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Genetic Diversity of Pestiviruses: Identification of Novel Groups and Implications for Classification

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The complete N^{pro} coding sequences were determined for 16 pestiviruses isolated from cattle, pig, and several wild ruminant species including reindeer, bison, deer, and bongo. Phylogenetic analysis enabled the segregation of pestiviruses into the established species bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, border disease virus (BDV), and classical swine fever virus (CSFV). For BVDV-1 five distinct subgroups were identified, while BVDV-2, BDV, and CSFV were each subdivided into two subgroups. The virus isolates from bongo and deer as well as one porcine virus isolate belong to BVDV-1. Interestingly, the isolates from reindeer and bison are distinct from the established pestivirus species. The N^{pro} sequences from these two viruses are more similar to BDV than to the other pestivirus species. Calculation of the pairwise evolutionary distances allowed a clear separation of the categories species, subgroup, and isolate only when the reindeer/bison viruses were considered as members of an additional pestivirus species. Furthermore, the entire E2 coding sequences of a representative set of virus isolates covering all recognized species and subgroups were studied. Segregation of pestiviruses based on the E2 region was identical with that obtained with the N^{pro} sequences. © 1999 Academic Press

INTRODUCTION

The genera *Pestivirus, Flavivirus,* and *Hepacivirus* constitute the family *Flaviviridae* (Pringle, 1999). For the genus Pestivirus the currently official nomenclature considers three species, namely bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV). In addition, a fourth pestivirus species comprising isolates from cattle and sheep has been described (Becher *et al.,* 1995; Pellerin *et al.,* 1994; Ridpath *et al.,* 1994). The *Flaviviridae* study group of the International Committee on Taxonomy of Viruses has recently proposed to term this additional species BVDV-2 and to include a pestivirus isolate from a giraffe as a tentative fifth species within the genus.

The pestivirus genome consists of a positive-stranded nonpolyadenylated RNA molecule of approximately 12.3 kb that contains one large open reading frame flanked by 5′ and 3′ noncoding regions (NCR) (Becher *et al.,* 1998a; Collett *et al.,* 1988; Meyers *et al.,* 1989; Ridpath and Bolin, 1995). In the virus-encoded polyprotein, the viral proteins are arranged in the following order (from the N to the C terminus): N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, (NS2), (NS3), NS4A, NS4B, NS5A, and NS5B [reviewed in Meyers and Thiel (1996) and Thiel *et al.* (1996)]; the abbreviation N^{pro} refers to an N-terminal autoprotease, and E^{rns} (ribonuclease soluble) refers to a structural glycoprotein with ribonuclease activity. The structural proteins are represented by the capsid protein C and three envelope proteins (E^{rns}, E1, and E2). The remaining proteins are presumably nonstructural (NS).

Different regions of pestiviral genomes have been employed to study their genetic diversity, including variable parts of the 5′ NCR, as well as the genes encoding C, E2, N^{pro}, and other nonstructural proteins. The four established pestivirus species BVDV-1, BVDV-2, BDV, and CSFV can be differentiated by comparison of both complete genomic sequences (Becher *et al.,* 1998a; Ridpath and Bolin, 1997) and partial sequences (Becher *et al.,* 1995, 1997; Harasawa, 1996; Hofmann *et al.,* 1994; Paton, 1995; Tijssen *et al.,* 1996; van Rijn *et al.,* 1997; Vilcek *et al.,* 1997). Short sequences derived from the highly conserved 5′ NCR can be used for the segregation of pestiviruses into the established species but are less suited to a further subdivision of the virus species into defined subgroups (Becher *et al.,* 1997). Alternatively, the genes encoding N^{pro} and E2 have been reported to represent useful targets for phylogenetic analyses of pestiviruses (Becher *et al.,* 1995, 1997; Tijssen *et al.,* 1996; van Rijn *et al.,* 1997; Vilcek *et al.,* 1997).

In previous studies, pestiviruses were segregated into genetic groups by the branching order of phylogenetic trees. In addition to this method, sequences can also be compared by the analysis of pairwise sequence similar-

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TABLE 1

Pestivirus Isolates

Virus isolate	Abbreviation	Year of isolation	Species of origin	Region of isolation	GenBank Accession No.	
					N^{pro}	E2
93-871	871	1993	Cattle	Germany	AF144462	—
721/96	721	1996	Cattle	Germany	AF144463	AF144609
93-519	519	1993	Cattle	Germany	AF144464	AF144610
RIT 4350	RIT	1984[a]	Cattle	?	AF144465	—
C86		1989	Cattle	United Kingdom	AF144466	AF144611
Giessen-1	Gi-1	1996	Cattle	Germany	AF104030	AF104030
Giessen-3	Gi-3	1997	Cattle	Germany	AF144467	—
230/98-K1	Gi-4	1997	Cattle[b]	Panama	AF144468	—
230/98-K2	Gi-5	1997	Cattle[b]	Panama	AF144469	—
230/98-K3	Gi-6	1997	Cattle[b]	Panama	AF144470	AF144612
SCP		1993	Sheep	Germany	U17149	AF144613
V360-Krefeld	V360	1996	Pig	Germany	AF144471	—
'Schweinfurt'	Schweinf.	1993	Pig	Germany	AF144472	—
95-4845 A	Deer-NZ1	1980	Deer	New Zealand	U80903	AF144614
'Deer'	Deer-GB1	1986	Deer	United Kingdom	U80902	AF144615
SH9		1991	Roe deer	Germany	AF144473	AF144616
V2486-Krefeld	Bongo	1997	Bongo[c]	Germany	AF144474	—
'Giraffe' (H138)	Giraffe-1	1967[d]	Giraffe	Kenya	U80907	AF144617
V60-Krefeld	Reindeer-1	1996	Reindeer[c]	Germany	AF144475	AF144618
V65-Krefeld	Bison-1	1996	Bison[c]	Germany	AF144476	AF144619

[a] Year of isolation unknown. First referenced by Lobmann *et al.* (1984).

[b] Isolated from fetal bovine serum.

[c] Captive ruminant housed in the Duisburg Zoo, Germany.

[d] Plowright, 1969, personal communication.

ities. One aim of this study was to define ranges of sequence similarities that correspond to the categories species, subgroup, and isolate. The establishment of such ranges of sequence similarity should be helpful when novel pestiviruses are studied. This report deals with the genetic heterogeneity of a number of new pestiviruses isolated from domestic and wild ruminant species. Pestiviruses from bongo, reindeer, and bison are characterized here for the first time. The results of our analysis led to the detection of novel subgroups within the species BVDV-1 and BVDV-2 and provided evidence for the presence of an additional major genetic group within the genus *Pestivirus.*

RESULTS AND DISCUSSION

Phylogenetic analysis of N^{pro} gene sequences

To characterize novel pestivirus isolates we initially concentrated on the N^{pro} coding region of the genome. For all pestiviruses analyzed here an RT-PCR assay using one primer pair allowed the amplification of the entire N^{pro} gene together with part of the 5′ NCR and the genomic region encoding C and part of E^{rns}. After molecular cloning in a bacterial vector, the N^{pro} coding consensus sequences were determined by sequencing both complementary strands of three independent clones.

The nucleotide and deduced amino acid sequences obtained were first aligned with corresponding published sequences of pestivirus reference strains (data not shown). For all pestiviruses, including the novel virus isolates presented here, the N^{pro} gene comprises 504 nucleotides. Evolutionary relationships of the N^{pro} genes were estimated by phylogenetic analysis using the neighbor-joining method (Felsenstein, 1985, 1993). In addition to the sequences obtained for the pestivirus isolates listed in Table 1, several published sequences of pestivirus reference strains were included. Bootstrap resampling of phylogenetic trees was carried out to test the robustness of the observed major clades and subgroups. Bootstrap values obtained for the single pestivirus species as well as for all identified subgroups within the single species were 100%. The phylogenetic tree shows five major branches corresponding to the pestivirus species BVDV-1, BVDV-2, BDV, CSFV, and an isolate from a giraffe (Fig. 1). Our analysis shows the presence of five different subgroups for BVDV-1, four of which have been previously described (Becher *et al.,* 1997). The novel BVDV-1 subgroup, termed 1d, comprises German pestivirus isolates from cattle (871, 721) and deer (SH9). BVDV vaccine strain C86 and the porcine isolate V360 belong to subgroup 1a, which also includes BVDV-1 NADL; BVDV vaccine strain RIT and the pestivirus isolate from a bongo belong to subgroup 1b, which also comprises BVDV-1 strains Osloss and CP7; bovine isolate 519 together with isolate Deer-NZ1 belongs to subgroup 1c;

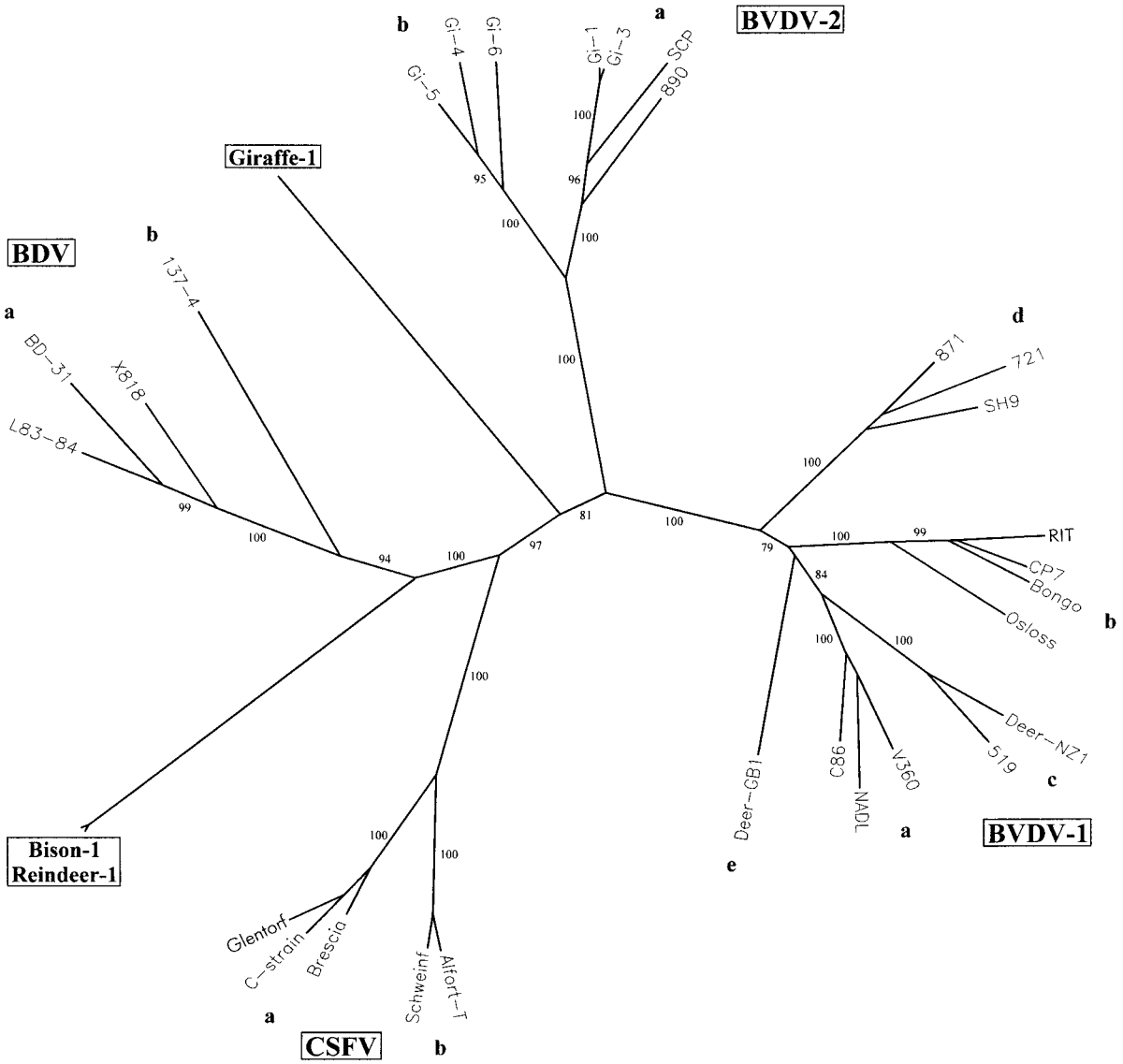

FIG. 1. Phylogenetic tree showing the genetic relationships of pestivirus strains used in this study. The dendrogram was constructed from the nucleotide sequences of the N^{pro} gene. Sequences from bovine strains 519, 871, 721, Gi-1, Gi-3, Gi-4, Gi-5, Gi-6, C86, and RIT, porcine strains 'Schweinfurt' and V360, and pestivirus strains SH9, Bongo, Reindeer-1, and Bison-1 were generated in this study. The corresponding nucleotide sequences of the N^{pro} genes of other pestivirus strains were obtained from the GenBank data library (for references see Becher *et al.,* 1997). Distances were calculated by the Kimura two-parameter method (Kimura, 1980) and used to construct the tree according to the neighbor-joining method (Felsenstein, 1993). Branch lengths are proportional to genetic distances. Numbers indicate the percentage of 1000 bootstrap replicates that support each labeled interior branch (Felsenstein, 1985; Hedges, 1992). The designations for isolates, subgroups, and species are indicated along branches.

isolate Deer-GB1 represents the first member of subgroup 1e. With respect to BVDV-2, two subgroups can be differentiated. All previously analyzed bovine and ovine BVDV-2 strains isolated in the United States, Canada, and Europe as well as the bovine pestivirus strains Gi-1 and Gi-3 isolated in Germany belong to subgroup 2a, while the BVDV-2 isolates from Panama can be placed into subgroup 2b (Fig. 1). As described earlier, BDV and CSFV can each be segregated into two subgroups (Becher *et al.,* 1997; Lowings *et al.,* 1996; Vilcek *et al.,* 1997). Interestingly, the two pestiviruses isolated from reindeer and bison were found to be clearly distinct from the five known pestivirus species (Fig. 1). The N^{pro} sequences from these two viruses are more similar to those of BDV than to the other pestivirus species.

Phylogenetic trees using the N^{pro} gene sequences and the unweighted pair-group arithmetic averaging (UPGMA) or maximum-likelihood methods had topologies identical to the tree obtained by the neighbor-joining method (data not shown). The branching order of phylogenetic trees based on the N^{pro} deduced amino acid sequences was identical to that based on the nucleotide sequences, but the former was supported by slightly lower bootstrap values (data not shown).

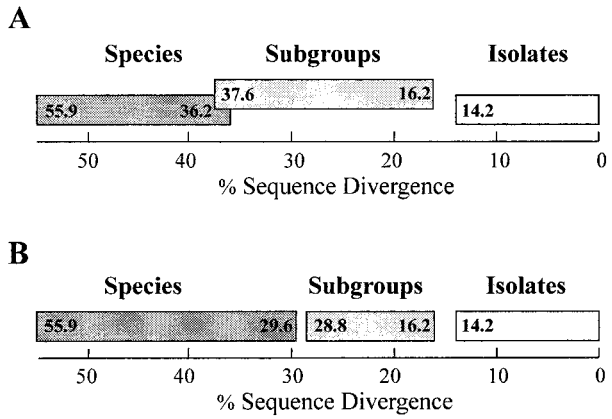

FIG. 2. Ranges of pairwise evolutionary distances among species, subgroups, and isolates of 32 N^{pro} gene sequences of the pestivirus strains included in the dendrogram shown in Fig. 1. (A) The ranges were calculated based upon the classification of strains Reindeer-1 and Bison-1 as a subgroup of BDV. Note that the ranges between species overlap with those between subgroups. (B) Classification of Reindeer-1 and Bison-1 as a separate species results in a clear separation of the categories species, subgroup, and isolate.

Classification by sequence similarity

The segregation of pestiviruses into defined genetic groups represents one important criterion for their classification. In previous phylogenetic analyses, the individual pestivirus species together with their subgroups were identified by the branching order of phylogenetic trees. In addition to this method, sequences can be compared by measures of pairwise sequence similarity. As previously stated, one aim of the present study was to define ranges of sequence divergence that correspond to the categories species, subgroup, and isolate.

For this purpose the pairwise evolutionary distances were calculated for the N^{pro} gene. With respect to the five established pestivirus species, the ranges of sequence divergence were <14.2% between virus isolates of one subgroup, 16.2–28.8% between subgroups, and 36.2–55.9% between species. Accordingly, the three categories species, subgroup, and isolate can be clearly distinguished by the calculated genetic distances. However, classification of the isolates Reindeer-1 and Bison-1 is problematic. The distances between these two isolates and BDV were between 29.6% and 37.6% with a calculated average value of 34.0% being intermediate between the ranges previously calculated between subgroups and between species. The divergence between the reindeer/bison viruses and the other pestivirus species was 39.1 to 50.7%. Based upon the classification of the reindeer/bison isolates as a subgroup of BDV, the pairwise distances between subgroups of one species (subgroup range: 16.2–37.6%) overlapped with distances between different species (species range: 36.2–55.9%) (Fig. 2A). Alternatively, the reindeer/bison isolates could be classified as a separate pestivirus species. This results in a clear separation of the ranges of distances for the three categories isolate (<14.2%), subgroup (16.2–28.8%), and species (29.6–55.9%) (Fig. 2B). Taken together, the results of our analysis suggest that the reindeer/bison isolates represent an additional sixth major genetic group within the genus *Pestivirus*. Furthermore, the establishment of defined ranges of pairwise sequence distances should be useful for the classification of novel pestivirus isolates.

Analysis of E2 gene sequences

Glycoprotein E2 represents one of the most variable proteins of pestiviruses, and this region has been used in previous studies concerning heterogeneity (Tijssen *et al.,* 1996; van Rijn *et al.,* 1997). The E2 genes of a representative set of pestiviruses covering all species and subgroups identified by analyses of the N^{pro} region were cloned and sequenced to study the variability of pestiviruses in a second region of the genome. While the N^{pro} genes of all pestiviruses analyzed here were successfully amplified by RT-PCR using one primer pair (see above), we did not succeed in generating one pair of primers suitable for the amplification of the respective E2 genes. The E2 genes of pestivirus strains 721, 519, C86, Gi-1, Gi-6, SCP, SH9, Deer-NZ1, Deer-GB1, Giraffe-1, Reindeer-1, and Bison-1 were amplified by RT-PCR using a limited set of forward primers (located in the E1 coding region) and of reverse primers (located in the region encoding p7 and NS2). The sequences obtained were compared with published E2 sequences of other pestivirus strains. An alignment of the deduced amino acid sequences showed 15 cysteine residues that are conserved among all pestiviruses (data not shown). Two additional cysteines are present at identical positions in E2 of all ruminant pestivirus strains but are not found in CSFV E2. Furthermore, insertions and deletions of one or two amino acids occurred at different locations of E2.

To study the genetic heterogeneity within the E2 region, a phylogenetic tree was generated using the neighbor-joining method for the nucleotide sequences encoding the entire E2. The topology of the E2-based tree (Fig. 3A) was identical to that obtained by phylogenetic analysis of the N^{pro} region. Statistical analyses of the E2-based tree showed that all groupings of species and subgroups were supported by high bootstrap values ($P < 0.95$). Phylogenetic trees obtained on the basis of the E2 deduced amino acid sequences using the neighbor-joining, UPGMA, and Fitch-Margoliash methods exhibited the same branching order. It can thus be concluded that subdivision of the genus *Pestivirus* into the defined species and subgroups as determined by the analysis of the N^{pro} region (Fig. 1) is supported by phylogenetic analysis of the E2 region.

Comparison of the E2 genes also showed that the pestiviruses from reindeer and bison can be grouped separately from all other pestiviruses and are more sim-

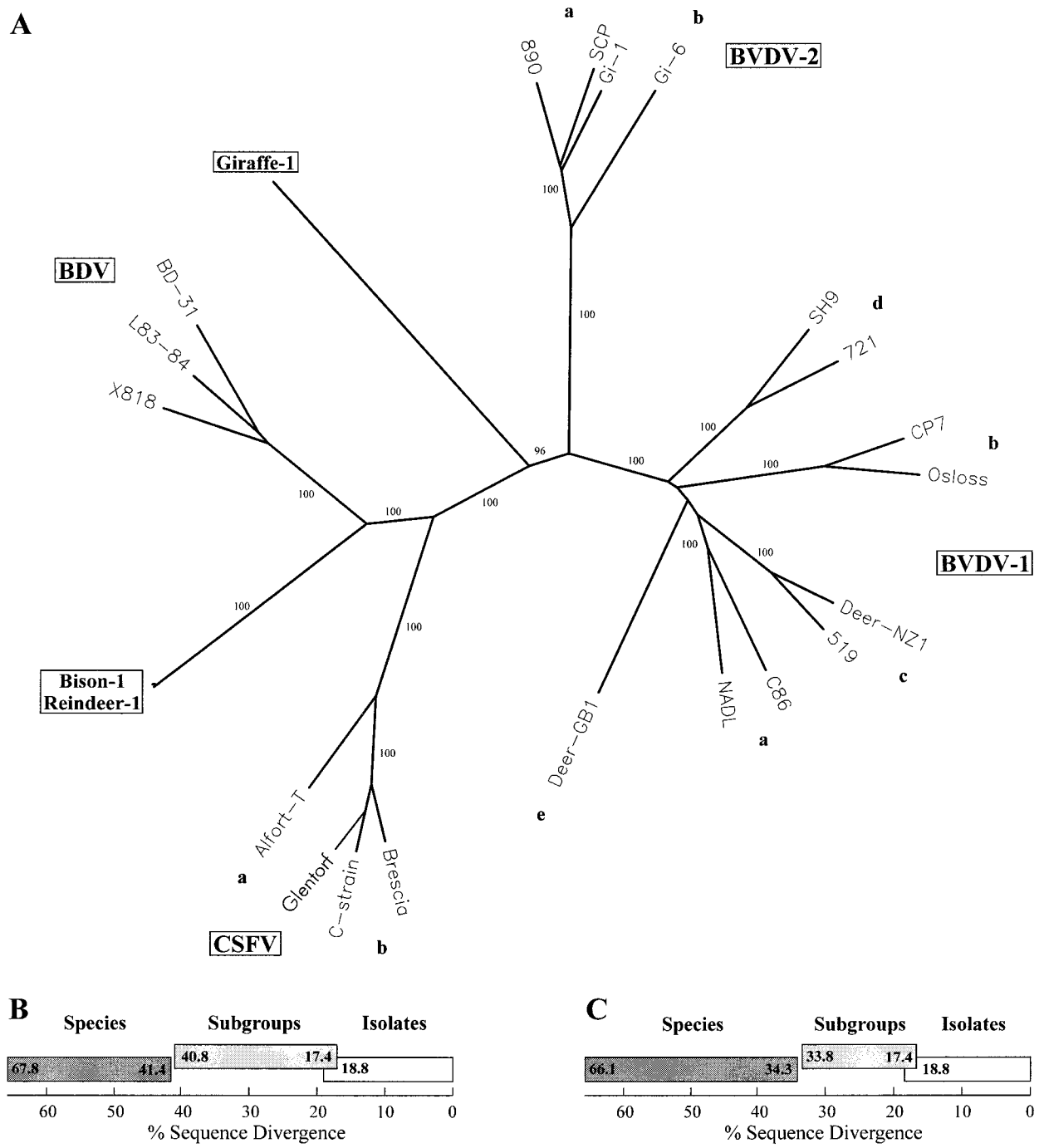

FIG. 3. Phylogenetic analysis of the E2 coding nucleotide sequences of 23 pestivirus strains. (A) Dendrogram showing the genetic relatedness of pestivirus strains. Sequences from bovine strains 519, 721, Gi-1, Gi-6, and C86, as well as from strains SCP, Deer-NZ1, Deer-GB1, SH9, Giraffe-1, Reindeer-1, and Bison-1 were generated in this study. The corresponding nucleotide sequences of the E2 genes of other pestivirus strains were obtained from the GenBank data library. Construction of the phylogenetic tree including bootstrap analysis was done as described in the legend to Fig. 1. (B, C) Ranges of pairwise distances among species, subgroups, and isolates based upon the classification of strains Reindeer-1 and Bison-1 as a subgroup of BDV (B) or, alternatively, as a separate species (C). In both cases the ranges between subgroups overlap with those between isolates.

ilar to BDV than to the other pestivirus species; this result is similar to that obtained with the N^{pro} sequences. For further analysis the pairwise evolutionary distances were calculated for the E2 coding sequences. When the reindeer/bison pestiviruses are classified either as an additional novel pestivirus species or as a subgroup of BDV, the range of the E2 distances between subgroups was separated from the distances between species, but overlapped with distances between isolates (Figs. 3B and 3C).

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                .         .         .         .         .         .         .         .         .
Deer-GB1  YPKCKPDYSYAIAKNDKVGLLGAEGLTTLWREYKPKMILQDALVKVRCKDGEFVHRKKCGKEARYLAILHTGALPTSVEFKKLFDKQKQE   90
´Deer´
NADL      HLD   EF      DERI Q       T K  S G K E TM IAW E  KLMYLQR TR T       R      V      GR
Osloss    L V   GFY      NEI P   T    Q Y  SDG R   TG V W  G  IKYLIT ER        R      V E II GKE
519         D R  F      DE I P    S   A KD S G R V TM VAW    Q TIMER AR        RR      V R    AKR
721       RLD    F       E I P       T Y  SDG Q S TM EA   N   IFVQR KT T       R      V E F  G  LA


                .         .         .         .         .         .         .         .         .
Deer-GB1  DIIEMENNFEFGLCPCDAKPLVKGKFNTTLLNGPAFQMVCPIGWTGSVS.CVLANEDTLETTVVQTYRRSRPFPHRQGCITHKILGEDLHD  180
´Deer´             G          W  ER  NNTGKW DY
NADL       VV  ND             I R                      T    TSF M   A    R     K          Q N      N
Osloss     VV  DD   L           R                      T  L HWS K   AM   R  K H    F       Q VI G  Y
519      N V  GD           R V R          A                 M       D         K   Y       Q VV
721        V  DD    AI    S  V R                           T   K   D A  R  K VV   N    V Q L     Y
```

FIG. 4. Alignment of the deduced amino acid sequences from the N-terminal half of E2. The E2 sequences of strains Deer-GB1, 519, and 721 determined in this study are compared with the published sequences of pestivirus strain 'Deer' (van Rijn *et al.,* 1997) and BVDV-1 strains NADL (Collett *et al.,* 1988) and Osloss (de Moerlooze *et al.,* 1993). Only differences from the Deer-GB1 sequence on the top line are indicated. Note that the sequence of strain 'Deer' published by van Rijn *et al.* (1997) differs from those of strain Deer-GB1 (determined in this study) by 12 amino acid residues; 11 differences are located in a highly conserved region of E2 (between amino acid positions 111 and 126).

With respect to previously analyzed pestiviruses, our grouping based on the N^{pro} and E2 genes is generally in agreement with those reported by others. However, there is one significant difference concerning the grouping of strain 'Deer.' By comparison of deduced amino acid sequences of the N-terminal half of E2 (encompassing 180 amino acids) it has been proposed that strain 'Deer' represents a separate genotype within the genus *Pestivirus* (van Rijn *et al.,* 1997). According to our analyses of both nucleotide and deduced amino acid sequences of the N^{pro} and E2 regions, strain 'Deer' clearly belongs to BVDV-1. A comparison of the 'Deer' sequence determined by us (termed Deer-GB1) with the published sequence of strain 'Deer' revealed only a single amino acid difference within the N-terminal 110 amino acids of E2; a short region of 16 amino acids follows with 11 differences between these two sequences; the remaining part with 54 amino acids exhibits 100% identity between the two sequences (Fig. 4). With the exception of the 'Deer' sequence published by van Rijn *et al.* (1997), the region including the above-mentioned differences is highly conserved among the five BVDV-1 subgroups (Fig. 4). Taken together, the results of our analysis strongly suggest that strain 'Deer' belongs to BVDV-1 and does not represent an additional pestivirus genotype.

In the present study, 16 novel pestivirus isolates from diverse host species were characterized at the molecular level. The results of our analysis suggested the presence of an additional pestivirus species represented by the isolates from reindeer and bison. Additional sequence information, serological investigations, animal experiments, etc., will show whether these viruses actually represent a distinct virus species within the genus Pestivirus. Furthermore, phylogenetic analyses resulted in the identification of novel subgroups within BVDV-1 and BVDV-2. It remains to be determined whether the presence of subgroups within a pestivirus species is linked to differences concerning virulence and cross protection. For BVDV-1 two distinct serotypes have recently been suggested, but the respective data were based only on sera against a single virus using a very limited number of BVDV-1 isolates (Toth *et al.,* 1999). Generation of sera raised against a representative set of pestiviruses by immunization with either whole virions or defined viral proteins, e.g., E2, and subsequent serological assays with a panel of virus isolates will show whether the segregation of pestiviruses into genetic groups and subgroups is mirrored by serological reactions. Such studies will help to determine whether vaccination with a single pestivirus strain can be expected to induce protective immunity against all known subgroups of the respective species.

MATERIALS AND METHODS

Virus isolates and cells. The bovine pestivirus isolates 519, 871, and 721 were obtained from field cases of mucosal disease, while isolate Gi-3 was from an animal with fever and thrombocytopenia. Gi-4, Gi-5, and Gi-6 represent virus contaminants isolated from fetal bovine sera (Greiner GmbH, Frickenhausen, Germany). In addition, the porcine pestivirus isolate V360 and pestivirus isolates from bongo (*Tragelaphus eurycerus*), reindeer (*Rangifer tarandus*), and European bison (*Bison bonasus*) are included in this study. The bongo virus was isolated from an aborted fetus; isolation of the pestivirus isolates from reindeer and bison was linked to abortion and fatal disease, respectively. The BVDV-2 strains Gi-1 (Becher *et al.,* 1999) and SCP (Becher *et al.,* 1995), the CSFV isolate 'Schweinfurt' (Kosmidou *et al.,* 1995), and the pestiviruses from deer (Becher *et al.,* 1997; Frölich and Hofmann, 1995) and giraffe (Plowright, 1969) have been described previously. BVDV vaccine strains RIT 4350 (Becher *et al.,* 1998b; Lobmann *et al.,* 1984) and C86 were obtained from Pfizer (Karlsruhe, Germany) and Intervet International (Boxmeer, The Netherlands), respectively. All pes-

tivirus isolates investigated in this study are listed in Table 1 together with their corresponding host species, year of isolation, and region of isolation. The BVDV strains RIT and C86 as well as the isolates 519, 871, 721, Gi-1, Giraffe-1, and SH-9 cause a cytopathic effect in tissue culture, while the other viruses listed in Table 1 are noncytopathogenic.

Pestiviruses were multiplied on Madin–Darby bovine kidney cells obtained from the American Type Culture Collection (Rockville, MD). Infection of cells was carried out as described previously (Becher *et al.,* 1997). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% horse serum. Cells and medium were tested regularly for the absence of pestiviruses by RT-PCR and immunofluorescence with monoclonal antibody 8.12.7 (directed against NS3), kindly provided by E. J. Dubovi (Cornell University, Ithaca, NY).

Oligonucleotides. Oligonucleotides were purchased from MWG Biotech GmbH (Ebersberg, Germany). Sequences of oligonucleotides and their polarities are as follows: OI W2 (5′ CGCGGATCCTGGTGGCCTTATGA 3′, sense), OI P2460 (5′ TAACAGGGGTACAAGG 3′, sense), OI P3400R (5′ CATGTATTGYTGGAAGTA 3′, antisense), OI P4700R (5′ TCATTTCTYACYTTGTGTTT 3′, antisense), OI R1400 (5′ GAGAAGACCAACTACACATG 3′, sense), OI RP7R1 (5′ CCAYTTCTTTAYKGGCTCATC 3′, antisense), OI 2P7R (5′ GTTGCCTATCATGACTATCTC 3′, antisense), OI SCPE2 (5′ AAGACCAGACTGGTGGCC 3′, sense), OI SCPE2R (5′ CCACAGTACGTACTTACC 3′, antisense), OI DeerE2R (5′ AGGYCHTYTGTTCTGATA 3′, antisense), and OI DeerGB3300 (5′ GATCACACCAAGTGAAGGAC 3′, sense); where H denotes A or C or T, K denotes G or T, and Y denotes C or T. The sense primers OI 100 (Becher *et al.,* 1998a) and OI P3400 (Becher *et al.,* 1999) as well as the antisense primers OI 35A (Becher *et al.,* 1994) and OI 1400R (Becher *et al.,* 1997) have been described previously.

RT-PCR. RNA preparation, RT, and PCR were done as described previously (Becher *et al.,* 1997). Primer OI 1400R and primer OI 100 were used for amplification of part of the 5′ NCR and the genomic region encoding N^{pro}, C, and part of E^{rns}. The E2 coding region was amplified by RT-PCR using (i) primer OI 35A and primer OI W2 (virus isolates 721, C86, SH9, and Giraffe-1); (ii) primer OI DeerE2R and primer OI W2 (virus isolates 519 and Deer-NZ1); (iii) primer OI 2P7R and primer OI SCPE2 (virus isolate Gi-6); (iii) primer OI RP7R1 and primer OI R1400 (virus isolates Reindeer-1 and Bison-1). With respect to isolate Deer-GB1 primer OI P3400R and primer OI P2460 were used for amplification of the 5′ portion of the E2 gene, while primer OI P4700R and primer OI DeerGB3300 enabled amplification of the 3′ part of the E2 gene. For analysis of the E2 genes of isolates SCP and Gi-1, RT-PCR assays with primer OI SCPE2R and primer OI SCPE2 as well as with primer OI P4700R and primer OI P3400 were performed. After amplification, the PCR products were characterized in agarose–ethidium bromide gels in Tris–acetate buffer.

Molecular cloning and nucleotide sequencing. The cDNA fragments obtained after RT-PCR were separated by agarose gel electrophoresis and purified using a Qiaex DNA Purification Kit (Qiagen). The respective cDNA fragments were cloned using a TA Cloning Kit (Invitrogen, De Schelp, The Netherlands). Nucleotide sequences were determined by cycle sequencing using a Thermo Sequenase Kit (Amersham Buchler, Braunschweig, Germany) and the DNA sequencer Li-Cor 4000 (MWG Biotech). All sequences were determined by sequencing both complementary strands of three independent cDNA clones. Sequence data from this article have been deposited with the EMBL/GenBank data libraries. The respective accession numbers are listed in Table 1.

Phylogenetic analysis. Computer analysis of sequence data was performed by using HUSAR (DKFZ, Heidelberg, Germany), which provides the GCG (Devereux *et al.,* 1984) and PHYLIP software packages (Felsenstein, 1985, 1993). Multiple sequence alignments of the nucleotide and deduced amino acid sequences were generated with programs PILEUP and CLUSTAL. Phylogenetic trees were constructed for the entire N^{pro} and E2 genes as well as the respective deduced amino acid sequence data sets using the neighbor-joining, UPGMA, and maximum-likelihood methods. The robustness of the phylogenetic analysis and significance of the branching order were determined by bootstrap analysis carried out on 1000 replicates using PHYLIP programs SEQBOOT and CONSENSE (Felsenstein, 1985, 1993; Hedges, 1992). Evolutionary distances between sequences were estimated by using the Kimura two-parameter method (Kimura, 1980).

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