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Genetic variation of Border disease virus species strains

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Summary

The 5′-untranslated region of *Pestivirus* strains isolated from domestic and wild animals were analysed to determine their taxonomic status according to nucleotide changes in the secondary genomic structure using the palindromic nucleotide substitutions (PNS) method. A total of 131 isolates out of 536 *Pestivirus* strains evaluated, were clustered as Border disease virus (BDV) species. The BDV strains were further divided into at least 8 genotypes or subspecies. Thirty-two isolates from small ruminants suffering from clinical symptoms of Border disease were clustered into bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2 and classical swine fever (hog cholera) virus species and also into the tentative BDV-2 species. Since the definition of an infectious disease is based primarily on a specific causative pathogen and taking into account the heterogeneity of the genus *Pestivirus*, clinical cases should be named according to the laboratory results. The PNS procedure could be useful for laboratory diagnosis of Border disease in domestic and wild ruminants.

Keywords

Border disease, Bovine viral diarrhoea, Classical swine fever, Genotyping, Hog cholera, Palindromic nucleotide substitutions, *Pestivirus*, Virus.

Virus della pestivirosi ovina: variazione genetica dei ceppi

Riassunto

Ceppi di Pestivirus *sono stati isolati da animali domestici e selvatici per l'analisi della regione non tradotta 5′. L'analisi ha avuto l'obiettivo di determinare lo stato tassonomico in base a variazioni nucleotidiche della struttura genomica secondaria utilizzando il metodo delle sostituzioni nucleotidiche palindromiche (PNS). Su 536 ceppi di* Pestivirus*, 131 isolati sono stati classificati come specie responsabili della pestivirosi ovina o* Border disease virus *(BDV). Questi ceppi sono stati ulteriormente suddivisi in 8 genotipi o sottospecie. Trentadue isolati di piccoli ruminanti con sintomi clinici di pestivirosi sono stati distinti nelle specie: diarrea virale bovina di tipo 1 (BVDV-1) e tipo 2 (BVDV-2), peste suina classica e nella specie provvisoria BDV-2. Poiché la definizione di malattia infettiva si basa principalmente su un agente patogeno specifico, tenendo in considerazione l'eterogeneità del genere* Pestivirus*, i casi clinici devono essere classificati in base ai risultati di laboratorio. La procedura PNS, pertanto, potrebbe essere utile per la diagnosi di laboratorio della pestivirosi ovina in ruminanti domestici e selvatici.*

Parole chiave

Border disease, Diarrea virale bovina, Genotipizzazione, Peste suina classica, *Pestivirus*, Sostituzioni nucleotidiche palindromiche (PNS), Virus.

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Introduction

Border disease virus (BDV) is a recognised species in the genus *Pestivirus* of the family *Flaviviridae* (13). Border disease affects mainly sheep and goats and has substantial loss-related economic implications worldwide. In addition to cattle, goats, sheep and pigs, ruminant pestiviruses have been isolated from many other wild ruminant species and serological surveys have demonstrated prior infection with pestiviruses in more than 40 species across the globe. The virus genome has a single-stranded, positive polarity RNA, composed of a sequence of about 12 500 nucleotides. It can be divided into three regions, as follows:

- a 5′-untranslated region (UTR)
- a single large open reading frame encoding a polyprotein
- a 3′-UTR.

The 5′-UTR is highly conserved among all members within the genus, thus being useful for the characterisation of genotypes. Primary structure analysis, by sequence alignment and construction of phylogenetic trees, is the most common method for the classification of the virus isolates.

The nucleotide substitutions occurring at the level of the 5′-UTR genomic region are particularly important, since positive-sense RNA viruses generally include regulatory motifs, which are indispensable for virus survival. In pestiviruses, the secondary structure of the 5′-UTR can be divided into four domains, A-D, with domain D encompassing two thirds in the 3′ region of the 5′-UTR predicted to fold into a complex stem-loop structure (10, 11, 21), critical region of the 5′-UTR, containing an internal ribosomal entry site (IRES), responsible for translational, transcriptional and replicational events. Thus, stable nucleotide variations at this level assume great importance in terms of virus evolutionary history. Nucleotide sequences at the variable loci, V1, V2 and V3, in the 5′-UTR of pestiviruses have been shown to be capable of forming a stable stem-loop structure peculiar to each *Pestivirus* species. Nucleotide substitutions in the stem regions always occur to maintain a stable stem-loop structure.

The observation of nucleotide variations among virus strains at the level of the three specific variable loci in the secondary structure of the 5′-UTR has been conceived as a simple and practical procedure for genotyping (23). The method, named 'palindromic nucleotide substitutions' (PNS) – with the term 'palindromic' intended as 'palindrome-like' and not referring precisely to the nucleotide sequence peculiarities of palindromes – provided essentially qualitative results with exact species classification of an isolate, clarifying species and genotype boundaries, due to the exclusive consideration of strategic and highly conserved regions and, consequently, helping to avoid unclear classification. According to the PNS method, in the genus *Pestivirus*, three genotypes have been described in the BDV species: BDV-1, BDV-2, and BDV-3, through the evaluation of 38 strains (16). The method was further improved by quantitative analysis on changes in the secondary structure (17).

The relatively high number of new deposited sequences of isolates from domestic and wild animals and the recent evidence of novel 'atypical' *Pestivirus* sequences, as for example the strains D32/00_'HoBi' (34) and Th/04_KhonKaen (27) isolated in cattle infected naturally in Brazil and Thailand, respectively, or the Bungowannah virus isolated from piglets in Australia (26) and, in particular, the recent reports on small ruminant atypical strains from clinical cases of Border disease from domestic and wild animals (8, 12, 36, 38, 39), motivated the necessity for an updated application of the PNS method.

Material and methods

A qualitative and quantitative evaluation of genomic sequence divergence, in terms of palindromic nucleotide base-pairing variations, was applied for taxonomical segregation of species through the evaluation of 536 genomic sequences. The nucleotide sequences in the 5′-UTR of *Pestivirus* strains of different

geographic origins and from different host species or contaminants of biological products, were obtained from the GenBank DNA database provided by authors or obtained in our laboratories (table available on request).

Nucleotide sequence secondary structures were predicted according to the algorithm of Zuker and Stiegler (43) using the Genetyx-Mac Version 10.1 program package (Software Development Co., Ltd, Tokyo). The minimum free energy was calculated using the method of Freier *et al*. (14). Relevant secondary structure regions at the 5′-UTR were used for genotyping based on the PNS method (17). According to PNS analysis based on changes in the secondary structure, the classification among BDV strains was completed by quantitative analysis. Base pair variations shared in the genus and at the level of the BDV species were identified as characteristic PNS. Within the BDV species, genotypes and subgenotypes were identified based on characteristic nucleotide base pairings. The nomenclature of genotypes was defined by alphabetical order to distinguish from numerical order of species definition and was ranked according to increasing divergence in the species, with reference to prevalent base pairs.

Results

According to the PNS method, through the evaluation of the nucleotide sequences in the 5′-UTR, 131 strains were shown to belong to the BDV species (Table I). A total of 32 small ruminant strains were clustered to other *Pestivirus* species, namely: bovine virus diarrhoea virus 1 (BVDV-1), BVDV-2, classical swine fever virus (hog cholera) (CSFV) and the tentative species BDV-2 (Table II). Strains originated from sheep (*Ovis aries*), goat (*Capra hircus*), Pyrenean chamois (*Rupicapra pyrenaica*), reindeer (*Rangifer tarandus*) and wisent (*Bison bonasus*). Table III summarises these 163 *Pestivirus* strains. Based on the 59 sequence variants detected, the PNS method in the 5′-UTR of BDV species revealed keys for virus identification at genus, species, genotype and subtype levels (Tables IV and V). The secondary structure variable loci in the 5′-UTR of the *Pestivirus* species are reported in

Table I
Border disease virus (BDV) species strains (*n* = 131) evaluated according to the palindromic nucleotide substitution method at the 5′-untranslated region of RNA

Species	Strain	Origin	Country	Accession	Reference
BDV	0502234	Sheep	Spain	EU711348	15
BDV	0501209-052GI	Sheep	Spain	DQ679902	5
BDV	06-F-0083	Sheep	France	EF693999	12
BDV	06-F-0299/357	Sheep	France	EF694000	12
BDV	06-F-0299/369	Sheep	France	EF694001	12
BDV	06-F-0299/420	Sheep	France	EF694002	12
BDV	06-F-0299/477	Sheep	France	EF694003	12
BDV	135 661	Sheep	United Kingdom	U65054	40
BDV	137/4	Sheep	United Kingdom	U65052	40
BDV	170 337	Sheep	United Kingdom	U65057	40
BDV	2112/99	Sheep	Spain	AY159513	24
BDV	33S	Sheep	Tunisia	AF462002	36
BDV	35	Sheep	Tunisia	AF462001	36
BDV	35T	Sheep	Tunisia	AF462000	36
BDV	37A	Sheep	Tunisia	AF461999	36
BDV	79248/01	Sheep	Spain	AY159515	24
BDV	80582/01	Sheep	Spain	AY159516	24
BDV	8320-22NZ	Sheep	New Zealand	U65063	40

Table I (contd)
Border disease virus (BDV) species strains (*n* = 131) evaluated according to the palindromic nucleotide substitution method at the 5´-untranslated region of RNA

Species	Strain	Origin	Country	Accession	Reference
BDV	8320-31NZ	Sheep	New Zealand	U65064	40
BDV	85-F-488	Sheep	France	EF693985	12
BDV	85-F-588	Sheep	France	EF693986	12
BDV	87877/01	Sheep	Spain	AY159517	24
BDV	89-F-5374	Sheep	France	EF693987	12
BDV	89-F-5415	Sheep	France	EF693988	12
BDV	90/8320/31	Sheep	United Kingdom	AF026769	Vilček *et al.* (unpublished)
BDV	90-F-6227	Sheep	France	EF693989	12
BDV	90-F-6335	Sheep	France	EF693990	12
BDV	90-F-6338	Sheep	France	EF693991	12
BDV	90-F-6339	Sheep	France	EF693992	12
BDV	91/5809	Sheep	United Kingdom	AF026768	Vilček *et al.* (unpublished)
BDV	91-F-6731	Sheep	France	EF988632	12
BDV	91-F-6732	Sheep	France	EF988633	12
BDV	91-F-7014	Sheep	France	EF693993	12
BDV	92-F-7119	Sheep	France	EF693994	12
BDV	93-F-7289	Sheep	France	EF693995	12
BDV	94-F-7446/1	Sheep	France	EF693996	12
BDV	94-F-7446/2	Sheep	France	EF693997	12
BDV	96-F-7624	Sheep	France	EF693998	12
BDV	A1263/2	Sheep	United Kingdom	U65027	40
BDV	A1870	Sheep	United Kingdom	U65028	40
BDV	A841/1	Sheep	United Kingdom	U65026	40
BDV	ARAN-1	Pyrenean chamois	Spain	AM765800	29
BDV	ARAN-2	Pyrenean chamois	Spain	AM765801	29
BDV	ARAN-3	Pyrenean chamois	Spain	AM765802	29
BDV	ARAN-4	Pyrenean chamois	Spain	AM765803	29
BDV	ARAN-5	Pyrenean chamois	Spain	AM765804	29
BDV	ARAN-6	Pyrenean chamois	Spain	AM765805	29
BDV	ARAN-7	Pyrenean chamois	Spain	AM765806	29
BDV	ARAN-8	Pyrenean chamois	Spain	AM765807	29
BDV	AV	Sheep	France	EF693984	12
BDV	BD31	Sheep	USA	U70263	33
BDV	BD ncp	Sheep	USA	Not deposited	9
BDV	BDV/Aydin/04-TR	Sheep	Turkey	AM418427	31
BDV	BDV/Burdur/05-TR	Sheep	Turkey	AM418428	31
BDV	BM01 isolate 5	Sheep	Tunisia	AY453630	36
BDV	BT2305	Sheep	Germany	EU637004	Schirrmeier *et al.* (unpublished)
BDV	BU1-C3	Sheep	Spain	DQ361068	38
BDV	BU1-C4	Sheep	Spain	DQ361069	38
BDV	BU-1CRA22	Sheep	Spain	DQ275622	39
BDV	C121	Sheep	Spain	DQ275625	39

Table I (contd)
Border disease virus (BDV) species strains (*n* = 131) evaluated according to the palindromic nucleotide substitution method at the 5´-untranslated region of RNA

Species	Strain	Origin	Country	Accession	Reference
BDV	C27	Sheep	Spain	DQ275623	39
BDV	C290	Sheep	Spain	DQ275624	39
BDV	CADI-1	Pyrenean chamois	Spain	AM905918	30
BDV	CADI-2	Pyrenean chamois	Spain	AM905919	30
BDV	CADI-3	Pyrenean chamois	Spain	AM905920	30
BDV	CADI-4	Pyrenean chamois	Spain	AM905921	30
BDV	CADI-5	Pyrenean chamois	Spain	AM905922	30
BDV	CADI-6	Pyrenean chamois	Spain	AM905923	30
BDV	CADI-7	Pyrenean chamois	Spain	AM905924	30
BDV	CADI-8	Pyrenean chamois	Spain	AM905925	30
BDV	CADI-9	Pyrenean chamois	Spain	AM905926	30
BDV	CADI-10	Pyrenean chamois	Spain	AM905927	30
BDV	CADI-11	Pyrenean chamois	Spain	AM905928	30
BDV	CADI-12	Pyrenean chamois	Spain	AM905929	30
BDV	CERDANYA-1	Pyrenean chamois	Spain	AM905930	30
BDV	CERDANYA-2	Pyrenean chamois	Spain	AM905931	30
BDV	CERDANYA-3	Pyrenean chamois	Spain	AM905932	30
BDV	CERDANYA-4	Pyrenean chamois	Spain	AM905933	30
BDV	Ch1Es	Sheep	Japan	D50816	22
BDV	Chamois1	Pyrenean chamois	Spain	AY738080	1
BDV	Chamois-Spain02	Pyrenean chamois	Spain	AY641529	25
BDV	Chemnitz	Sheep	Germany	EU637006	4
BDV	Colm24	Sheep	Spain	DQ361073	38
BDV	D1586/2	Sheep	United Kingdom	U65034	40
BDV	G1305	Sheep	United Kingdom	U65035	40
BDV	G2048	Sheep	United Kingdom	U65036	40
BDV	Genzkow 701	Sheep	Germany	EU636999	Schirrmeier *et al.* (unpublished)
BDV	Gifhorn	Pig	Germany	EU636997	Schirrmeier *et al.* (unpublished)
BDV	Gifhorn-sh	Sheep	Germany	EU637007	Schirrmeier *et al.* (unpublished)
BDV	isard4606	Pyrenean chamois	France	EU637005	Schirrmeier *et al.* (unpublished)
BDV	J1004	Sheep	Germany	EU637001	Schirrmeier *et al.* (unpublished)
BDV	JH2816	Sheep	United Kingdom	U65037	40
BDV	K1729/3	Sheep	United Kingdom	U65038	40
BDV	L83/L84	Sheep	Germany	U17144	2
BDV	L991	Sheep	United Kingdom	U65039	40
BDV	LA1108	Sheep	Germany	EU637000	Schirrmeier *et al.* (unpublished)
BDV	LE31C2	Sheep	Spain	DQ361072	38
BDV	Lot21	Sheep	Tunisia	AF461998	36
BDV	M3	Sheep	Spain	DQ275626	39
BDV	Moredun cp	Sheep	United Kingdom	U65022	40
BDV	Moredun ncp	Sheep	United Kingdom	U65023	40

Table I (contd)
Border disease virus (BDV) species strains (*n* = 131) evaluated according to the palindromic nucleotide substitution method at the 5´-untranslated region of RNA

Species	Strain	Origin	Country	Accession	Reference
BDV	Orlu-Etagne	Pyrenean chamois	France	DQ898291	32
BDV	Orlu-ORL 2004 02 C	Pyrenean chamois	France	EU477593	Dubois *et al.* (unpublished)
BDV	Orlu-R36	Pyrenean chamois	France	DQ898294	32
BDV	Orlu-R41	Pyrenean chamois	France	DQ898295	32
BDV	Orlu-S24	Pyrenean chamois	France	DQ898292	32
BDV	Orlu-S36	Pyrenean chamois	France	DQ898293	32
BDV	Q1488/1	Sheep	United Kingdom	U66042	40
BDV	Q1488/6	Sheep	United Kingdom	U65043	40
BDV	Q1673/2	Sheep	United Kingdom	U65044	40
BDV	R1292/01	Sheep	Switzerland	AY081182	6
BDV	Rentier Rudolph	Reindeer	Germany	AB122086	16
BDV	RM	Sheep	Tunisia	AY583307	Thabti *et al.* (unpublished)
BDV	Rocco	Sheep	Spain	DQ361067	38
BDV	SN1T	Sheep	Tunisia	AF461997	36
BDV	SN2T	Sheep	Tunisia	AF461996	36
BDV	SN3G	Sheep	Tunisia	AY583306	Thabti *et al.* (unpublished)
BDV	ST1405	Sheep	Germany	EU637002	Schirrmeier *et al.* (unpublished)
BDV	ST1507	Sheep	Germany	EU637003	Schirrmeier *et al.* (unpublished)
BDV	Stolpe	Sheep	Germany	EU636998	Schirrmeier *et al.* (unpublished)
BDV	T1789/1	Sheep	United Kingdom	U65045	40
BDV	T1802/1	Sheep	United Kingdom	U65046	40
BDV	V1414	Sheep	United Kingdom	U65047	40
BDV	V2377/12	Sheep	United Kingdom	U65048	40
BDV	V2536/2	Sheep	United Kingdom	U65049	40
BDV	V3196/1	Sheep	United Kingdom	U65050	40
BDV	VFMIII	Sheep	Spain	DQ361071	38
BDV	V-TOB	Cattle	Australia	U80906	3
BDV	Wisent Casimir	Wisent	Germany	AB122085	16
BDV	X818	Sheep	Australia	AF037405	2
BDV	ZA1-1115	Sheep	Spain	DQ361070	38

BDV Border disease virus

Table II
Small ruminant *Pestivirus* strains (*n* = 32) evaluated according to the palindromic nucleotide substitution (PNS) method at the 5´-untranslated region of RNA and clustered in Border disease virus (BDV-2) tentative species and other *Pestivirus* species different from BDV

Species	Strain	Origin	Country	Accession	Reference
BVDV-1	1041/01	Sheep	Spain	AY159542	24
BVDV-1	114 817	Sheep	United Kingdom	U65053	40
BVDV-1	7535	Sheep	Sweden	U65060	40
BVDV-1	7546	Sheep	Sweden	U65061	40
BVDV-1	7548	Sheep	Sweden	U65062	40
BVDV-1	A553	Sheep	United Kingdom	U65025	40
BVDV-1	B1056	Sheep	United Kingdom	U65029	40
BVDV-1	D1120/1	Sheep	United Kingdom	U65032	40
BVDV-1	D1432/P	Sheep	United Kingdom	U65033	40
BVDV-1	D771/1	Sheep	United Kingdom	U65030	40
BVDV-1	D861	Sheep	United Kingdom	U65031	40
BVDV-1	Q1161/1	Sheep	United Kingdom	U65040	40
BVDV-1	Q1161/2	Sheep	United Kingdom	U65041	40
BVDV-1	Weybridge	Sheep	United Kingdom	U65024	40
BVDV-2	098	Sheep	Tunisia	AF462004	Thabti *et al.* (unpublished)
BVDV-2	119	Sheep	Tunisia	AF462003	Thabti *et al.* (unpublished)
BVDV-2	167 237	Sheep	United Kingdom	U65055	40
BVDV-2	168 149	Sheep	United Kingdom	U65056	40
BVDV-2	173 157	Sheep	United Kingdom	U65058	40
BVDV-2	175 375	Sheep	United Kingdom	U65059	40
BVDV-2	59386	Sheep	United Kingdom	U17146	2
BVDV-2	63	Sheep	Tunisia	AF462005	Thabti *et al.* (unpublished)
BVDV-2	BD-78	Sheep	United States	U18330	35
BVDV-2	BM01 isolate 11	Sheep	Tunisia	AF462006	Thabti *et al.* (unpublished)
BVDV-2	C413	Sheep	United States	AF002227	Chen & Berry (unpublished)
BVDV-2	Lees	Sheep	United Kingdom	U65051	40
CSFV	5440/99	Sheep	Spain	AY159514	24
New taxon	712/02	Goat	Italy	AJ829444	8
New taxon	LA/91/05	Sheep	Italy	FM163381	20
New taxon	TO/121/04	Sheep	Italy	AM900848	20
New taxon	LA/82/04	Sheep	Italy	FM163383	20
New taxon	LA/26/04	Sheep	Italy	FM163382	20

BVDV bovine viral diarrhoea virus CSFV classical swine fever (hog cholera) virus

Table III
Summary of *Pestivirus* strains (*n* = 163) evaluated according to the palindromic nucleotide substitution method at the 5´-untranslated region of RNA

Species	No. of strains	Host	Geographic origin
BDV	131	Sheep, Pyrenean chamois, cattle, pig, reindeer, wisent	Australia, France, Germany, Japan, New Zealand, Spain, Switzerland, Tunisia, Turkey, United Kingdom, United States
BDV-2*	5	Sheep, goat	Italy
BVDV-1	14	Sheep	Spain, Sweden, United Kingdom
BVDV-2	12	Sheep	Tunisia, United Kingdom, United States
CSFV	1	Sheep	Spain

BDV Border disease virus BVDV bovine viral diarrhoea virus CSFV classical swine fever (hog cholera) virus
* tentative species

Table IV
Palindromic nucleotide substitutions characteristic to the Border disease virus (BDV) and Border disease virus type 2 (BDV-2) species
The position of base pairings is defined by numbering from the bottom of the variable locus

Genus	Locus	Characteristic PNS markers
Pestivirus	V1	Absence in position 22 - size of V1 21 bp (exception U) C C bulge in position 11 A-U in position 10 C-G in position 8 (exceptions U*G, U-A and G G bulge) U-A in position 7 (exception G-C and A A bulge) A in position 6 (exception G) U*G in position 5 U in position 5 right nucleotide G-C in position 4
	V2	GGGGU loop (exception GGGGC) C-G in position 8 (exception U*G)
Species	Locus	Characteristic PNS markers
BDV	V1	G-C or A-U in position 15 (exceptions C U and A C bulges)
	V3	U C and U U bulges or U*G in position 7 (exceptions A-U, U-A and C C, A C, C U and C A bulges)
BDV-2 tentative species (Italian ovine isolates)	V1	U-A or C A bulge in position 15
	V3	G*U or G G bulge in position 8
BDV genotypes	Locus	Characteristic PNS markers
BDV-a	V1	A-U or C U bulge in position 9 A A or A G bulges in position 18 (exception G G bulge)
	V2	A-U in position 1
	V3	A A, G A or A C bulges in position 8
BDV-b	V1	G-C in position 9; G-C or G G bulge in position 18 G*U or G G bulge in position 20
	V2	G*U in position 1
	V3	U-A or C A bulge in position 8
BDV-c	V1	G-C or U C bulge in position 20 U or U U bulge in position 21
	V2	A C bulge in position 1
	V3	C C bulge in position 7
BDV-d	V1	G-C or A-U in position 9 U*G, G-C, G*U or G G bulge in position 18 (exception A G bulge)
	V2	G*U, G-C in position 1 (exceptions A-U and C U bulge)
	V3	U-A, C-G, U*G, A A or C A bulges in position 8
BDV-e	V1	U-A, C-G or U*G in position 16
	V3	C U bulge in position 1; G*U or U U bulge in position 2
BDV-f	V3	U-A in position 2; U*G in position 7; U or C in position 8
BDV-g	V1	G-C in position 3; U-A or C-G in position 16
	V3	G-C in position 4
BDV-h	V2	G-C in position 5
	V3	G-C or A C bulge in position 2; C-G in position 7; U U bulge in position 9 U U or C U bulge in position 10

BDV Border disease virus
PNS palindromic nucleotide substitution

Table V
Palindromic nucleotide substitutions characteristic to the Border disease virus subgenotypes
The position of base pairings is defined by numbering from the bottom of the variable locus

BDV genotype	Sub-genotype	Locus	Characteristic PNS markers
BDV-a	BDV-a1	V1	U in position 7 right nucleotide; A-U in position 12; A-U or C U bulge in position 15
	BDV-a2	V1	G in position 7 right nucleotide; G-C in position 12 (exception C C bulge); G-C in position 15
	BDV-a3	V1	A in position 7 right nucleotide; G-C in position 12; A C bulge in position 15
BDV-b	BDV-b1	V1	G*U in position 16
		V3	U-A in position 7
	BDV-b2	V1	A-U in position 16; G in position 21
		V3	C A bulge in position 7
BDV-d	BDV-d1	V1	G-C, G G or A G bulges in position 18
		V3	C-G in position 6
	BDV-d2	V1	G G bulge in position 18
		V3	C U bulge in position 6
	BDV-d3	V1	U in position 7 right nucleotide; C-G in position 17; G*U in position 18
		V3	C-G in position 6; A-U or A C bulge in position 7
	BDV-d4	V1	U*G in position 18
		V3	C-G, C A or A G bulges in position 6
BDV-f	BDV-f1	V1	G-C in position 1
		V2	U-A or C-G in position 3
		V3	A-U in position 6
	BDV-f2	V1	A-U in position 1
		V2	A-U or A C bulge in position 3
		V3	A C, U U or C C bulges in position 1
BDV-g	BDV-g1	V1	G*U in position 12
		V3	C-G in position 3
	BDV-g2	V1	A-U or A C bulge in position 12
		V3	U-A in position 3

BDV Border disease virus
PNS palindromic nucleotide substitution

Figure 1. Base pairings characteristic of the genus (PNS genus-specific), the characteristic base pairings of the BDV genotypes (PNS genotype-specific) and base pairings characteristic of subtype-specific PNS are represented in Figure 2. The position of base pairings was defined by numbering from the bottom of the secondary structures.

The resultant BDV species was heterogeneous (Table VI). Figure 3 shows the graphic representation of the variation of divergence values among strains within the species, ranging from low values obtained by comparing homogeneous sequences in the species, to high values indicating the presence of strain sequences genetically distant from classical BDV. The strains were divided into at least 8 genotypes or subspecies by phylogenetic analysis at the variable regions in the 5′-UTR. Classical ovine BDV isolates were clustered in genotype BDV-a. Genotypes BDV-b and BDV-c included ovine isolates from Spain and France, respectively. Genotype BDV-d included all the Spanish and French isolates from Pyrenean chamois and the hyper virulent ovine strain AV. Strains from wisent and reindeer belonged to genotype BDV-e. Tunisian sheep isolates and the pig isolate Gifhorn were included in genotypes BDV-f and BDV-g, respectively. The strains Aydin/04-TR

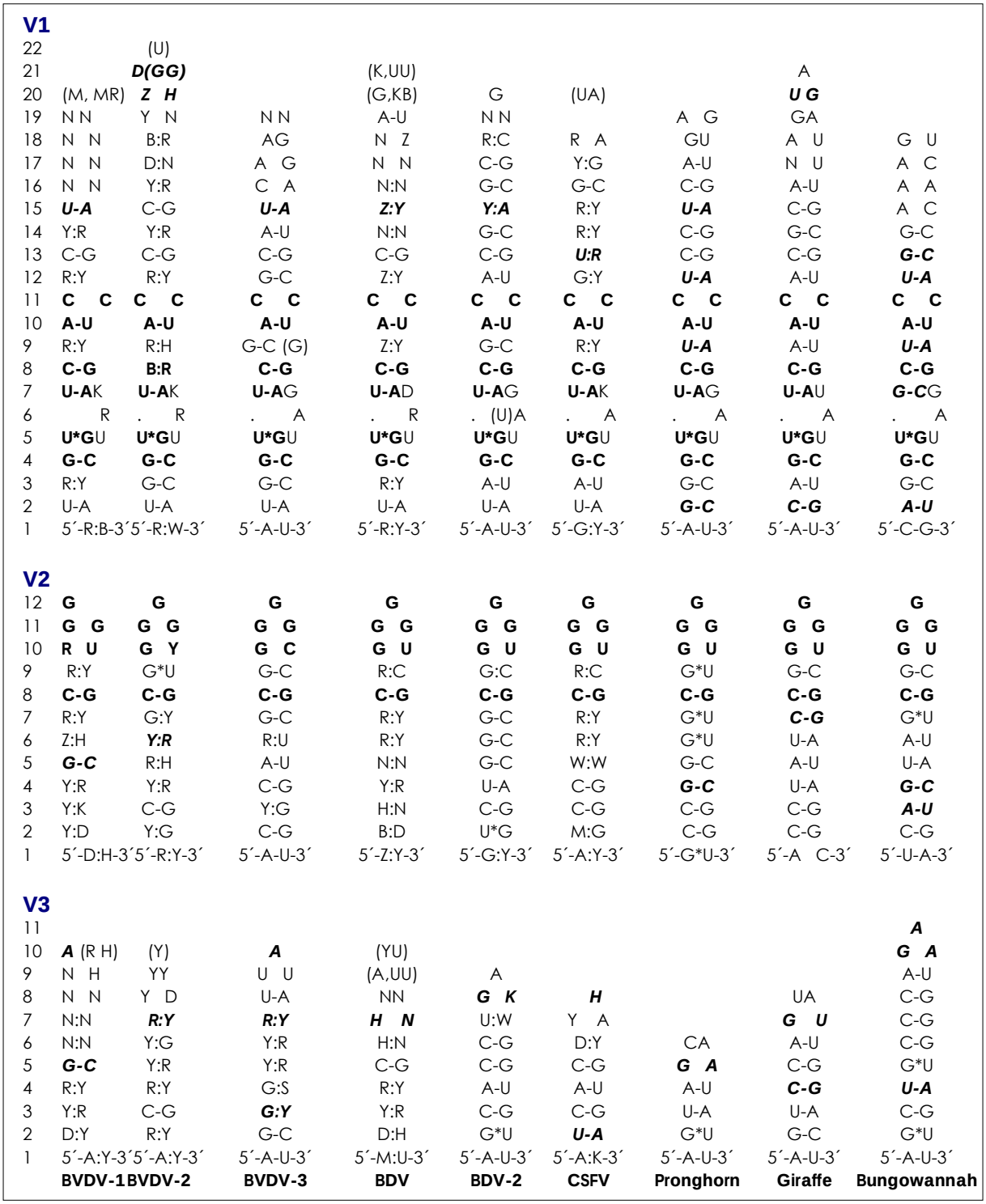

Figure 1
V1-V3 palindromic loci in the 5′-untranslated region of the genus *Pestivirus* species
Base pairings characteristic of the genus (palindromic nucleotide substitution [PNS] genus-specific) are shown in bold
The characteristic base pairings of the species bovine viral diarrhoea virus 1 (BVDV-1), BVDV-2, Border disease virus (BDV), classical swine fever (hog cholera) virus (CSFV) and the new proposed taxons BVDV-3, Border disease virus 2 (BDV-2), Giraffe, Pronghorn and Bungowannah (PNS species-specific) are represented in bold and italics
The position of base pairings is defined by numbering from the bottom of the secondary structures
Watson-Crick base pairings are indicated by a dash (–)
Tolerated pairings in secondary structure are indicated by an asterisk (*)
Interchangeable base pairings are indicated by a colon (:)
M = A or C; R = A or G; W = A or U; S = C or G; Y = C or U; K = G or U; Z = A or C or G; H = A or C or U; D = A or G or U; B = C or G or U; N = A or C or G or U

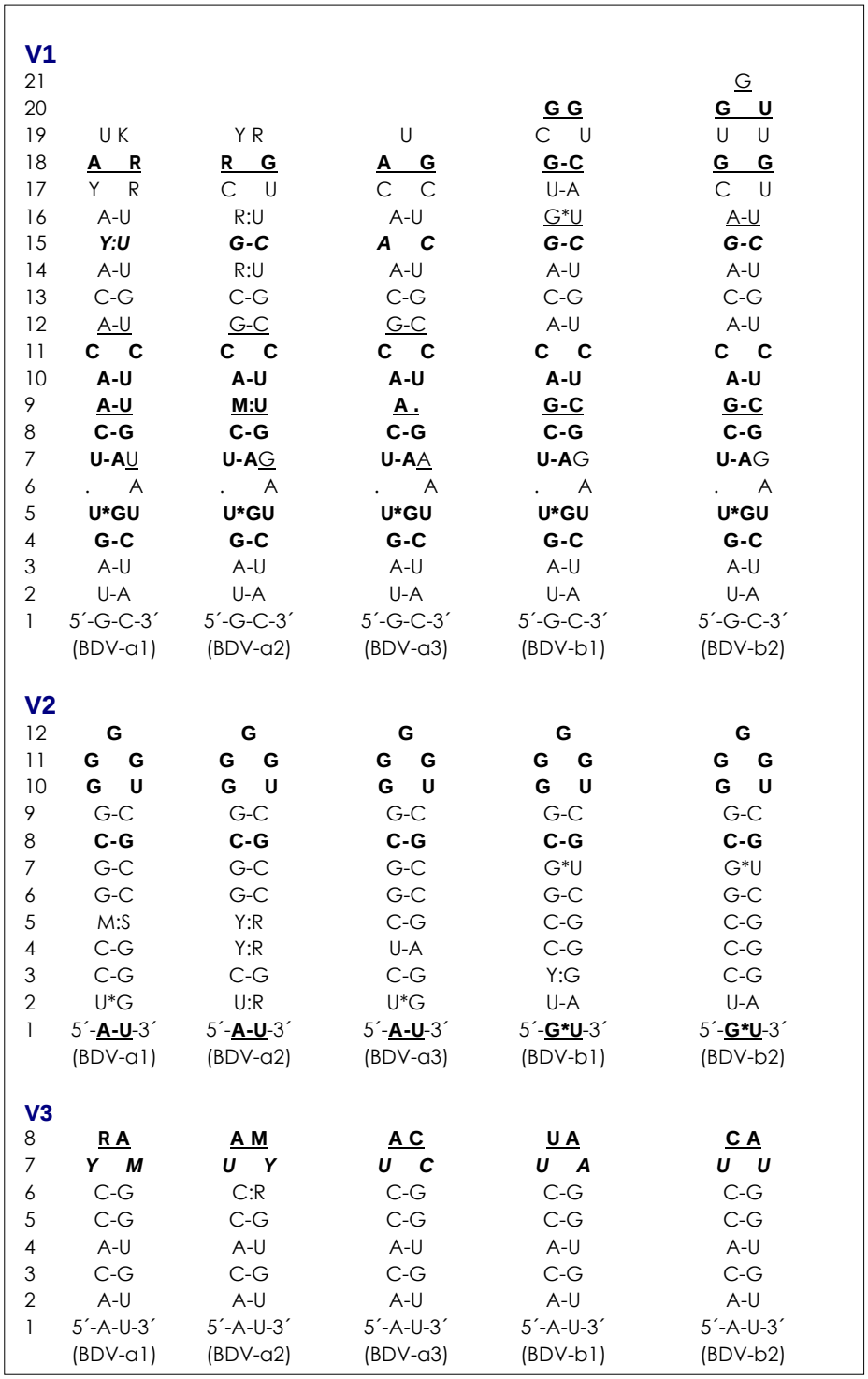

Figure 2
V1, V2 and V3 palindromic loci in the 5′-untranslated region of the Border disease virus *Pestivirus* species
Base pairings characteristic of the genus (palindromic nucleotide substitution [PNS] genus-specific are shown in bold
The characteristic base pairings of the Border disease virus (BDV) species (PNS species-specific) are represented in bold and italics
The characteristic base pairings of the BDV species genotypes (PNS genotype-specific) subgenotypes (PNS subgenotype-specific) are represented in bold and are underlined and are underlined, respectively
Watson-Crick base pairings are indicated by a dash (–)
Tolerated pairings in secondary structure are indicated by an asterisk (*)
Interchangeable base pairings are indicated by a colon (:)
M = A or C; R = A or G; W = A or U; S = C or G; Y = C or U; K = G or U; H = A or C or U; D = A or G or U

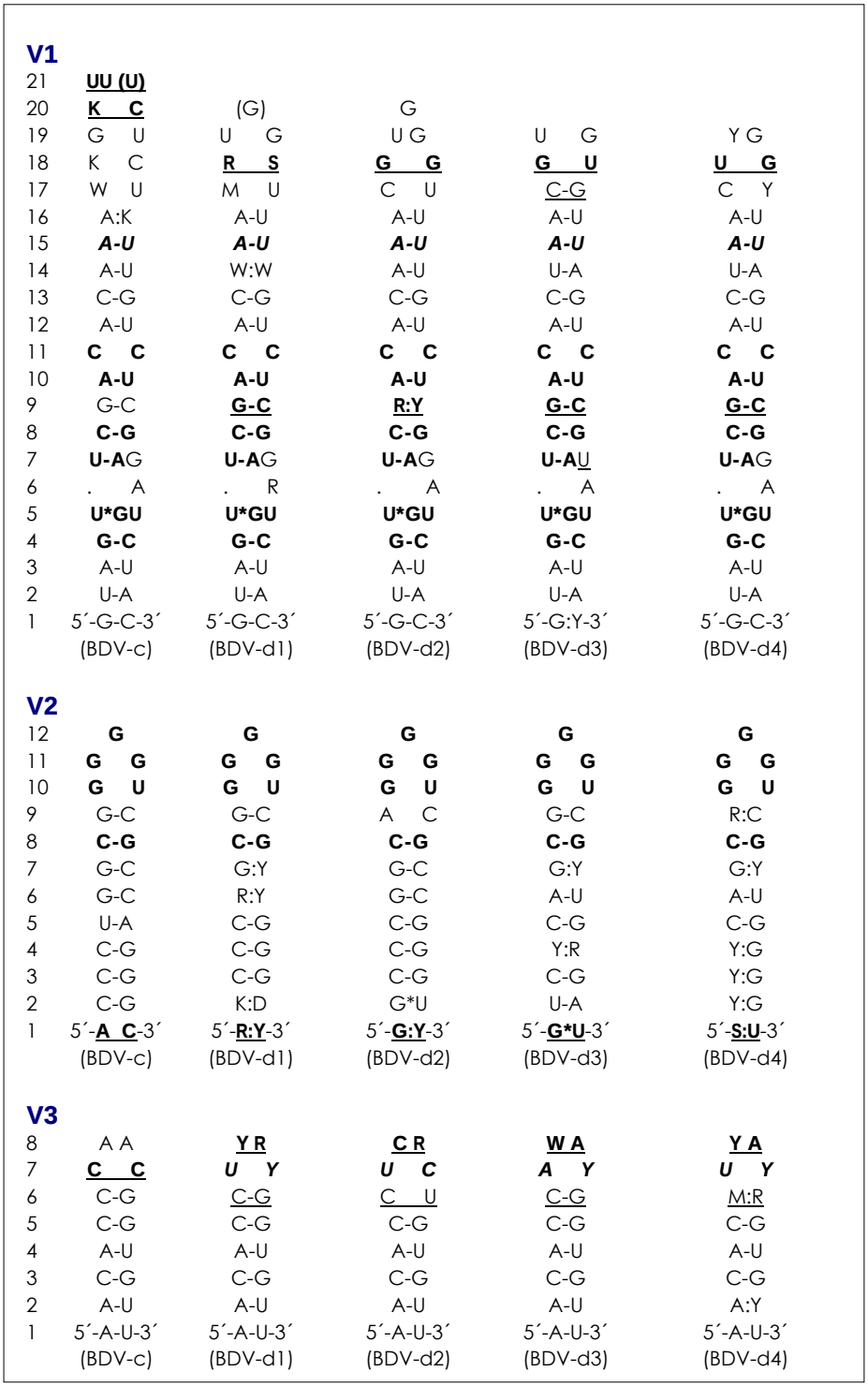

Figure 2 (contd)

V1, V2 and V3 palindromic loci in the 5´-untranslated region of the Border disease virus *Pestivirus* species

Base pairings characteristic of the genus (palindromic nucleotide substitution [PNS] genus-specific are shown in bold

The characteristic base pairings of the Border disease virus (BDV) species (PNS species-specific) are represented in bold and italics

The characteristic base pairings of the BDV species genotypes (PNS genotype-specific) subgenotypes (PNS subgenotype-specific) are represented in bold and are underlined and are underlined, respectively

Watson-Crick base pairings are indicated by a dash (–)

Tolerated pairings in secondary structure are indicated by an asterisk (*)

Interchangeable base pairings are indicated by a colon (:)

M = A or C; R = A or G; W = A or U; S = C or G; Y = C or U; K = G or U; H = A or C or U; D = A or G or U

V1

19	G	G K	A U	U C	U R	A A
18	Y G	G G	A G	A G	A G	A C
17	R:A	U-A	U-A	C C	C U	U C
16	**Y:R**	G-C	G-C	**U-A**	**Y:R**	G-C
15	***A-U***	***G-C***	***G-C***	***G*U***	***A:Y***	***G-C***
14	A-U	A-U	W:W	G-C	G-C	M:K
13	C-G	C-G	C-G	C-G	C-G	C-G
12	G-C	R:U	A-U	G*U	A:Y	G-C
11	**C C**	**C C**	**C C**	**C C**	**C C**	**C C**
10	**A-U**	**A-U**	**A-U**	**A-U**	**A-U**	**A-U**
9	R:Y	R:Y	A-U	G-C	G-C	A-U
8	**C-G**	**C-G**	**C-G**	**C-G**	**C-G**	**C-G**
7	**U-A**G	**U-A**G	**U-A**G	**U-A**G	**U-A**G	**U-A**G
6	. A	. A	. A	. A	. A	. A
5	**U*GU**	**U*GU**	**U*GU**	**U*GU**	**U*GU**	**U*GU**
4	**G-C**	**G-C**	**G-C**	**G-C**	**G-C**	**G-C**
3	A-U	A-U	A-U	**G-C**	**G-C**	R:Y
2	U-A	U-A	U-A	U-A	U-A	U-A
1	5′-G-C-3′	5′-G-C-3′	5′-A-U-3′	5′-G-C-3′	5′-G-C-3′	5′-G-C-3′
	(BDV-e)	(BDV-f1)	(BDV-f2)	(BDV-g1)	(BDV-g2)	(BDV-h)

V2

12	**G**	**G**	**G**	**G**	**G**	**G**
11	**G G**	**G G**	**G G**	**G G**	**G G**	**G G**
10	**G U**	**G U**	**G U**	**G U**	**G U**	**G U**
9	G-C	G-C	G-C	G-C	G-C	G-C
8	**C-G**	**C-G**	**C-G**	**C-G**	**C-G**	**C-G**
7	G-C	G-C	G-C	G-C	G:Y	R:U
6	G-C	G-C	G-C	G-C	G-C	G-C
5	U-A	A-U	A-U	U-A	U-A	**G-C**
4	C-G	C-G	C-G	C-G	C-G	U-A
3	C-G	Y:R	A:Y	C-G	Y:G	C-G
2	Y:G	Y:G	U*G	U*G	Y:G	C-G
1	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′
	(BDV-e)	(BDV-f1)	(BDV-f2)	(BDV-g1)	(BDV-g2)	(BDV-h)

V3

10						**Y U**
9				A		**U U**
8	A W	**U**	**Y**	U G	K C	M U
7	***U Y***	**U G**	**U G**	***C U***	***U U***	**C-G**
6	C-G	A-U	H:Y	C-G	C-G	C-G
5	C-G	C-G	C-G	U*G	C-G	C-G
4	A-U	A-U	A-U	**G-C**	**G-C**	A-U
3	C-G	C-G	C-G	C-G	U-A	Y:G
2	**K:U**	**U-A**	**U-A**	A-U	A-U	**R:C**
1	5′-**C U**-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′	5′-A-U-3′
	(BDV-e)	(BDV-f1)	(BDV-f2)	(BDV-g1)	(BDV-g2)	(BDV-h)

Figure 2 (contd)
V1, V2 and V3 palindromic loci in the 5′-untranslated region of the Border disease virus *Pestivirus* species
Base pairings characteristic of the genus (palindromic nucleotide substitution [PNS] genus-specific are shown in bold
The characteristic base pairings of the Border disease virus (BDV) species (PNS species-specific) are represented in bold and italics
The characteristic base pairings of the BDV species genotypes (PNS genotype-specific) subgenotypes (PNS subgenotype-specific) are represented in bold and are underlined and are underlined, respectively
Watson-Crick base pairings are indicated by a dash (–)
Tolerated pairings in secondary structure are indicated by an asterisk (*)
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M = A or C; R = A or G; W = A or U; S = C or G; Y = C or U; K = G or U; H = A or C or U; D = A or G or U

Table VI
Relation among Border disease virus species genotypes

Genotype	Divergence value mean/divergence (%)						
BDV-b	11.75/97.50						
BDV-c	11.58/100	11.16/100					
BDV-d	11.31/85.31	10.09/62.50	12.33/91.66				
BDV-e	8.60/59.17	14.91/100	12.00/100	14.34/98.96			
BDV-f	11.52/96	13.90/100	15.40/100	16.06/98.75	13.70/100		
BDV-g	11.86/84	15.10/100	14.00/100	14.36/96.25	12.86/100	16.08/100	
BDV-h	14.15/100	18/100	19/100	19.03/100	14.66/100	14.30/100	17.40/100
	BDV-a	BDV-b	BDV-c	BDV-d	BDV-e	BDV-f	BDV-g

BDV Border disease virus
PNS palindromic nucleotide substitution

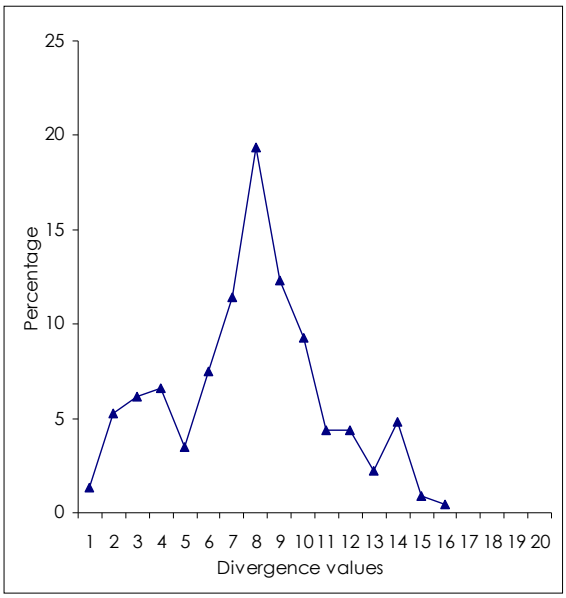

Figure 3
Determination of the level of heterogeneity within the Border disease virus species in the genus *Pestivirus* (palindromic nucleotide substitution method)
The divergence values between single strain sequences were obtained by comparing base pairing from aligned secondary structure sequences, helping for the characterisation and clustering of specific strains
The borderline strains Aydin/04-TR and Burdur/05-TR (31), showing high divergence values in the species, have been excluded in the construction of the graph
Species divergence limit value: 13

and Burdur/05-TR (31), isolated from sheep in Turkey, genotype BDV-h, were located on a borderline within the species. Their sequences showed qualitative similarities with' the BDV species, sharing the specific PNS species markers, but with high divergence values, thus, candidates for reclustering as a separate group in the genus.

With the exception of genotypes BDV-c, BDV-e and BDV-h, which were homogeneous, the other genotypes were further divided in subgenotypes. In particular, genotype BDV-d revealed four distinct subtypes, namely:

- BDV-d1 with ovine strains isolated in France and Spain
- BDV-d2 with the ovine French strains including the hyper virulent strain AV
- BDV-d3 including ovine strains from Spain
- BDV-d4 specific for Spanish and French isolates from Pyrenean chamois.

The relation between BDV and CSFV species appeared very clearly (Figure 4) when comparing BDV with other *Pestivirus* species to determine the genetic relatedness among *Pestivirus* species. The computing of the divergence by comparing sequences from both species showed very low values (mean 14.48) when compared to those obtained with other *Pestivirus* species (from 16.30 with BDV-2, to 25.98 with Bungowannah) (Table VII). Interestingly, the sheep isolates from Tunisia (strains 33S, 35, 35T, Lot21, SN1T, SN3G, SN2T, 37A, RM and BM01 isolate 5) reported by Thabti *et al*. (36) and the French strains 91-F-6731 and 91-F-6732 (12) shared the CSFV characteristic U-A base pairing in position 2 in V3. The two French strains that resulted were multi-related with CSFV showing high sequence similarities and low divergence values. These ambiguous strains, sharing common sequence characteristics with both

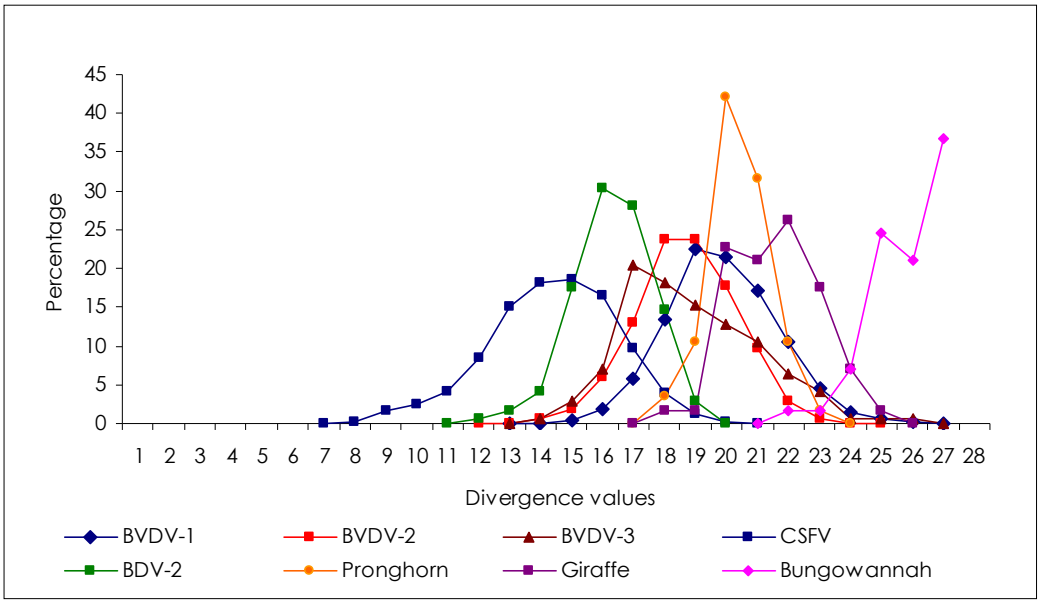

Figure 4
Determination of the genetic relatedness among *Pestivirus* species (palindromic nucleotide substitution method)
The comparison of classical swine fever virus (CSFV) strain 5′-untranslated region sequences with those from other species showed a very clear relation between CSFV and Border disease virus species, with very low divergence values (mean: 14.48)
Ambiguous strains, sharing common sequence characteristics with both species (multi-related strains), could be clustered in the species showing the lowest divergence values

Table VII
Relation between Border disease virus (BDV) and Border disease virus type 2 (BDV-2) species with other identified species within the genus *Pestivirus*

Species	BDV		BDV-2	
	Divergence (%)	Divergence value mean	Divergence (%)	Divergence value mean
BVDV-1	100	19.70	99.62	17.86
BVDV-2	100	18.73	100	17.79
BVDV-3	100	18.83	100	19.33
BDV	27.89	11.22	97.74	16.30
BDV-2	97.74	16.30	0	4.5
CSFV	69.30	14.48	100	17.13
Pronghorn	100	20.39	100	17.00
Giraffe	100	21.66	100	20.33
Bungowannah	100	25.98	100	24.66

BDV Border disease virus
BVDV bovine viral diarrhoea virus
CSFV classical swine fever (hog cholera) virus

species (multi-related strains), could be clustered in the BDV species showing the lowest divergence values.

The ovine strains BD non-cytopathic (ncp) from United States (9), L83/L84 from Germany (2), R1292/01 from Switzerland (6) and strain V-TOB isolated from cattle in Australia (3), all reported as BDV, showed sequences corresponding to BDV species, but could not be genotyped using the PNS method due to the absence of initial portions of the 5′-UTR sequence.

Discussion

The PNS analysis in the 5′-UTR demonstrated a rational and simple approach for viral investigations. Secondary structures predicted at the variable regions in the 5′-UTR showed typical PNS which were useful for classification or genotyping of BDV. The PNS at the three variable loci (V1, V2 and V3) in the 5′-untranslated region (UTR) of the *Pestivirus* genome were considered for taxonomical segregation of the species, through the evaluation of 536 strains. On the basis of qualitative and quantitative secondary structure characteristics, species were identified within the genus, determining genetic distances between species isolates, clarifying borderline and multi-related sequences and characterising and clustering the *Pestivirus* strains showing unexpected genomic sequences. A total of 9 genomic groups have been identified in the genus, as follows:

- BVDV-1
- BVDV-2
- BDV
- CSFV
- Pronghorn
- Giraffe
- BVDV-3 (HoBi group)
- BDV-2 (Italian small ruminant isolates)
- Bungowannah.

The observation made on the nucleotide sequences of the three variable loci at the level of the 5′-UTR genomic region of BDV strains led to the identification of consensus motifs shared by all species. The characteristic PNS were identified at genus, species, genotype and subtype levels, respectively. The PNS characteristics of the species were included in the stem-loop secondary structure. Characteristic base pairings were not always identifiable for each genotype or subtype. However, a clear identification was obtained using specific combinations of base pairings in the sequence. These base pairings were non-specific when considered separately.

A total of 131 strains of the BDV species were classified using the PNS genotyping method at the 5′-UTR of the viral RNA. Another 32 strains isolated from small ruminants suffering from Border disease were clustered into the *Pestivirus* species that were different from BDV. The BVDV-1 species included 14 strains. A total of 12 strains were clustered in the BVDV-2 species. The ovine strains 098, 119 and 63 from Tunisia (Thabti *et al*., unpublished findings) were clustered within the BVDV-2 species, group BVDV-2A, constituting a separate genotype in addition to the four genotypes, BVDV-2a, BVDV-2b, BVDV-2c, and BVDV-2d described previously (18). The ovine strain 5440/99 (24) belonged to the CSFV species. Strains 712/02 (8), LA/91/05, LA/82/04, LA/26/04 and TO/121/04 (20) were clustered in the tentative BDV-2 species.

The classification among *Pestivirus* species strains according to PNS analysis based on changes in the secondary structure was compared with those based on the 5′-UTR primary structure, performed through alignment and construction of phylogenetic trees. The results were generally comparable (17, 19). In particular, new taxons were defined due to specific base pairings, despite the limited number of allocated strains. This corresponded to the observations made by other authors, evaluating the 5′-UTR or other genomic regions (7, 26, 34, 41). However, some atypical strains were related to controversial taxonomical clustering, in particular for sequences isolated from small ruminants (Table VIII). The strains Aydin/04-TR and Burdur/05-TR (31), isolated from sheep in Turkey, reported by the author as a new genotype of BDV and clustered in genotype BDV-h, according to the PNS method, were considered a separate species within the genus *Pestivirus* and named Turkey by other authors (20). The sheep isolates from Tunisia (strains 33S, 35, 35T, Lot21, SN1T, SN3G, SN2T, 37A, RM and BM01 isolate 5) reported by Thabti *et al*. (36), associated with clinical cases caused by iatrogenic and natural infections, represented an interesting intermediate group of pestivirus that is genetically close to CSFV but antigenically related to BDV. They have been reported by the author as members of a new genotype of the BDV species. Two French ovine isolates, (91-F-6731 and 91-F-6732) (12),

Table VIII
Comparison of clustering of virus strains related with Border disease according to palindromic nucleotide substitution method and other methods based on primary sequence analysis

Strain	PNS		Valdazo-Gonzalez *et al.* (38, 39)		Thabti *et al.* (36)		Dubois *et al.* (12)		Liu *et al.* (28)		De Mia *et al.* (8)		Oguz-oglu *et al.* (31)		Marco *et al.* (29)		Giam-marioli *et al.* (20)	
	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G	S	G
D1586/2	BDV	a.1	BDV	1a			BDV	1										
137/4	BDV	a.1	BDV	1a			BDV	1b							BDV	1		
T1802/1	BDV	a.2	BDV	1b					BDV	1								
91/5809	BDV	a.2	BDV	1b							BDV						BDV	1
Moredun cp	BDV	a.2	BDV	1b							BDV						BDV	1
8320-31NZ	BDV	a.2	BDV	1b			BDV	1										
Moredun ncp	BDV	a.2	BDV	1b	BDV		BDV	1			BDV				BDV	1		
X818	BDV	a.2	BDV	1b	BDV		BDV	1a	BDV	1					BDV	1		
BD31	BDV	a.3	BDV	1b	BDV		BDV	1a	BDV	1								
C27	BDV	b.1	BDV	4			BDV	4										
ZA1-1115	BDV	b.2	BDV	4b														
92-F-7119	BDV	c					BDV	6										
06-F-0299/369	BDV	c					BDV	6									BDV	6
06-F-0299/477	BDV	c					BDV	6									BDV	6
91-F-7014	BDV	c					BDV	6									BDV	6
94-F-7446/1	BDV	c					BDV	6									BDV	6
89-F-5415	BDV	d.1					BDV	5									BDV	5
C121	BDV	d.1	BDV	4			BDV	4a									BDV	4
BU-1CRA22	BDV	d.1	BDV	4			BDV	4b										
Rocco	BDV	d.1	BDV	4b											BDV	4		
LE31C2	BDV	d.1	BDV	4a			BDV	4a							BDV	4		
AV	BDV	d.2					BDV	5									BDV	5
96-F-7624	BDV	d.2					BDV	5									BDV	5
2112/99	BDV	d.3	BDV	4a							BDV						BDV	4
M3	BDV	d.3	BDV	4			BDV	4										
Chamois1	BDV	d.4	BDV	4a			BDV	4	BDV	4	BDV				BDV	4	BDV	4
Chamois-Spain02	BDV	d.4	BDV	4a											BDV	4		
ARAN-1	BDV	d.4													BDV	4		
Rentier Rudolph	BDV	e	BDV	2	BDV		BDV	2a	BDV	2					BDV	2	BDV	2
Wisent Casimir	BDV	e	BDV	2			BDV	2a	BDV	2	BDV				BDV	2	BDV	2
91-F-6732	BDV	f.1					Tns											
91-F-6731	BDV	f.1					Tns											
37A	BDV	f.2			BDV	N												
35	BDV	f.2			BDV	N	Tns											
35T	BDV	f.2			BDV	N												
SN2T	BDV	f.2	Tns		BDV	N					BDV						Tns	
RM	BDV	f.2	Tns		BDV	N												
SN1T	BDV	f.2			BDV	N			TSV									
BM01 isolate 5[(a)]	BDV	f.2	Tns		BDV	N	Tns				BDV						Tns	
33S	BDV	f.2			BDV	N	Tns		TSV									
Lot21	BDV	f.2			BDV	N												
SN3G	BDV	f.2			BDV	N					BDV						Tns	

Table VIII (contd)
Comparison of clustering of virus strains related with Border disease according to palindromic nucleotide substitution method and other methods based on primary sequence analysis

Strain	PNS		Valdazo-Gonzalez *et al.* (38, 39)		Thabti *et al.* (36)		Dubois *et al.* (12)		Liu *et al.* (28)		De Mia *et al.* (8)		Oguz-oglu *et al.* (31)		Marco *et al.* (29)		Giam-marioli *et al.* (20)	
	S	G	S	G	S	G	S	G	S		S	G	S	G	S	G	S	G
Gifhorn	BDV	g.1							BDV	3								
06-F-0083	BDV	g.2					BDV	3										
85-F-588	BDV	g.2					BDV	3									BDV	3
90-F-6227	BDV	g.2					BDV	3									BDV	3
90-F-6338	BDV	g.2					BDV	3									BDV	3
Burdur/05-TR	BDV	h[b]											BDV	7			Trk	
Aydin/04-TR	BDV	h[b]											BDV	7			Trk	
BM01 isolate 11[c]	BVDV -2A	2e							TSV									
TO/121/04	BDV-2																BDV	7
LA/91/05	BDV-2																BDV	7
LA/82/04	BDV-2																BDV	7
LA/26/04	BDV-2																BDV	7
712/02	BDV-2		BDV				NBC				BDV	N					BDV	7

PNS palindromic nucleotide substitution
S species
G genotype
BDV Border disease virus
Tns Tunisian
Trk Turkey
TSV Tunisian sheep virus
N new
NBC not BDV cluster

a) Accession number AY453630
b) borderline
c) Accession number AF462006

showed high genetic similarity with the Tunisian strains. The author indicated the appurtenance of both groups of strains to a novel species named Tunisian. Other studies reported the Tunisian strains as a separate species (20, 28, 38, 39), or belonging to the BDV species (8). The application of PNS segregated these isolates in the BDV species, based on the divergence values when compared to CSFV and BDV sequences, despite the sharing of CSFV-specific base pairing U-A in position 2 in V3. In particular, the Tunisian and French isolates constituted a homogeneous group with a divergence mean of 5.5 within the group. The strains showed lower divergence values with BDV than with CSFV, mean 12.07 and 12.72, respectively. Furthermore, strains 712/02 (8), LA/91/05, LA/82/04, LA/26/04 and TO/121/04 (20) isolated from small ruminants in Italy and reported as genotypes of BDV species, were clustered as new taxon in the genus *Pestivirus*, showing a divergence value mean of 16.30 with the BDV species.

In our study, four strains, all reported as BDV, could not be genotyped using the PNS method due to the absence of initial portions of the 5′-UTR sequence. The design of the primers for 5′-UTR could avoid the lack of important sequence fractions and related difficulties in the application of identification procedures.

Recent reports have demonstrated confirmed cases of isolation of ruminant *Pestivirus* and disease with significant population impacts on free-ranging animals (29, 32, 39). An outbreak of a previously unreported disease, associated with a new pestivirus belonging to the BDV virus cluster, was reported in Southern chamois (*Rupicapra pyrenaica*) in the Catalan Pyrenees (north-eastern Spain) in 2001 and 2002, and was apparently responsible for a

population decrease of 40%-45% in the areas most affected (29). These recent reports indicate the potential for an emerging disease in wild animal populations, with a severe impact on sensitive populations. Furthermore, the role of wild animals as reservoirs that could spread the disease to domestic populations might raise the risk of substrate for mutational processes that could result in increased virulence. It is interesting to note the genetic relatedness between the strains reported in the Pyrenean chamois and the hypervirulent strain AV, both clustered in the same genotype 4.

According to the World Organisation for Animal Health (OIE: *Office International des Épizooties*), the BDV genetically related pestiviruses BVDV-1 and CSFV are included in the list of diseases of importance to international trade. Article 1.2.1. of the *Terrestrial animal health code* (42) provides the criteria for the inclusion of a disease in the OIE list. Basic criteria are international spread, significant spread within naive populations, zoonotic potential and emerging diseases. BDV might also be considered, taking into account the fact that the disease is cosmopolitan and can cause significant morbidity.

Due to the global importance of BDV and the difficulties encountered in the control of the disease, it is therefore important to understand the genetic aspects of the virus and the evolutionary history. The application of the PNS method might represent an additional tool that will be useful to determine the genetic variations among virus strains. Similarly, studies of characteristic groups within a limited geographic area, in wild animal species or in animals suffering from specific clinical symptoms, might reveal interesting virus molecular aspects. The identification of viral types or subtypes based on genetic changes should improve our understanding of the virus and might provide markers for biological differences, such as virulence. Previous observations on the BVDV-2 species have indicated the possibility of identifying virulence markers in the 5′-UTR secondary structure (18, 37). The manual searching of relevant base pairings and direct observations of the sequences, nevertheless, still remain the major limitations of the method. Further efforts are required to develop the PNS method as a fully computerised procedure for easy access and rapid testing with reliable results.

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