
68	02PB1		Thailand	2002	AY297116			US
69	03RB1		Thailand	2003	AY297124			EU-1
70	VR-2332		USA	1989	U87392			US
71	HP		USA		AY754345			US
72	MN-184		USA		AY656992			US
73	EuroPRRS		USA	1999	AY366525			EU-1
74	Ingelvac PRRS MLV		USA	Vaccine	AF020048			US
75	Ingelvac PRRS ATP		USA	Vaccine	AY656991			US
76	PrimePac PRRS		USA	Vaccine	AF066384			US