Phylogeny-Based Evolutionary, Demographical, and Geographical Dissection of North American Type 2 Porcine Reproductive and Respiratory Syndrome Viruses[▽]

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Type 2 (or North American-like) porcine reproductive and respiratory syndrome virus (PRRSV) was first recorded in 1987 in the United States and now occurs in most commercial swine industries throughout the world. In this study, we investigated the epidemiological and evolutionary behaviors of type 2 PRRSV. Based on phylogenetic analyses of 8,624 ORF5 sequences, we described a comprehensive picture of the diversity of type 2 PRRSVs and systematically classified all available sequences into lineages and sublineages, including a number of previously undescribed lineages. With the rapid growth of sequence deposition into the databases, it would be technically difficult for veterinary researchers to genotype their sequences by reanalyzing all sequences in the databases. To this end, a set of reference sequences was established based on our classification system, which represents the principal diversity of all available sequences and can readily be used for further genotyping studies. In addition, we further investigated the demographic histories of these lineages and sublineages by using Bayesian coalescence analyses, providing evolutionary insights into several important epidemiological events of type 2 PRRSV. Moreover, by using a phylogeographic approach, we were able to estimate the transmission frequencies between the pig-producing states in the United States and identified several states as the major sources of viral spread, i.e., “transmission centers.” In summary, this study represents the most extensive phylogenetic analyses of type 2 PRRSV to date, providing a basis for future genotyping studies and dissecting the epidemiology of type 2 PRRSV from phylogenetic perspectives.

Porcine reproductive and respiratory syndrome virus (PRRSV) is an economically important virus which infects swine and causes reproductive failure in sows and respiratory problems in growing pigs. As a member of the *Arteriviridae* family (15, 47, 59, 66), PRRSV has a positive-sense RNA genome of approximately 15 kb that carries eight overlapping open reading frames (ORFs), designated ORFs 1a, 1b, and 2 to 7 (15, 47). Among these ORFs, ORF5, encoding the major envelope glycoprotein, is an ideal candidate for phylogenetic tree construction, because it exhibits marked genetic variation within its relatively short length.

PRRSV can be classified into two genotypes: type 1 (EU-like), comprising mainly European strains and represented by the prototype strain Lelystad (75); and type 2 (NA-like), comprising mainly North American strains and represented by the prototype strain VR-2332 (14). Although clinical diseases are similar following infections with these viruses, they differ significantly in terms of antigenic properties (18, 74) and genetic content (42, 48, 51). This has sparked hot debates on the evolutionary history and divergence time of these two geno-

types (24, 25, 29, 58), but no substantial consensus has been reached.

Classification and epidemiology of type 2 PRRSV. Clinical disease due to type 2 PRRSV was first recorded in 1987 in the United States (30, 35). Although PRRSV antibody-positive sera were identified retrospectively back to the mid-1980s in Iowa (31) and to 1979 in eastern Canada (31), viruses were never detected in the sera. During its short history, the virus has gained remarkable diversity (34, 49), and it now occurs in most commercial swine industries throughout the world (10, 12, 41, 69, 77). There are a number of important epidemiological events in the history of type 2 PRRSVs, and their origins remain mysterious, including the emergence of an MN184-related cluster (28), “acute PRRS”/“abortion storm” (3, 4, 36), and highly pathogenic Chinese strains (58).

Despite the wealth of sequence data in the databases, there has been no satisfactory classification system to cover the diversity of all available type 2 PRRSV sequences. Nonetheless, a framework was constructed by some Asian research groups, who were seeking to genotype their own strains (72, 77). Their work reveals the presence of the following four potential lineages: (i) sequences closely related to prototype strain VR-2332; (ii) sequences having the RFLP184 pattern, including Canadian and Thai field isolates as well as the newly emerged MN184-related sequences (28); (iii) sequences isolated in the “abortion storm” starting in the latter half of 1996 (36) and sequences closely related to the PrimePac vaccine strain (72);

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and (iv) several Asian sequences that bear significant diversity and are distantly related to the North American isolates (72, 77). The major limitations of these genotyping studies are the incomplete coverage of available sequences and the lack of a reference sequence set that can be used to guide future genotyping studies.

Problems of live attenuated vaccines. In an attempt to reduce the impact and transmission of type 2 PRRSV, tremendous effort has been directed to the development of vaccines. However, despite the availability of different types of vaccines, the disease still remains difficult to control. Current available live attenuated vaccines for type 2 PRRSV include MLV (Ingelvac PRRS MLV; Boehringer Ingelheim, Germany) and ATP (Ingelvac PRRS ATP; Boehringer Ingelheim, Germany). These vaccines are effective in reducing clinical signs and the duration of viral shedding (6, 7, 11, 26, 38, 45, 50), but they are not likely to completely prevent infection (37, 44). Also, the vaccine efficacy tended to drop significantly upon heterologous challenge (6, 44, 50, 54, 64). Moreover, the safety of live attenuated vaccines poses more serious problems. Vaccine viruses have been demonstrated to persist in vaccinated pigs and to spread to nonvaccinated pigs (46, 53, 71), indicating their ability to circulate in the field. Using restriction fragment length polymorphism (RFLP) typing, it was shown that the prevalence of vaccine strains and vaccine derivatives reached 10% in Quebec (39) and >33% in Ontario (5). In addition, vaccine derivatives might not share the same attenuated phenotype as their parental types, and reversion to a virulent type has been observed in the field (36, 52, 55).

Long-distance dissemination of PRRSV. PRRSV can be transmitted over long distances via pathways that are inherent features of modern swine production, notably the transportation of pigs and the use of contaminated semen. Over the last 15 years, multiple-site production systems (where pigs of different age groups are reared at separate locations) have been a preferred model for pig production in the United States. These systems require pig transport between locations, and in 2001, it was estimated that 27 million pigs born in the United States (approximately a quarter of production) were transported interstate (65). Over a million pigs were shipped from North Carolina to Iowa alone, and there are also substantial flows of weaned or feeder pigs from Canada into the United States' corn belt, particularly Minnesota. These industry activities obviously provide constant opportunity for long-distance dissemination of PRRSV. Another important mechanism of long-distance PRRSV transmission is through contaminated semen during the process of artificial insemination (76). PRRSV is shed in the semen of experimentally infected boars for up to 43 days postinfection, and viral RNA may exist even longer (13, 67), indicating that semen can be important in long-distance PRRSV transmission. In the United States, the relative importance of semen as a vehicle of PRRSV dissemination has declined due to biosecurity interventions (particularly air filtration) and intensive surveillance of boar studs, which preclude viral infection of boars and thus prevent contamination of semen (63). These two pathways, particularly animal movement, remain important mechanisms that can shorten the spatial and temporal distances present for the viruses and allow them to spread more efficiently and thoroughly.

Motivations of this study. As mentioned above, with the rapid growth of sequence deposition in the databases (e.g., for ORF5 of type 2 PRRSV, there are >8,500 sequences), these large data sets contain a wealth of information needed to understand the viral epidemiology of PRRSV; on the other hand, the size of these data sets represents a technical challenge for veterinary researchers in genotyping their own sequences. This study therefore attempts to perform the following: (i) to systematically classify all available type 2 PRRSV sequences (as lineages and sublineages) in order to establish a reference sequence set for future genotyping studies, (ii) to investigate the origins of several epidemiological events for type 2 PRRSVs (as mentioned above) by coalescence analyses of the lineages and sublineages, and (iii) to investigate the interstate viral transmission network in the United States in order to shed light on the mode of long-distance dissemination of PRRSV in the United States.

MATERIALS AND METHODS

Data sets. All available complete ORF5 gene sequences of type 2 PRRSVs were downloaded from GenBank and the PRRSV Database (<http://prrsdb.org/>) in January 2009. These sequences were comprised of 8,624 worldwide field samples, 3 live attenuated vaccine strains (MLV, ATP, and PrimePac), and 1 laboratory attenuated strain (Abst-1). Most of the field samples were from North America and were sequenced as part of routine diagnostic service by the Minnesota Veterinary Diagnostic Laboratory, the South Dakota Animal Disease Research & Diagnostic Laboratory, and the Iowa State University Veterinary Diagnostic Laboratory (deposited in the PRRSV Database). The group at the University of Hong Kong is one of the PRRSV Database contributors and contributed 131 sequences. The remaining sequences were provided primarily by a variety of research laboratories worldwide, including laboratories in Canada, Mexico, and the United States for North America; China, South Korea, Japan, and Thailand for Asia; and Austria, Denmark, Italy, and Poland for Europe.

Phylogeny construction and lineage classification. The ORF5 nucleotide sequences ($n = 8,624$) covering the first 199 codons (597 nucleotides [nt]) were aligned in MUSCLE v3.6 (22), using default settings, with minor manual adjustments. The alignment was then subjected to recombination screening, using Recombination Detection Program v2 (43). Eight potential recombinants were identified from the ORF5 alignment. However, it was difficult to determine whether they were naturally occurring recombinants or laboratory artifacts, because each recombinant type had only one representative sequence, which was evidence not in favor of its circulation in the field. These potential recombinant sequences were removed, generating an alignment of 8,616 sequences in the whole data set. To classify type 2 PRRSVs into lineages and sublineages, an overall phylogeny, based on 550 sequences representing the principal diversity of the whole data set, was constructed using a Bayesian Markov chain Monte Carlo (BMCMC) method implemented in MrBayes v3.2 (62). A general time-reversible nucleotide substitution model with 4 categories of gamma-distributed rate heterogeneity and a proportion of invariant sites ($GTR + \Gamma_4 + I$) was used. The posterior distribution of trees and model parameters were summarized from Markov chain Monte Carlo sampling over 10 million generations, during which trees were sampled every 250 generations. We used two independent runs to ensure that these runs would converge to the same result (standard deviation of the split frequencies, <0.01). The final inference of the tree was summarized from both runs, with the initial 10% of samples discarded as burn-in.

We classified the lineages according to the following procedures. First, topologically distinct monophyletic clusters (i.e., posterior probability of >90%) were manually identified as the initial classification. Second, the whole data set (the original sample size, i.e., $n = 8,616$) was divided into sub-data sets according to the initial classification. Third, intra-sub-data-set diversity (i.e., average pairwise genetic distance) was calculated for each sub-data set. Sub-data sets with a diversity level of >11% were further divided into smaller monophyletic clusters. Finally, nine monophyletic lineages, generally with intralinear diversity levels of <11% (with one exception that is discussed in Results), were then established as the final classification. Sublineages within lineages were identified using the same procedures, with a cutoff of 7%. In this way, the whole data set was divided into sub-data sets at the lineage ($n = 9$) and sublineage ($n = 37$) levels. For each of the lineage and sublineage data sets, we constructed Bayesian phylogenies as

FIG. 1. Classification and evolutionary history of all type 2 PRRSV lineages. (Right) Overall phylogeny constructed using MrBayes, which was midpoint rooted and partitioned into lineages by dotted lines. Information for each lineage is indicated to the side of the phylogeny, including diversity of the lineage, its distribution within the United States, its international distribution, and some well-known isolates. For clarity, only the bootstrap values for selected and well-supported nodes are shown. (Left) Summary of coalescence analyses of the overall and lineage data sets, performed in BEAST. The summary is based on the backbone structure of maximum clade credibility (MCC) trees reconstructed in BEAST, with unsupported nodes collapsed together to prevent uncertainty. Each box represents a well-supported lineage. The left boundary of the box is aligned with the emergence time of the lineage, whereas the right boundary of the box is aligned with the sampling time of the most recent sample. The

described previously. The sublineage phylogenies were later used in the geographical analyses. All of the reference data sets (including the overall data set, the 550-sequence representative data set, and lineage and sublineage data sets) are available upon request.

Time of emergence and demographic history. We estimated the time of emergence and demographic history of various data sets by using a Bayesian molecular clock and a coalescence-based method implemented in BEAST v1.4.8 (20). This method uses the sampling time (year) of the noncontemporaneous sequences to calibrate the molecular clock and estimates the time to the most recent common ancestor (tMRCA) and the demographic history based on the jointly constructed Bayesian phylogenies. The analyses were performed on dated sequences selected from the whole data set, 6 lineage data sets, and 15 sublineage data sets. Other data sets were not analyzed because they either had an insufficient number of dated sequences or were limited in diversity and time span. Vaccine strains and their associated sublineages or clusters were excluded from the analyses.

For each analysis in BEAST, we allowed enough generations of MCMC sampling for the effective sample size for every estimated parameter to exceed 200, and the burn-in period was determined visually by plotting likelihood versus generation in Tracer v1.4 (61). We used a GTR + Γ_4 + I model for nucleotide substitution, with model parameters estimated separately on codon positions 1 and 2 versus position 3. In order to relax the constraint of uniformity on the evolutionary rate, a relaxed clock model was used (19). This model allowed the substitution rate of each branch to be drawn independently from a log-normal distribution, and it showed a significantly better fit for all PRRSV data sets than the strict clock model did ($\log_{10}$ Bayes factor of >10).

The demographic histories of PRRSV lineages and sublineages were reconstructed by estimating the changes of relative genetic diversity through time, using a flexible model known as a Bayesian skyline plot (BSP) implemented in the BEAST program (21, 60). The relative genetic diversity is directly proportional to the effective population size (N_e) if the analyzed data set (i.e., a lineage or sublineage, in this case) does not have a significant subdivision of population (i.e., panmixis could be assumed). Alternatively, if the analyzed population is subdivided, then the relative genetic diversity reflects both the population size and the complexity of the population structure (i.e., changes of population size and population subdivision are indistinguishable) (9). In our analyses, 10 grouped intervals were used, and the uncertainty of the estimation was reflected by 95% highest-posterior-density (HPD) intervals.

Virus geographical structure and transmission between states in the United States. To investigate the significance of viral population structuring by geographic states, we estimated the association index (AI) (73) and the Fitch parsimony score (PS) (23), using the BaTS software package (56). To account for phylogenetic uncertainty, both statistics were summarized over the posterior distribution of sublineage phylogenies generated earlier by MrBayes. Asian sublineages were not included in the analyses unless they contained sequences from North America. Vaccine sublineages were also not included due to low resolution at the backbones of their phylogenies. We then compared the calculated value of each statistic with its null distribution obtained by 1,000 random reassignments. A significantly smaller value indicated a strong geographic association.

To characterize the interstate virus transmission dynamics, the migration frequencies among major swine-producing states in the United States were estimated based upon the same sublineage phylogenies used in geographic structure analyses. For each of the data sets, we assigned the states from which sequences were isolated to their corresponding tips in the phylogeny. The states of internal nodes were then estimated from tree topology and tip states by use of parsimony criteria, an algorithm allowing the fewest migration events (i.e., change of state) to occur, using the PAUP* software package, version 4beta10 (68). Based on the distribution of states along the phylogeny, we calculated the numbers of migration events between all pairs of states, with direction (e.g., from Minnesota to Iowa). The above analyses were performed for all sampled Bayesian topologies (generated earlier from MrBayes) for each of the data sets, and the final estimates are presented as migration frequencies averaged over all sampled Bayesian topologies. These averaged numbers then were organized to form a matrix

TABLE 1. Pairwise interlineage genetic distance comparisons for ORF5 nucleotide sequences of type 2 PRRSVs

Lineage	% Difference for lineage							
	1	2	3	4	5	6	7	8
2	14.4							
3	15.6	17.4						
4	12.7	14.6	14.1					
5	14.9	14.8	15.2	11.8				
6	16.6	16.8	18.0	15.1	13.2			
7	13.6	13.5	14.2	10.1	10.2	13.0		
8	14.8	14.2	16.0	13.1	12.1	14.0	11.0	
9	15.5	15.4	16.3	13.2	12.9	13.5	10.4	11.3

showing the interstate migration frequencies of PRRSV for each of the data sets. The picture of PRRSV interstate traffic was revealed after summation of all matrices calculated from all relevant sublineage data sets.

RESULTS AND DISCUSSION

Classification. Based on the criteria described in Materials and Methods, we divided the overall phylogeny into 9 monophyletic lineages, all of which were supported by $>90\%$ posterior probabilities, except for lineage 9 (76%) (Fig. 1, right panel). These lineages generally have intralinear diversities below 11%, with the exception of lineage 3 (11.3%), which could not be split meaningfully into smaller lineages, as it consists of only 49 taxa. These lineages are genetically distinct, as evidenced by an interlineage diversity of at least 11%, if lineage 7, which consists of only 14 taxa, is not taken into account (Table 1; also see our supplemental material at http://evolution.hku.hk:16080/prrsv_jvi10/Supplemental_figure1.pdf). Over 85% of all sequences fell into four large lineages, including lineage 9 ($n = \sim 2,800$), lineage 1 ($n = \sim 2,000$), lineage 5 ($n = \sim 1,500$), and lineage 8 ($n = \sim 1,400$), while the remainder fell into 5 small lineages with sample sizes ranging from 14 to 115 (Fig. 1). Within each of the 4 large lineages, sublineages were classified by the same procedure, using a cutoff of 7% (Fig. 2 to 4; also see our supplemental material [described above]). These sublineages generally have intrasublineage diversities below 7% and intersublineage diversities above 7%, with only a few exceptions (see our supplemental material). Note that these intracluster diversity cutoffs were chosen arbitrarily, based on several initial attempts of lineage and sublineage assignments. We believe that the current classification system with the above chosen cutoffs reasonably represents the principal diversity of all available sequences of type 2 PRRSV. For these lineages and sublineages, we have summarized the details in Fig. 1 to 4.

Geographic distributions. In the overall phylogeny, North American strains dominated all lineages except for two Asian-specific lineages (lineages 3 and 4 in Fig. 1). These two Asian

shaded area inside the box corresponds to the periods when sequences were sampled. The black curve inside the box is a BSP, and the gray curves represent upper and lower 95% HPD values. The y axis of each BSP is relative genetic diversity, represented on a $\log_{10}$ scale. For some of the lineages, BSP was not performed because of limited sample size or a serious deviation from clock behavior (vaccine-associated lineages). In the cases of vaccine lineages, we used histograms instead, which revealed the sampling size during the course of their history. The y axis of each histogram shows the number of samples.

FIG. 2. Classification and evolutionary history of lineage 1 PRRSVs. The Bayesian phylogeny is divided into sublineages. Figure descriptions are as described in the legend to Fig. 1.

lineages are paraphyletic, and their times of emergence were estimated to be around the late 1980s, which might represent separate and early introductions of PRRSV into Asia from North America, if we assume that the last common ancestor of type 2 PRRSV was in North America (58). Aside from these two lineages, other international sequences sampled from outside North America were all buried within the diverse population of North American sequences (Fig. 1 to 4) and likely

represent relatively recent introductions of PRRSV from North America. The majority of these international sequences belonged to lineage 5, whereas the remainder were present in other lineages. For example, lineage 1 had several Thai sequences clustered with early Canadian sequences (Fig. 2); lineage 8 contained highly pathogenic Chinese strains and their relatives (Fig. 3); and lineages 8 and 9 had several Italian isolates which were distributed separately along the phylogeny

FIG. 3. Classification and evolutionary history of lineage 8 PRRSVs. Figure descriptions are as described in the legend to Fig. 2.

(Fig. 3 and 4), indicating independent introductions of PRRSV from the United States to Italy.

Vaccine-associated lineages and sublineages. We located three live attenuated vaccines as well as their parental strains in the overall phylogeny (Fig. 1, right panel). First, vaccine strain MLV (Boehringer Ingelheim, Germany) and its parental strain, VR-2332, belonged to sublineage 5.1, along with a large number of international sequences. The intralinear diversity of sublineage 5.1 was substantially less than that of other non-vaccine-associated sublineages, despite its huge number of

geographically and temporally distant samples ($n > 1,400$) (Fig. 1, right panel). Such a difference might imply the presence of a large number of vaccine-related sequences in sublineage 5.1, although it was often difficult to distinguish vaccine descendants from wild-type viruses circulating in the field. Second, vaccine strain ATP (Boehringer Ingelheim, Germany) and its parental strain, JA142, belonged to sublineage 8.9, which contained around 400 sequences with a relatively low level of diversity, resembling that of sublineage 5.1. Third, vaccine strain PrimePac and its parental strain, Neb-1, be-

FIG. 4. Classification and evolutionary history of lineage 9 PRRSVs. Figure descriptions are as described in the legend to Fig. 2.

longed to lineage 7. Unlike ATP and MLV, PrimePac's associated lineage was made up of only a few sequences ($n = 14$). PrimePac was withdrawn from the market in 2000 due to a patent infringement. This factor and the possibility that the parental wild-type virus and the vaccine itself had low fitness may have prevented the lineage from becoming widespread, as observed for other vaccine-associated lineages.

The safety of live attenuated PRRSV vaccines has been questioned since the detection of MLV vaccine revertants which caused productive problems similar to those of wild-type PRRSV (41, 52, 55). In our analyses, we identified two large vaccine-associated sublineages, with the majority of their sequences being closely related to MLV (sublineage 5.1) and ATP (sublineage 8.9). These sequences may be the vaccine strains themselves (vaccine reisolates), descendants of the vaccine viruses, or wild-type and related viruses (i.e., VR-2332 and JA142). After conservatively removing the first category by excluding sequences identical to or within a 2-nucleotide difference from those of the vaccine strains, the remaining categories still constituted significant portions (85% and 79% for sublineages 5.1 and 8.9, respectively). Considering the limited diversity of the sequences versus the time span and sample amount for both sublineages 5.1 and 8.9, we postulate that the majority of these sequences are descendants of the vaccine strains rather than wild-type or related viruses. The attenuation of vaccine strains reduces their replication in pigs (33) and thus may limit the establishment of effective transmission chains of wild-type or related viruses. In the vaccine-associated sublineage phylogenies (data not shown), there were a number of well-supported small clusters that might reflect the small-scale transmission of the vaccine viruses in the field if they are not wild-type viruses. No significant conclusions can be drawn on the virulence of these vaccine descendants due to limitations in the report on virulence.

Overview of the evolutionary history of type 2 PRRSV. The estimation in BEAST obtained by the relaxed molecular clock model yielded an average substitution rate for ORF5 of 9.6×10^{-3} , with a 95% credible interval of 8.7×10^{-3} to 1.1×10^{-2} substitution/site/year. In combination with the substitution rate, we estimated the tMRCA for all type 2 PRRSVs to be close to the year 1979, with 95% HPD values of 1977 to 1982. This result was consistent with the date of the earliest reported evidence of PRRSV in pigs, i.e., 1979 (8), which fell within the HPD values of our estimates. By the end of the 1980s, major lineages of type 2 PRRSV had emerged and become established (Fig. 1), although the earliest sequences were not sampled until the early 1990s. Moreover, it is worth noticing that within its 30 years of evolutionary history, the type 2 PRRSV ORF5 gene reached an average diversity of 12.5%, with the largest pairwise distance being as high as 27.8%. Such rapid evolution caused difficulties in virus diagnosis and vaccine protection, and for these works to be kept on pace with virus evolution, continuous surveillance and sequencing of field samples are required.

Evolutionary history of lineage 1 and origin of the MN184-related cluster. Lineage 1 PRRSVs, including viruses with the RFLP184 pattern (28), appeared early in the literature as Canadian isolates, most of which were from Quebec (16, 17, 57). In the phylogeny of lineage 1, these sequences from Quebec

occupy the basal position (Fig. 2), implying that this lineage might be of Canadian origin.

Our BSP estimation suggests that lineage 1 experienced a period of exponential increases in relative genetic diversity from 2000 to 2004 that coincided with a new wave of PRRSV outbreaks and was the major cause of the current diversity (Fig. 1). One of the new outbreaks was recorded as the "sudden appearance of seemingly novel virulent PRRSV" (28) in late 2001 and was represented by MN184 variants and other samples observed in sublineage 1.9 (Fig. 2). These MN184 variants had a significantly modified genome, which was illustrated mainly by substantial deletions in the *nsp2* region (28). When these deletion mutants emerged is a mystery. Our phylogeny suggested that sublineage 1.9 was most closely related to sublineage 1.8 (Fig. 2, right panel), and their divergence was estimated to be 4 to 8 years before the emergence of MN184-related sequences (Fig. 2, left panel). Therefore, in order to trace the evolutionary origin of deletion mutants in sublineage 1.9, it will be useful to examine isolates in sublineage 1.8 (Fig. 2) and to determine if they have the same *nsp2* arrangement as MN184 variants. Moreover, in addition to the MN184 series outbreak, there were several rapid expansions in relative diversity in 2000 to 2004 for other sublineages, whose consequences are still not clear (Fig. 2, left panel).

Prevalence of lineages 8 and 9 and emergence of "acute PRRS" in 1996. In the overall phylogeny, lineages 6 to 9 form a well-supported clade, with a common ancestor emerging in the early to mid-1980s (Fig. 1). Lineages 6 and 7 diverged earlier in this clade, followed by lineages 8 and 9 (late 1980s), which developed into two prevalent clusters that include more than half of all field samples. Lineages 8 and 9, which include the majority of the PRRSV sequences in the database, have been responsible for several outbreaks in the field (Fig. 3 and 4). However, the presence of these two lineages was barely recognized until the "abortion storm" started in 1996, when they appeared as "acute PRRS" that swept through vaccinated (MLV and/or PrimePac) herds in Iowa and many other states in the United States (3, 4, 36). Since the sequences sampled from this epidemic belong to different clusters in lineages 8 and 9 (Fig. 3 and 4), "acute PRRS" may have been due to simultaneous outbreaks by multiple sublineages. Moreover, independent outbreaks of "acute PRRS" could also be visualized through BSP, which showed that lineages 8 and 9 experienced significant expansion in relative diversity through the mid- to late 1990s (Fig. 1, left panel). However, the sudden appearance of "acute PRRS" in 1996, even though earlier viruses in these lineages had been described (2), suggested a possible change in virulence, perhaps due to a reorganized viral genome (deletion or recombination), coinfection with other pathogens, such as PCV2, or changes in the management or physiology of the host. The hypothesis of a virulence shift is also supported by a recent PRRSV outbreak in China, in which variations in virulence occurred between closely related field isolates (32, 40, 70, 78).

Origin of the highly pathogenic Chinese cluster. A devastating outbreak of highly pathogenic PRRSV strains was first reported in China in 2006 (70). Our phylogeny placed this highly pathogenic Chinese cluster in sublineage 8.7, which consists only of sequences sampled from China that emerged around the mid-1990s (Fig. 3). Moreover, our BSP estimation

FIG. 5. Interstate PRRSV directional transmission frequencies for 10 states in the United States. The viral outflows from six states, namely, Minnesota (A), Iowa (B), North Carolina (C), Nebraska (D), Oklahoma (E), and Texas (F), are demonstrated on the maps. Frequencies of transmission from one state to another are divided into 5 categories, corresponding to the thickness of the connecting line. (G) Pairwise transmission frequencies between states, with the number of asterisks indicating the relative extent of transmission.

of sublineage 8.7 suggested that its population underwent a transition from being constant to an expansion in 2006, which is consistent with the first reported outbreak in China. These results suggest that the highly pathogenic Chinese cluster is unlikely to have originated from a previously unknown lineage but is more likely to have originated from lineage 8 strains that were circulating in China for about 10 years before the outbreak in 2006.

Transmission network between states in the United States.

We summarized the geographical distribution of PRRSVs within 10 major pig-producing states in the United States, all of which had a sample size of >100. At the lineage level, viruses in lineages 5, 8, and 9 were established in all 10 states. In contrast, other lineages were distributed only regionally. Among these, lineage 1 PRRSVs could hardly be found in more southern regions, such as Oklahoma and Texas, despite the huge diversity and sample size of this lineage. Sublineages were typically associated with 1 to 3 states, among which Minnesota, Iowa, or both were regularly present. Moreover, the geographical association analyses revealed a strong population subdivision within geographic states for all of the nonvaccine sublineages ($P < 0.00001$). This indicated that the PRRSV population was not likely to be panmictic within the United States, even though interstate migration was frequent (Fig. 5).

The interstate PRRSV migration frequencies are summarized in Fig. 5. By examining the outflow frequency for each state, we identified three important geographical “transmission centers,” namely, Iowa, Minnesota, and Oklahoma (Fig. 5A, B, and E). Multiple PRRSV strains appeared to spread frequently from these states to many other states. Iowa plays a central role because its viruses were introduced recurrently to all nine other states (Fig. 5B). The remaining states were not just receiving sites. Their local strains also were transmitted to other states repeatedly, but within a narrower range. For example, viruses frequently spread from North Carolina to Iowa and Minnesota (Fig. 5C), and the frequency of viral inflow was much higher than the efflux from these two states (Fig. 5G). On the other hand, South Dakota was more likely to receive PRRSV from Iowa and Minnesota than to be a site of distribution outward (Fig. 5G).

Our phylogeographic analyses reveal, for the first time, an interstate PRRSV traffic network in the United States. Within this network, direct viral transmissions between neighboring states and nonneighboring states are observed, indicating that PRRSV transmission is not limited by geographical distance. This conclusion is consistent with previous results showing that genetic similarity of PRRSV isolates fails to correlate with their geographical distance (27). The result also indicates that long-distance spread is a frequent process for PRRSV, which is not surprising given the potential transmission routes via transportation (65) and artificial insemination (76). Moreover, it is interesting that long-distance transmissions are not evenly distributed among major swine-producing states, and this is potentially related to asymmetric pig flow among geographic regions (for example, North Carolina is a large net exporter of weaned pigs, while other states are predominantly importers). These relationships will be examined in more detail in future work.

Implications of the genotyping system. Although ORF5 phylogeny has been used frequently to investigate genetic relation-

ships (2, 27, 34, 36) and for genotyping (1, 10, 12, 70, 72, 77), its full picture was barely revealed due to limited sample size or biased selection of representative sequences. In this study, based on analyses of all available sequences in the databases, we described 9 well-defined lineages for type 2 PRRSVs. This is the first time that their diversity has been characterized fully and their relationships systematically investigated (i.e., classification into lineage and sublineages). Moreover, we identified two lineages (lineage 2 and lineage 6) whose sequences had never been described. To this end, we emphasize the importance of a well-defined genotyping system for type 2 PRRSVs. With the rapid growth of sequence deposition in the databases, it would be technically difficult for veterinary researchers to genotype a sequence by reanalyzing all sequences in the databases. This study aimed to establish a set of reference sequences with well-defined genotypes (as lineages and sublineages) which fully cover the genetic diversity of all sequences in the database and can be used as a standard data set for future genotyping studies. More importantly, it has been demonstrated that currently available live attenuated PRRSV vaccines fail to completely prevent heterologous infection or eliminate the wild-type virus upon infection (6, 44, 50, 54, 64). The establishment of a well-organized genotyping system could not only facilitate the correct selection of related vaccines but also shed light on directions for future vaccine development.

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