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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

Brief Report

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Summary. The putative membrane (M) protein (ORF 6) and nucleocapsid (N) protein (ORF 7) genes of five U.S. isolates of porcine reproductive and respiratory syndrome virus (PRRSV) with differing virulence were cloned and sequenced. To determine the genetic variation and the phylogenetic relationship of PRRSV, the deduced amino acid sequences of the putative M and N proteins from these isolates were aligned, to the extent known, with other PRRSV isolates, and also other members of the proposed arterivirus group including lactate dehydrogenase-elevating virus (LDV) and equine arteritis virus (EAV). There was 96–100% amino acid sequence identity in the putative M and N genes among U.S. and Canadian PRRSV isolates with differing virulence. However, their amino acid sequences varied extensively from those of European PRRSV isolates, and displayed only 57–59% and 78–81% identity, respectively. The phylogenetic trees constructed on the basis of the putative M and N genes of the proposed arterivirus group were similar and indicated that both U.S. and European PRRSV isolates were related to LDV and were distantly related to EAV. The U.S. and European PRRSV isolates fell into two distinct groups, suggesting that U.S. and European PRRSV isolates represent two distinct genotypes.

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Porcine reproductive and respiratory syndrome (PRRS) has been devastating the swine industry in North America since 1987 and in Europe since 1990

[9, 23, 32]. The causative agent of the syndrome, the porcine reproductive and respiratory syndrome virus (PRRSV), has been isolated and characterized [3, 4, 30]. Both U.S. and European PRRSV isolates are tentatively classified as members of the newly proposed arterivirus group, which includes lactate dehydrogenase-elevating virus (LDV), equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) [5, 18, 20, 24]. These positive-strand RNA viruses resemble togaviruses morphologically but are distantly related to coronaviruses and toroviruses on the basis of genome organization and gene expression [15, 24, 25, 26, 28]. The members of this group infect macrophages and contain a nested set of 6 to 7 subgenomic mRNAs in infected cells [5, 6, 8, 13, 14, 18, 20, 21, 24]. However, no serological cross-reaction has been found among PRRSV, LDV and EAV [9]. The PRRSV is a positive-strand RNA virus with a genome of about 15 kb that contains eight open reading frames (ORFs) [20]. ORFs 1a and 1b are predicted to encode viral RNA polymerase, whereas ORFs 2 to 6 probably encode viral membrane-associated (envelope) proteins. The ORF 6 is predicted to encode the membrane (M) protein based on its similar characteristics with the ORF 6 of EAV and LDV, and the M protein of mouse hepatitis virus and infectious bronchitis virus [5, 18, 20]. The product of ORF 7 is extremely basic and hydrophilic, and is predicted to be the viral nucleocapsid (N) protein [5, 17, 18, 20].

Although the syndrome caused by PRRSV appears to be similar in the U.S.A. and Europe, several recent studies have described phenotypic, antigenic, genetic and pathogenic variations among PRRSV isolates in the U.S.A. and in Europe [1, 2, 18, 27, 31]. The European isolates grow preferentially in swine alveolar macrophage (SAM) cultures and replicate to a very low titer in other culture systems [30, 31, 32]. The U.S. isolates replicate well in SAM as well as in three continuous cell lines, CL2621, MARC-145 and CRL 11171 [3, 4, 11, 18]. Phenotypic differences among U.S.A. isolates were also observed, as not all PRRSV isolates isolated on SAM can replicate on CL2621 cell line [1] and some isolates show delayed onset of the cytopathic effect in infected cells ([2], Meng and Paul, unpubl. obs.]. A high degree of antigenic variation among PRRSV isolates was also reported, four European isolates resembled each other closely, but differed from the U.S. isolates, whereas three U.S. isolates differed antigenically from each other [31]. Animals seropositive for European isolates were found to be seronegative for U.S. isolate VR 2332 [2]. The genetic differences between U.S. and European isolates are striking, especially as they are considered to be the same virus [18]. The amino acid sequence identity of ORFs 5, 6 and 7 between U.S. isolate VR 2385 and the European isolate Lelystad virus (LV) is only 54%, 78% and 58%, respectively [18]. Similar observations were also reported when comparing the ORF 7 of a Canadian isolate IAF-exp91 and another U.S. isolate VR 2332 with LV [17, 22]. However, the 3' terminal 5 kb nucleotide sequences of two European isolates [5, 20] and portion of the N genes from seven Spanish PRRSV isolates [29] are almost identical. The existence of apathogenic or low-pathogenic strains has been suggested among isolates [27]. The U.S. isolate VR 2385 is highly pathogenic compared to European LV, and

differences in pathogenicity among U.S. isolates were also observed (Halbur, Paul, Meng and Lum, unpubl. obs.). These studies suggest that the PRRSV isolates in North America and in Europe are antigenically and genetically heterogeneous, and that different genotypes or serotypes of PRRSV may exist.

To further determine the genetic variation and the phylogenetic relationship of PRRSV, the putative M and N genes of five additional U.S. PRRSV isolates with differing virulence were cloned and sequenced. Phylogenetic trees based on the putative M and N genes of seven U.S. PRRSV isolates (six Iowa isolates and one Minnesota isolate), one Canadian PRRSV isolate, two European PRRSV isolates and other members of the proposed arterivirus group, including LDV and EAV, were constructed. The PRRSV isolates used in this study, designated as VR 2385 [18], ISU-22, ISU-55, ISU-79, ISU-1894 and ISU-3927, were isolated from pig lungs obtained from different farms in Iowa during PRRS outbreaks. A continuous cell line ATCC CRL 11171, was used to isolate and propagate these viruses. All viruses used in this study were biologically cloned by three rounds of plaque purification. Pathogenicity studies in caesarean-derived colostrum-deprived (CDCD) pigs showed that VR 2385, ISU-22 and ISU-79 were highly pathogenic and produced from 50 to 80% consolidation of the lung tissues in experimentally-infected five-week-old CDCD pigs necropsied at 10 days post inoculation, whereas ISU-55, ISU-1894 and ISU-3927 were low-pathogenic and produced only 10 to 25% consolidation of lung tissues (Halbur, Paul, Meng and Lum, unpubl. obs.).

Monolayers of ATCC CRL 11171 cells were infected with different PRRSV isolates at the seventh passage at a m.o.i. of 0.1. Total intracellular RNA was isolated from infected cells by a guanidinium isothiocyanate method (Stratagene). The quality of RNA from each isolate was determined by Northern blot hybridization (data not shown) with a cDNA probe generated from the extreme 3′ end of VR 2385 isolate [18]. cDNA was synthesized from total intracellular RNA by reverse transcriptase using random primers and amplified by polymerase chain reaction (PCR) as described previously [19]. Primers for RT-PCR were designed on the basis of VR 2385 sequence which amplified the entire protein coding regions of the M and N genes (5′ primer: 5′ GGGGATCCAGA GTTTCAGCGG 3′; 3′ primer: 5′GGGAATTCACCACGCATTC 3′). Unique restriction sites (EcoRI and BamHI) at the termini of the PCR products were introduced. A PCR product with the expected size of about 900 bp was obtained from all virus isolates (data not shown). Southern blot hybridization was then used to confirm the specificity of the amplified products. The ^{32}P-labeled cDNA probe from VR 2385 hybridized with the RT-PCR products from all virus isolates (data not shown). The PCR products of the M and N genes from all PRRSV isolates were then digested with EcoRI and BamHI, purified and cloned into vector pSK + [19]. Plasmids containing the full length M and N genes were sequenced with an automated DNA Sequencer (Applied Biosystem, Inc.). Three to four cDNA clones from each virus isolate were sequenced with universal and reverse primers as well as other virus-specific sequencing primers (PP288: 5′GCGGTCTGGATTGACGAC 3′; PP289: 5′ GACTGCTAGGGCTTCTGC

3′; DP966: 5′AATGGGGCTTCTCCGG 3′). The sequences were combined and analyzed by MacVector (International Biotechnologies, Inc) and GeneWorks (IntelliGenetics, Inc.) computer programs. Phylogenetic analyses were conducted with the aid of the PAUP software package version 3.1.1 (David L. Swofford, Illinois Natural History Survey, Champaign, IL). PAUP employs the maximum parsimony algorithm to construct phylogenetic trees. The nucleotide sequence data reported in this paper have been deposited with GenBank database under the accession numbers U18748 (ISU-1894), U18749 (ISU-22), U18750 (ISU-3927), U18751 (ISU-55) and U18752 (ISU-79).

Analysis of the nucleotide sequences encoding the putative M and N proteins of the five U.S. PRRSV isolates indicated that, like LV [20] and VR 2385 [18], the M and N genes of all the five U.S. isolates overlapped by 8 base pairs (bp) (Fig. 1). The AACC motif located 13 nucleotides upstream of the N gene is believed to be the leader-body junction site for subgenomic RNA 7 during PRRSV transcription [18, 21]. Numerous substitutions in the nucleotide sequence were distributed randomly throughout the M and N genes in all the five isolates when compared to VR 2385 (Fig. 1). There is no correlation between the virus virulence and the genetic variation in the M and N genes among these U.S. isolates analyzed. Most of the substitutions are third base silent mutation when converted to amino acid sequences (Fig. 2). Insertions and deletions were found in the nucleotide sequences of the M and N genes between the U.S. isolates and LV, but not among the U.S. isolates (Fig. 1). The deduced amino acid sequences of the M and N genes from the five U.S. PRRSV isolates were then aligned with the corresponding sequences of two other U.S. isolates, Minnesota isolate VR 2332 [22], only N gene) and Iowa isolate VR 2385 [18], one Canadian PRRSV isolate IAF-exp91 [17], only N gene), two European PRRSV isolates, LV [20] and PRRSV isolate 10 (PRRSV-10) [5], two LDV strains, LDV-C [7] and LDV-P [14], and EAV [6] (Fig. 2). The amino acid sequence of the N gene among the eight North American PRRSV isolates were highly conserved (Fig. 2b), and displayed 96–100% amino acid sequence identity (Table 1). However, the N protein of all North American PRRSV isolates shared only 57–59% amino acid sequence identity with that of the two European isolates when the inserted amino acids of the European isolates were included in the alignment (Table 1), suggesting that the North American and the European isolates may represent two different genotypes. The M protein of all the U.S. isolates with varying virulence was also highly conserved, and displayed higher sequence similarity with the M proteins of the two European isolates (Fig. 2a), ranging from 78 to 81% amino acid identity (Table 1). The N gene of all the

Fig. 1. Comparison of the nucleotide sequences of the putative M (ORF 6) and N (ORF 7) genes of the LV and the six U.S. PRRSV isolates with varying virulence. The VR 2385 nucleotide sequence is shown on top, and only differences are indicated. The start codons are indicated by (+1 >), and the termination codons are indicated by asterisks (*). Deletions are indicated by (−), and the two larger insertions in the N gene of LV are indicated by (∧). The leader-body junction site motif, AACC, upstream of N gene is underlined

```
            +1>M
VR2385      ATGGAGTCGTCCTTAGATGACTTCTGTCATGATAGCACGGCTCCACAAAAGGTGCTCTTGGCGTTTTCTATTACCTACACGCCAGTGATGATATATGCCC 100
ISU-1894    ....G.....................C........T....................T........................................... 100
ISU-22      ....G...........................C.......................T........................................... 100
ISU-79      ....G......................T.......T....................T.....A.......................A............. 100
ISU-55      ....G.....................C.............................T........C.................................. 100
ISU-3927    ....G.......C....C.....T..CA............................T..........................G..............T. 100
LV          ....G--A.-G.C....C..T..T..CA.C...CCT.TC..CG........C.CG.GC.A..C...AGC..C..A.....A..TA.A........C....  97

VR2385      TAAAGGTGAGTCGCGGCCGACTGCTAGGGCTTCTGCACCTTTTGGTCTTCCTGAATTGTGCTTTCACCTTCGGGTACATGACATTCGTGCACTTTCAGAG 200
ISU-1894    ............................................A....................................................... 200
ISU-22      .G..........................................A....................................................... 200
ISU-79      ............................................A.T........C..............................A............. 200
ISU-55      ....A..A....................................A.......A............................................... 200
ISU-3927    .......A....................................A.T..T..............T..T...........................G.... 200
LV          .T......TCA...........C..G.....GT.....A.CC.AA.A..T.....C...T.C..T..A.....A..........AT.....T.....ATC 197

VR2385      TACAAATAAGGTCGCGCTCACTATGGGAGCAGTAGTTGCACTCCTTTGGGGGGTGTACTCAGC--CATAGAAACCTGGAAATTCATCACCTCCAGATGCC 298
ISU-1894    ...............................................................--................................... 298
ISU-22      ...............A...............................................--................................... 298
ISU-79      ...............................................................--................................... 298
ISU-55      C.....C........................................................--................................... 298
ISU-3927    C.......G...........................C.....T..C.................--................................... 298
LV          C..C..CCGT.....A..T..CC....G..T..T..C.--.CCT.C..T.......TTA....TT..C...GT.A.....G..T.....T.........A 295

VR2385      GTTTGTGCTTGCTAGGCCGCAAGTACATTCTGGCCCCTGCCCACCACGTTGAAAGTGCCGCAGGCTTTCATCCGATTGCGGCAAATGATAACCACGCATT 398
ISU-1894    .................................................................................................... 398
ISU-22      .................................................................................................... 398
ISU-79      .................................................................................................... 398
ISU-55      .............................T..............................................A....................... 398
ISU-3927    ....................................................G............................................... 398
LV          .A.....T.GC..T.....GCGA....................T.....A........T.....TC.C..T..A.CT.A...GTC..G.....GA....A 395

VR2385      TGTCGTCCGGCGTCCCGGCTCCACTACGGTCAACGGCACATTGGTGCCCGGGTTAAAAAGCCTCGTGTTGGGTGGCAGAAAAGCTGTTAAACAGGGAGTG 498
ISU-1894    ......................................................G............................................. 498
ISU-22      ......T...............................................G............................................. 498
ISU-79      ......................................................G............................................. 498
ISU-55      ..............................T.......................G................................C............ 498
ISU-3927    ..............................T.......................G.G....................A............G......... 498
LV          C.CT..GA.AAAG.....ACTA..AT.A..G........TC.A..A..A..AC.TCGG.........C....C....A.CG...........GA...... 495

               -13          +1>N    ***(M)
VR2385      GTAAACCTTGTTAAATATGCCAAATAACACCGGCA-AGCAGCAGAAGAGAAAGAA---------------GGGGGATGGCCAGCCAGTCAATCAGCTGTG 582
ISU-1894    ...........C.................A.....-...................---------------.............................. 582
ISU-22      ...........C.................A...T.-...................---------------.............................. 582
ISU-79      ...........C.................A.....-...................---------------.............................. 582
ISU-55      .............................A.....-............A......---------------.............................. 582
ISU-3927    .............................A.....-............A......---------------...........................C.. 582
LV          ..T.....C..C..G....G.CGG...A.A.--..G...--.......A......AAGTACAGCTCCGAT....A...................A..... 591
                                                                   ^^^^^^^^^^^^^^^

VR2385      CCAGATGCTGGGT--AA-GATCATCGCTCACCAAAACCAGTCCAGAGGCAAGGGACCGGGAAAGAAAAATAAGAAGAAAAACCCGGAGAAGCCCCATTTC 679
ISU-1894    .............--..-............G......................................C.............................T 679
ISU-22      ............C--..-............G.....T..............................................................T 679
ISU-79      .............--..-.........C..G...........T........................................................T 679
ISU-55      .............--..-............G......................................C.............................T 679
ISU-3927    ...A.........--..-.........C..G.................T..................................................T 679
LV          ....T........GC..T...A.AGT.C..G.---G....--..-.CCT.G.....---..C..---GCC..A........G..T........A.....T 679

VR2385      CCTCTAGCGACTGAAGATGATGTCAGACATCACTTTACCCCTAGTGAGCGTCAATTGTGTCTGTCGTCAATCCAGACCGCCTTTAATCAAGGCGCTGGGA 779
ISU-1894    ...................................C..............G................................................. 779
ISU-22      ..................................................G................................................. 779
ISU-79      ..................................................G.......................A..T...................... 779
ISU-55      ...................................C...T..G.......G..........................A....................A. 779
ISU-3927    ...................................C.....C........G..........................T...........G.......... 779
LV          ..C..G..TG..........CA..C.G..C...C.C....AG.C...A..CTCCC.C..CT..CAA..G........G..T..C...........A..A. 779

VR2385      CTTGCACC-CTGTCAGATTCAGGGAGGATAAGTTACACTGTGGAGTTTAGTTTGCCTACGCATCATACTGTGCGCCTGATCCGCGTCACAGCATCACCC- 877
ISU-1894    ........-...............................................A..................T.......................- 877
ISU-22      ........-..........................................................................................- 877
ISU-79      ........-..................................................................T.......................- 877
ISU-55      ....T...-...............................................G...................................G......- 877
ISU-3927    .C..T.T.-..A............................................G.......................T........G..C......- 877
LV          ..-..GT.G..T...TCCAGC....A.G.C....TTCAG..T.......TGC....GGTTGC......A...........T.....G..TT.TA..T..G 878

                             ***(N)
VR2385      TCAG-CA----------TGA   886
ISU-1894    ....-..----------...   886
ISU-22      ....-..----------...   886
ISU-79      ....-..----------...   886
ISU-55      ....-..----------...   886
ISU-3927    ....-..----------...   886
LV          C...T..GGGTGCAAGT.A.   898
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(a)

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VR2385      MESSLDDFCHDSTAPQKVLLAFSITYTPVMIYALKVSRGRLLGLLHLLVFLNCAFTFGYMTFVHFQSTNKVALTMGAVVALLWGVYSAIETWKFITSRCR 100
ISU-1894    .G..............................................I................................................... 100
ISU-22      .G..............................................I................................................... 100
ISU-55      .G..............................................I................................................... 100
ISU-79      .G.......Y......................................I.............M..................................... 100
ISU-3927    .G.......N......................................I................E...R.............................. 100
LV          .G-G.....N.PI.A..LV.........I..:..............I.I....S.......Y.......R....L............FT.S.........  99
PRRSV-10    .G-G.....N.PI.A..LV.........I.................I.I....S.......Y.......R....L............FT.S.........  99
LDV-C       .G-G.-E..DQTSWY.-IFI...L....IA..S...F..T.A.IVNIFI.I..CVS.V.LMYH.-SV..TI..SL...I..V..I.TLVKIVDWLVI...  96
LDV-P       .G-G.-E..DQTSWY.-I.I...L....IA..S...F..T.A.IVNIFI.I..CVS.V.LMYH.-SV..T...SL...I..V..I.TLVKIVNWMVL...  96

VR2385      LCLLGRKYILAPAHHVESAAGFHPIAANDNHAFVVRRPGSTTVNGTLVPGLKSLVLGGRKAVKQGVVNLVKY-AK 174
ISU-1894    ........................................................................-.. 174
ISU-22      ........................................................................-.. 174
ISU-55      ........................................................................-.. 174
ISU-79      ........................................................................-.. 174
ISU-3927    ...................................................R.....K..............-.. 174
LV          ..C...R..............L.S.S.SG.R.YA..K..L.S.........R.....KR...R.........-GR 173
PRRSV-10    ..C...R..............L.S.S.SG.R.YA..K..L.S.........R.....KR...R.........-GR 173
LDV-C       ..F...S.....PS..DTSD.RQSLTTSLTT.....K....L...Q...DFQR.....K...SK.A...L..VS. 171
LDV-P       ..F...S.....PS..DTSD.RQSLTTSSTT.....K....L...Q...DFQR.....K...SK.A...L..VS. 171
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(b)

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VR2385      MPNNTGKQQKRKK-------GDGQPVNQLCQMLGKIIAHQNQSRGKGPGKKNKKKNPEKPHFPLATEDDVRHHFTPSERQLCLSSIQTAFNQGAGTCTLS 93
ISU-1894    ....N........-------..................Q............................................................. 93
ISU-22      ....N........-------..................Q............................................................. 93
ISU-79      ....N........-------..................Q............................................................. 93
ISU-3927    ....N.....K..-------..................Q..........................................................I.. 93
ISU-55      ....N.....K..-------..................Q....................................SG....................... 93
VR2332      ....N...TEE..-------..................Q............................................................. 93
IAF-exp91   ....N.R...K..-------..................Q............................................................. 93
LV          .---A..N.SQ..KKSTAPM.N..........L..AM.KS.R.---QPR.GQA...K.........A...I...L.QT..S...Q...........AS.. 94
PRRSV-10    .---A..N.SQ..KKSTAPM.N..........L..AM.KS.R.---QPR.GQA...K.........A...I...L.QT..S...Q...........PS.. 94
LDV-C       .SQ.KK.GGQN.--------.AN.---..N.LISALLRNAG.--N..K.Q.K..-Q.-.L...M.GPS.L..VM..N.V.M.R..LV.L....G.Q...V 85
LDV-P       .SQ.KK.SGQN.--------.AN.---..N.LINALLRNAG.--N..K.Q.K..-Q.-.L...M.GPS.L..VM..N.V.M.R..LV.L....G.Q...V 85
EAV         .ASRRSRP.AASF-------RN.R--RRQPTSYNDLLRMFG.-------MRVR.PPAQPTQAII.EPG.L..DLNQQ..ATLS.NV.RF.MI.H.SL.-A 83
                         ^^^^^^^

VR2385      DSGRISYTVEFSLPTHHTVRLIRVTASP----SA 123
ISU-1894    ............................----.. 123
ISU-22      ............................----.. 123
ISU-79      ............................----.. 123
ISU-3927    ..........................P.----.. 123
ISU-55      ............................----.. 123
VR2332      ............................----.. 123
IAF-exp91   .......A....................----.. 123
LV          S..KV.FQ...M..VA.........STSASQGAS 128
PRRSV-10    S..KV.FQ...M..VA.........STSASQGAS 128
LDV-C       ...G.NF..S.M....A.....NAS.NS----.. 115
LDV-P       ...G.NF..S.M....A.....NAS.NS----.. 115
EAV         .A.GLT...SW-V..KQIQ.KVAPP.G.------ 110
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Fig. 2. Alignment of amino acid sequences of the M (**a**) and N (**b**) genes of the proposed arterivirus group. The EAV M gene sequence was omitted because the relatively low sequence identity with PRRSV and LDV requires gaps in the alignments. The M gene sequences of U.S. PRRSV isolate VR 2332 and Canadian isolate IAF-exp91 are not available. VR 2385 sequences are shown on top, and only differences are indicated. Deletions are indicated by (–), and the two larger insertions in the N gene of the European PRRSV isolates are indicated by (∧). The alignments were performed with a GeneWorks program (IntelliGenetics, Inc.). The following parameters (default value) were used. Cost to open a gap is 5, cost to lengthen a gap is 25, minimum diagonal length is 4, and the maximum diagonal offset is 10

Table 1. Pairwise comparison of the amino acid sequences among the putative nucleocapsid and membrane proteins of members of the proposed arterivirus group

Virus	Virus												
	VR2385	ISU-22	ISU-55	ISU-79	ISU-1894	ISU-3927	VR2332	IAF-exp91	LV	PRRSV-10	LDV-P	LDV-C	EAV
VR2385	***	98[b]	96	98	98	96	96	96	57	57	49	49	22
ISU-22	99[a]	***	98	100	100	98	98	98	57	57	49	49	23
ISU-55	99	100	***	98	98	97	96	97	59	59	49	49	23
ISU-79	98	99	99	***	100	98	98	98	57	57	49	49	23
ISU-1894	99	100	100	99	***	98	98	98	57	57	49	49	23
ISU-3927	96	97	97	97	97	***	96	97	59	59	49	49	23
VR2332	N/A	N/A	N/A	N/A	N/A	N/A	***	96	57	57	50	49	22
IAF-exp91	N/A	N/A	N/A	N/A	N/A	N/A	N/A	***	59	59	49	49	23
LV	78	79	79	79	79	81	N/A	N/A	***	99	41	40	23
PRRSV-10	78	79	79	79	79	81	N/A	N/A	100	***	41	40	23
LDV-P	50	51	51	51	51	51	N/A	N/A	53	53	***	98	23
LDV-C	49	50	50	50	50	50	N/A	N/A	52	52	96	***	24
EAV	16	16	16	16	16	15	N/A	N/A	17	17	16	17	***

[a]The values in the table are the percentage identity of amino acid sequences

[b]Nucleocapsid protein comparisons are presented in the upper right half, and membrane protein comparisons are presented in the lower left half

N/A Not available

North American PRRSV isolates shared 49–50% amino acid sequence identity with that of the two LDV strains, whereas the two European PRRSV isolates shared only 40–41% amino acid identity with that of the LDV strains when the inserted amino acids of the European PRRSV isolates were included (Table 1). Two regions of amino acid sequence insertions, "KKSTAPM" and "ASQG", were found in the N protein of the two European PRRSV isolates when compared to the eight North American PRRSV isolates as well as two LDV strains and EAV (Fig. 2b). These results indicated that the U.S. PRRSV isolates were more closely related to LDV than were the European PRRSV isolates, and that PRRSV may have undergone divergent evolution in the U.S. and in Europe before their association with PRRS was recognized in swine [22]. The M and N genes of the North American and European PRRSV isolates shared only 15–17% and 22–24% amino acid sequence identity with those of EAV, respectively.

The sequence homology of PRRSV with LDV and EAV suggests that these viruses are closely related and may have evolved from a common ancestor [22, 24]. The evolutionary relationships of PRRSV with other members of the proposed arterivirus group were determined on the basis of the amino acid sequence of the M and N genes. Bootstrapped parsimony (1000 replicates) with branch-and-bound search option were performed to find the tree with shortest length (most parsimonious). The trees were rooted by assuming EAV as the outgroup for the N genes and LDV as the outgroup for the M genes. The phylogenetic tree drawn for the N gene is essentially the same as that drawn for the M gene (Fig. 3). The PRRSV isolates fall into two distinct groups. All the North American PRRSV isolates thus far sequenced are closely related and formed one group. The two European PRRSV isolates are closely related and comprised another group. Recently, portions of the N gene from seven Spanish PRRSV isolates have been determined, and displayed 96–97% sequence homology with the corresponding region of LV [29]. This again indicates that the European PRRSV isolates are highly conserved. Both the U.S. and European PRRSV isolates are related to LDV strains and distantly related to EAV (Fig. 3). The North American and European PRRSV isolates represent two distinct genotypes (Fig. 3).

A striking feature of RNA viruses is their rapid evolution resulting in extensive sequence variation [12]. Direct evidence for recombination between different positive-strand RNA viruses has been obtained [16]. Western equine encephalitis virus appears to be an evolutionarily recent hybrid between Eastern equine encephalitis virus and another alphavirus closely related to Sindbis virus [10]. Thus, the emergence of PRRSV and its close relatedness to LDV and EAV is not surprising. Although the capsid or nucleocapsid protein has been used for construction of evolutionary trees of many positive-strand RNA viruses, proteins with conserved sequence motifs such as a RNA-dependent RNA polymerase or a RNA replicase, would be more suitable for phylogenetic studies [12]. Further sequence information of both North American and European PRRSV isolates would facilitate the evolutionary studies of PRRSV.

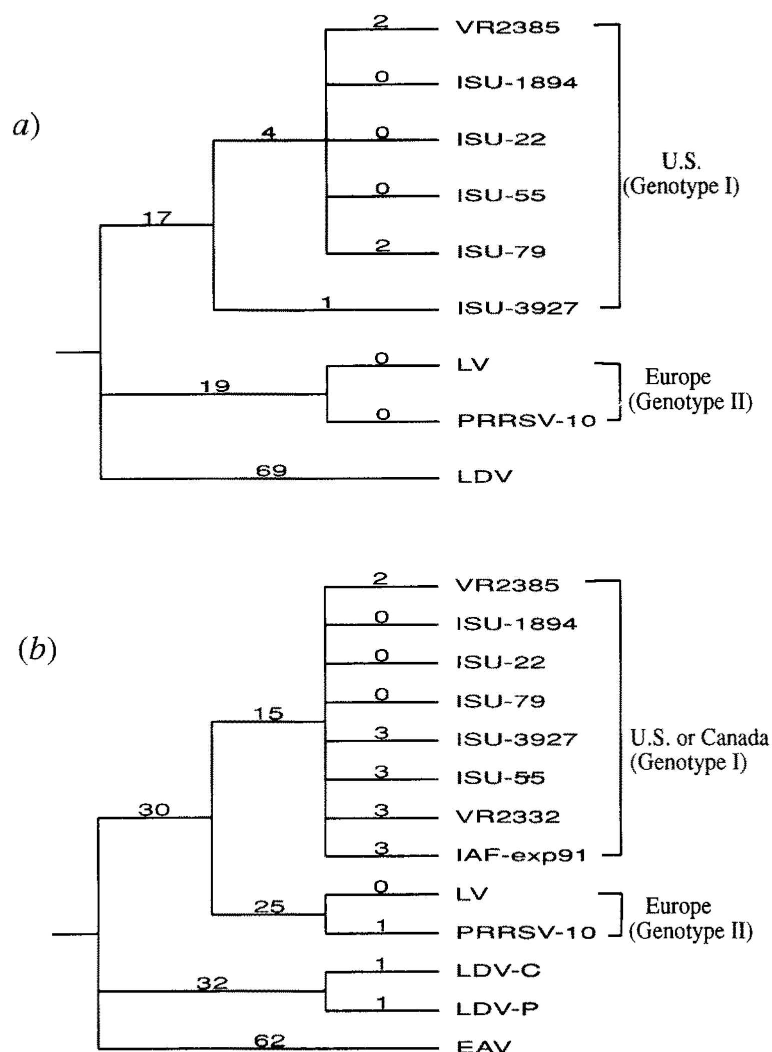

Fig. 3. Phylogenetic trees based on the amino acid sequences of the M (**a**) and N (**b**) genes of the proposed arterivirus group. The trees were constructed by maximum parsimony methods with the aid of the PAUP software package version 3.1.1. The trees with the shortest length (most parsimonious) were found by implementing the bootstrap (1000 replicates) with branch-and-bound search option. The trees were rooted by assuming EAV as the outgroup for the N genes and LDV as the outgroup for the M gene. The branch lengths (number of amino acid substitutions) are given above each branch

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