Porcine reproductive and respiratory syndrome viruses (PRRSV) are divided into North American and European types, which show about 40% difference in their amino acid sequences. The divergence time of these two types has been estimated to be about 1980 from epidemiological data. This suggested that PRRSV have evolved at a higher evolutionary rate (order of $10^{-7}/$site/year) compared with other RNA viruses of $10^{-5}$ to $10^{-6}/$site/year. Here, to test the evolutionary history of PRRSV speculated by the epidemiological background, we estimated the divergence time and evolutionary rate of PRRSV with molecular evolutionary analysis. Estimated divergence time (1972–1988) corresponded well to that estimated by the epidemiological data, and the evolutionary rate $(4.71–9.8) \times 10^{-5}$ of PRRSV was indeed the highest among RNA viruses so far reported. Furthermore, we inferred important sites for the adaptation in order to examine how PRRSV have adapted to swine since they emerged. The adaptive sites were located not only in the epitopes related to immunity but also in the transmembrane regions including a signal peptide. In particular, the adaptive sites in the transmembrane regions were considered to affect compatibility to the host cell membrane. We conclude that PRRSV were transmitted from another host species to swine in about 1980 and have adapted to swine by altering the transmembrane regions.

Key words: PRRSV, evolutionary rate, divergence time, positive selection, emerging virus and transmembrane region.

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Materials and Methods
Constructing a Phylogenetic Tree of the Order Nidovirales

The order Nidovirales can be divided into two families, Arteriviridae and Coronaviridae. PRRSV belong...
Conserved region A

| Strain       | Accession Number |
|--------------|-----------------|
| PRRSV-A      | AJ271965         |
| PRRSV-E      | AF304460         |
| LDEV         | AF029248         |
| EAV          | AF391541         |
| SHFV         | M95169           |
| BCov         | X52374           |
| HCov         | X53459           |
| MHV          | L13298           |
| AIBV         | AF180391         |
| TGEV         | AF046869         |
| BEV          | M96262           |

FIG. 1.—Conserved regions among the order Nidovirales. Three conserved regions of ORF1b among the order Nidovirales were detected by the dot plot method. An amino acid alignment was constructed for each conserved region.

to the Arteriviridae family (Cavanagh 1997). Despite the difference in genome sizes, the genome organizations of the order Nidovirales are remarkably similar to each other (De Vries et al. 1997). In the present study, in order to construct a phylogenetic tree of Nidovirales and to estimate the evolutionary position of PRRSV, we used the amino acid sequences of ORF1b because ORF1b was reported to be conserved among the order Nidovirales (Bredenbeek 1990; Snijder and Meulenberg 1998). The ORF1b sequences for transmissible gastroenteritis virus (TGEV), human coronavirus (HcoV), murine hepatitis virus (MHV), bovine coronavirus (BCV), avian infectious bronchitis virus (AIBV), Berne virus (BEV), equine arteritis virus (EAV), lactate dehydrogenase–elevating virus (LDEV), simian hemorrhagic fever virus (SHFV), PRRSV-A, and PRRSV-E were collected from the International Nucleotide Sequence Database (INSD: DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank). The amino acid sequence accession numbers for TGEV, HcoV, MHV, BCV, AIBV, BEV, EAV, LDEV, SHFV, PRRSV-A, and PRRSV-E are AJ271965, AF304460, AF029248, AF391541, M95169, X52374, X53459, L13298, AF180391, AF046869, and M96262, respectively. The conserved regions in ORF1b were detected by the DotPlot program, DOTTER (Sonnhammer and Durbin 1995), and a multiple alignment of the conserved regions among the viruses was made by ClustalW (Thompson, Higgins, and Gibson 1994). The phylogenetic tree was constructed by the maximum likelihood (Felsenstein 1981) and Neighbor-Joining methods (Saitou and Nei 1987) using MOLPHY version 2.3 and PHYLIP program package, respectively. The Jones-Taylor-Thornton (JTT) model was used for constructing the maximum likelihood tree, whereas Kimura’s equation was used to estimate the evolutionary distances for constructing the Neighbor-Joining tree. The robustness of the topology of each tree was examined by the bootstrap method.

The Synonymous Substitution Rate and Divergence Time of PRRSV

The nucleotide sequences of the whole envelope region (ORFs 3, 4, and 5) of PRRSV-A were collected from the INSD. PRRSV-A strains whose years of isolation were known were used in the present study. Wild strains isolated after 1995 were excluded from the analysis because they included vaccine-derived strains. The accession number and the isolation time of each sequence used are shown in Supplementary Materials. The synonymous substitution rate and divergence time of PRRSV were estimated by both the distance-based method and the maximum likelihood method.

In the distance-based method, the nucleotide sequences of PRRSV-A, PRRSV-E, and LDEV were aligned by
ClustalW. A phylogenetic tree was constructed by the maximum likelihood method using the HKY (gamma) model (PAUP* version 4.0b) (Hasegawa, Kishino, and Yano 1985). To estimate the most recent ancestral sequence for PRRSV-A and PRRSV-E, LDEV was used as the out-group in the phylogenetic tree because LDEV is the closest virus to PRRSV among the known viruses of Arteriviridae. The ancestral sequence was estimated by the nucleotide model of the likelihood approach (PAML version 3.13) (Yang, Kumar, and Nei 1995). The number of synonymous substitutions between the ancestral sequence and each PRRSV-A sequence was estimated by the Nei-Gojobori model (Nei and Gojobori 1986). The year of isolation and the synonymous distance were plotted for each viral sequence in the two-dimensional space, and both the synonymous substitution rate of PRRSV-A and the divergence time between PRRSV-A and PRRSV-E were estimated by the least squares method (Suzuki, Yamaguchi-Kabata, and Gojobori 2000). The standard error of the divergence time was estimated by the bootstrap method under the assumption that the topology of the phylogenetic tree was correct. We constructed 500 sets of sequence alignments by randomly sampling each codon from the original alignment (Nei and Kumar 2000).

In the maximum likelihood method, the nucleotide sequences of PRRSV-A and PRRSV-E were aligned by ClustalW. Using PRRSV-E as the out-group, a phylogenetic tree was constructed by the Neighbor-Joining method. Then, we estimated both the synonymous substitution rate of PRRSV-A and the divergence time of PRRSV using the software PAML including TIPDATE algorithm (Rambaut 2000).

### Nucleotide Sequence Data for Inferring Positively Selected Sites in the Genes Encoding the Outside Component of the Virion

To detect positively selected sites in the genes encoding the outer component of the PRRSV virion, ORFs 3, 4, 5, and 6 of PRRSV-A strains were collected from the INSD. Sequences including undetermined nucleotides and gaps were eliminated from the present analysis. Consequently, the numbers of sequences used for ORF3, ORF4, ORF5, and ORF6 were 31, 30, 141, and 41, respectively. A multiple alignment was made for each coding region using ClustalW. Positively selected amino acid sites were identified using the method of Suzuki and Gojobori (1999; Suzuki, Gojobori, and Nei 2001). In this method, a phylogenetic tree was constructed by the Neighbor-Joining method using the number of synonymous substitutions. The ancestral sequence was inferred at each node of the phylogenetic tree using the maximum parsimony method (Hartigan 1973). Then, the average numbers of synonymous (sS) and nonsynonymous (sN) sites and the total numbers of synonymous (cS) and nonsynonymous (cN) substitutions throughout the phylogenetic tree were estimated for each codon site. The probability (P) of obtaining the observed or more biased numbers of synonymous and nonsynonymous substitutions was computed for each codon site, assuming a binomial distribution. In the computation, sS/(sS + sN) and sN/(sS + sN) were used as the probabilities of the occurrence of synonymous and nonsynonymous substitutions, respectively. The number of synonymous substitutions per synonymous site (ds) and

### Table 1

**Rate of Synonymous Substitution and Divergence Time of PRRSV by Distance-Based Method and Maximum Likelihood Method**

|                          | Distance-Based Method | Maximum Likelihood Method |
|--------------------------|-----------------------|----------------------------|
| Rate of synonymous substitution | $(7.7 \pm 2.1) \times 10^{-2}$ | $(4.74 \pm 0.3) \times 10^{-2}$ |
| Divergence time of PRRSV | $1986 \pm 1.8$ | $1973 \pm 1.3$ |
that of nonsynonymous substitutions per nonsynonymous site (dn) were estimated by cS/sS and cN/sN, respectively. The transmembrane and signal peptide regions of the envelope genes (ORFs 3, 4, 5, and 6) were estimated by TMPRED version 2.0 (Hofmann and Stoffel 1993).

Results and Discussion

The Phylogenetic Tree of Nidovirales

Three major conserved genomic regions (A, B, and C) among the order Nidovirales, including the families Arteriviridae and Coronaviridae, were identified for constructing the phylogenetic tree of the order Nidovirales, and amino acid alignments of the conserved regions were constructed (fig. 1). From the alignments, the phylogenetic tree of the order Nidovirales was constructed by the Neighbor-Joining method (fig. 2). The topology of the phylogenetic tree was the same as that constructed by the maximum likelihood method. The closest virus species to PRRSV was LDEV.

The Synonymous Substitution Rate and Divergence Time of PRRSV

Using LDEV as the out-group, the divergence position between PRRSV-A and PRRSV-E was identified using the whole envelope genes (fig. 3a). The rate of synonymous substitution and the divergence time of PRRSV were examined by both the distance-based and maximum likelihood methods (table 1 and fig. 4). The rate of PRRSV was estimated to be $7.7 \times 10^{-2}$ and $4.74 \times 10^{-3}$/synonymous site/year by the distance-based and maximum likelihood methods, respectively. Assuming that the rate estimated for three ORFs can be extrapolated to the whole genome, the divergence times are 1986 ± 1.8 and 1973 ± 1.3 according to the distance-based and maximum likelihood methods, respectively. Epidemiologically, there are two hypotheses for the divergence time. One is that PRRSV diverged in approximately 1980, the other is that PRRSV diverged well before 1980s as far back as approximately 1900. Our results strongly supported the former hypothesis because the estimated divergence times were 1972–1988. However, our results could not identify with confidence that the two major types of PRRSV diverged at approximately the same time as PRRSV appeared in the United States, as the maximum likelihood divergence time indicates an earlier divergence time. We conclude that PRRSV diverged approximately within 10 years before PRRSV spread in the United States.

Moreover, we examined whether the data we used had recombination events or not. In the present study, we used

![Fig. 4.—Divergence time was calculated by the least squares method. Values on the vertical axis indicate the synonymous distance from the ancestral node to each strain of PRRSV-A. Values on the horizontal axis indicate the year of isolation. A blue solid circle indicates each PRRSV-A strain. The red line was calculated from the original sequence data. Each blue line was obtained by the bootstrap method. A detailed explanation is presented in the Materials and Methods.](image-url)
three ORFs (ORF3, 4, and 5) for estimating the evolutionary rate. Phylogenetic trees for ORFs 3, 4, and 5 were independently constructed by the Neighbor-Joining method with 1,000 bootstrap replicates. Branches supported by more than 50% bootstrap values were all shared among three trees (fig. 3b). The high correlation of the phylogenetic trees among the three ORFs indicates a lack of recombination events in the data.
The rate of synonymous substitution estimated here was the highest among RNA viruses so far reported (Ellen et al. 1996; Suzuki and Gojobori 1998; Jenkins et al. 2002). We suggest two hypotheses for the extraordinarily rapid rate of PRRSV evolution, given the assumption that the rate of synonymous substitution is approximately equal to the mutation rate. The first is that the rate of replication error for PRRSV is extraordinarily high, and the second is that the number of replications per unit time (replication frequency) for PRRSV is extraordinarily high. To investigate support for these hypotheses, we compared the rate of replication error with the synonymous substitution rate among five RNA viruses, including PRRSV, as shown in table 2. In table 2, the replication error rate of PRRSV was estimated as follows. We first estimated the error rate per passage from the number of nucleotide substitutions occurring among passages (Yuan et al. 2001). Then, the replication error rate was estimated by the passage error rate divided by the time required for viral budding (Dea et al. 1995). The rates of replication error for other four RNA viruses were collected from literature (Holland et al. 1990; Schrag, Rota, and Bellini 1999; Stech et al. 1999; Escarmis et al. 2002). We also estimated the rates of synonymous substitution for these four RNA viruses (table 2). The results show that replication error rate is approximately equal between PRRSV and other RNA viruses. Even RNA viruses that have rates of synonymous substitution that are very different from PRRSV have replication error rates that are almost the same as that of PRRSV. It follows that the main source for the high synonymous substitution rate of PRRSV is the high replication frequency of PRRSV.

There are some factors that can increase the replication frequency of PRRSV. PRRSV infect swine via the air, and swine continues to release the virus for half a year (Albina 1997). Viruses infected by air and inducing acute and persistent infection for long periods have been reported to have a rapid evolutionary rate (Hanada, Suzuki, and Gojobori 2004). PRRSV can also easily spread because of the dense population in swine facilities. Furthermore, as a reason why the speed of the spread could be accelerated, most swine do not have immunity against PRRSV because the virus only recently emerged in swine. Thus, it is considered that PRRSV not only repeated replications within each infected host but also quickly infected other individuals surrounding the infected host.

Positively Selected Sites of Envelope Genes

The positively and negatively selected amino acid sites in ORFs 3, 4, 5, and 6 of PRRSV-A are summarized in figure 5 and table 3. Figure 5 shows that ds exceeded dn at more than half the amino acid sites (470/810, 58.0%) and that negative selection was detected at more than 25% of all amino acid sites (216/810, 26.7%). Nevertheless, we detected sites in which dn exceeded ds. Among the sites where dn exceeded ds, we called a site where the value (1 – P) exceeded 0.95, a positively selected site (table 4). In figure 5, there are several sites where dn exceeded ds in the regions experimentally recognized as the B-cell epitopes of ORFs 3, 4, and 5 (Olekiewicz et al. 2001; Ostrowski et al. 2002; Pflegemann, Rowland, and Faaberg 2002), although (1 – P) did not exceed 0.95 at these sites. The ectodomain identified in ORF6 by TMPRED contained a positively selective site. This site may be susceptible to attack by the immune system. PRRSV may have escaped from the immune system of swine through replacements of such amino acid sites. These adaptive sites may also provide important information for developing vaccines for PRRSV because antibodies against the regions including adaptive sites could become ineffective after amino acid replacement at these sites. To develop an effective vaccine against PRRSV, it will be necessary to make a vaccine highly expressing the regions related to immunity but not those including adaptive sites (Suzuki 2004).

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| ORF | Average dn/ds Ratio | Number of Positively Selected Sites | Number of Negatively Selected Sites |
|-----|---------------------|------------------------------------|------------------------------------|
| ORF3 | 0.307 | 0 | 51 |
| ORF4 | 0.242 | 0 | 35 |
| ORF5 | 0.791 | 17 | 54 |
| ORF6 | 0.133 | 1 | 40 |

Table 4 Positively Selected Sites

| ORF  | Positions | dn/ds Ratio | Value (1 – P) |
|------|-----------|-------------|---------------|
| ORF5 | 3  | 3.59 | 0.99 |
|      | 11 | 1.88 | 0.99 |
|      | 13 | 6.13 | 0.99 |
|      | 14 | 3.39 | 0.99 |
|      | 20 | 4.72 | 0.99 |
|      | 21 | 2.22 | 0.95 |
|      | 32 | 23.68 | 0.96 |
|      | 42 | 10.48 | 0.99 |
|      | 95 | 6.94 | 0.99 |
|      | 106 | 6.47 | 0.99 |
|      | 112 | 5.91 | 0.99 |
|      | 121 | 2.86 | 0.96 |
|      | 139 | 3.23 | 0.98 |
|      | 164 | 1.89 | 0.95 |
|      | 179 | 6.46 | 0.99 |
| ORF6 | 63 | 2.58 | 0.99 |

Note: ds values could not be calculated, because of ds = 0.
Therefore, the positive selection of the membrane regions in ORF5 is considered to be important for adaptation of PRRSV to the membrane of swine cells. In fact, PRRSV emerged so suddenly in swine only about 20 years ago and may have not yet adapted completely to swine cells. Given these circumstances, we speculate that PRRSV transferred from another host to swine in about 1980 and adapted to swine cells by altering the transmembrane regions of ORF5.

One of the potential problems of the Suzuki and Gojobori method (1999) for detecting positively selected amino acid sites was that the multiple substitutions were not taken into account in the estimation of the numbers of synonymous and nonsynonymous substitutions. However, as long as the nucleotide sequences compared are relatively closely related, the number of multiple substitutions would be sufficiently small so that the results obtained appear to be reliable (Saitou 1989). Indeed, in the present analysis, the branch lengths of the phylogenetic tree were very small (on average, 0.031, 0.029, 0.018, and 0.025 per synonymous site in ORFs 3, 4, 5, and 6, respectively).

It should also be noted that it is possible that the hitchhiking effect caused by strong positive selection as indicated above raised the rate of synonymous substitution for PRRSV. However, it appears that the increase in synonymous substitution is less affected by the hitchhiking effect than the effect of replication frequency because it has been reported that the hitchhiking effect minimally affects synonymous substitution (Birky and Walsh 1988).

Conclusions

In conclusion, PRRSV were transmitted from another host to swine in about 1980, and the virus explosively increased among domesticated swine. During this process, the synonymous substitution rate became the highest among RNA viruses so far reported. We identified positively selected sites in the transmembrane regions that do not affect adaptation to the host immune system but nevertheless affect adaptation to the host cell membrane for PRRSV.

Supplementary Material

Supplementary data are available at Molecular Biology and Evolution online www.molbiolevo.org.

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