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Pestivirus bovis Species Taxonomy and Genomic Sequence Characteristics

Massimo Giangaspero[1,2*], **Shuqin Zhang**[3]

[1]Parliamentary Assembly of the Mediterranean, City of San Marino, Republic of San Marino
[2]Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy
[3]Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, China
Email: *massimo.giangaspero@pam.int

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Abstract

Pestivirus bovis viruses are pathogens of bovines and other domestic and wild ruminant species, causing economic losses on zootechnics. Investigations on genetic characteristics are required to clarify the epidemiology of the species genotypes and to elucidate specific aspects at molecular level, such as the ways of diffusion, transmissibility, virulence or mechanisms to escape host defense. In order to study the taxonomy of the species, with particular focus on genotype b, deposited 5'-UTR and Npro sequences of strains have been evaluated by the primary and secondary structure analysis, by comparison with molecular characteristics of previously reported virus isolates in the genus *Pestivirus*. The strains were clustered into genotypes and sub genotypes, showing the heterogeneity of the *P. bovis* species. In relation to geographic distribution, genotype b resulted in a cosmopolitan distribution. Other genomic clusters were related to restricted regions. Genotype b showed a wider host spectrum, in contrast to the other groups. Cattle were the most common host in all the genotypes of the *P. bovis* species. The application of genetic characterization methods is essential to clarify the taxonomical status of strains and should not be limited to a single procedure or the evaluation of primary sequence structure, especially of highly variable parts of the genome. The analysis of the secondary structure of relevant genomic sequences may help to discriminate atypical and genetically different clusters. Furthermore, information obtained by molecular investigations provides essential elements on circulating virus populations and their distribution in animal hosts, with direct implications on control and prophylaxis strategies. The occurrence of *P. bovis* sub genotype b2 in mosquitoes deserves particular attention. Other investigations will be necessary to confirm the phenomenon and understand its extent, which has the potential to exponentially enhance the diffusion of the virus.

Keywords

Pestivirus bovis, Sequence Characteristics, Taxonomy

1. Introduction

Pestivirus bovis (*Pestivirus A*, Bovine viral diarrhea virus type 1-BVDV-1) is an established species of the genus *Pestivirus* of the family *Flaviviridae* [1]. The virus causes relevant economic damage on zootechnic worldwide, responsible for different clinical diseases in cattle and other ruminants, affecting respiratory, gastrointestinal and reproductive systems. To reduce its impact on livestock production, vaccination and culling of carriers are recommended, introducing voluntary programs for disease eradication. In recent years, from simple voluntary small scale eradication campaigns organized by local farmer's associations, various laws were issued by competent authorities to introduce specific rules related to the health risk management of selected major pathogens, considering also BVDV. In the European Union, communicable diseases, for which compulsory detailed legal measures for the prevention and control have been issued, were listed in Article 5 and Annex II of the Regulation 429/2016, the "Animal Health Law", in force since April 2021 [2]. Subsequently, different implementing Regulations and European Commission Delegated Acts have been promulgated as legal framework to regulate the defined important diseases. In this framework, BVDV was included in the Annex II of Reg 2016/429, as amended by Commission Delegated Regulation (EU) 2018/1629 [3]. Based on the Implementing Regulation 2018/1882, the listed diseases were classified and related susceptible species were specified to defined targeted animal populations, with a view to regulating official eradication programs recognized at Union level [4]. According to this Regulation, all listed diseases fall within definitions of Category A (diseases absent in EU and potential introduction to be prevented), B (diseases to be eradicated at Union level) or C [4], where category C disease means a listed disease which is of relevance to some EU Member States. BVDV was classified as disease of categories C, D (needed to prevent spreading) and E (need for surveillance within the Union), and the *Bison* ssp., *Bos* ssp., *Bubalus* ssp. Species: the related target animal populations. Therefore, BVDV was also considered for programs to be approved by the European Commission, along with historical pathogens, such as *Brucella abortus*, *melitensis* and *suis* and *Mycobacterium tuberculosis* complex or rabies virus, object of long-term prophylactic and control campaigns in Europe. The Commission Delegated Regulation 2020/689 laid down rules for control strategy of major listed diseases (surveillance, eradication programs, and disease-free status) [5]. National competent authorities were empowered to design disease control strategy of optional eradication programs for BVD (Regulation 2020/689, Art 16, point f). Bovine animals kept in establishments were defined as target animal population for BVD eradication programs (Regulation 2020/689, Art 17, point d). This implies that operators of establishments where animals from targeted animal populations are kept must comply with specific obligations in respect to eradication programs for BVDV to obtain and maintain disease-free status (Reg 2020/689 Art. 18). The requirements ordered by the competent authority include surveillance of the disease, avoid compromission of health status due to transport or introduction of animals or products, vaccination of kept animals, control measures if the disease is suspected or confirmed and any additional measures decided by the competent authority. In certain cases, vaccines are forbidden by the competent authority to avoid a disturbance of serological monitoring due to seroconversion induced by immunization in the framework of eradication programs. The eradication programs were undertaken to obtain disease-free status, according to the procedure defined by the Commission Implementing Regulation (EU) 2021/620 laying down rules for the approval of the disease-free and non-vaccination status [6]. Recently, between 2022 and 2024, the European Commission approved eradication programs for BVD, to be implemented in the whole territory of Ireland and in parts of Germany, according to the Implementing Regulations 2022/1218 and 2024/2032, amending the Implementing Regulation 2021/620 as regards the approval of eradication programs (annex VII, part II) and published in the Official Journal of the European Union [7] [8]. In 2022, the whole territory of Austria, Denmark, Finland and Sweden obtained the disease-free status from BVD, granted by the European Commission, according to Regulation 2022/1218 (annex VII, part I) [7]. Based on the same EU Regulation, the disease-free status from BVD was also granted to various administrative territories (bundesland) of Germany. In light of the economic importance and the regulatory framework, it is therefore of utmost importance to implement effective fight against BVDV which relies on serological and antigenical laboratory diagnostic, identification and elimination of immune tolerant individuals and vaccination. Giving that, as for other pathogens, the laboratory testing tools and vaccinal products are prepared using the antigenic substrate of selected viruses [9]-[11], it essential to improve the understanding of virus characteristics to establish effective control and preventive measures.

The virus was widely studied. The complete genome of the *P. bovis* strains is a single-stranded, positive polarity RNA virus of about 12,500 nucleotides in size [12], which comprises one large open reading frame (ORF) encoding about 3900 amino acids, for the production of four structural (C, Erns, E1, and E2) and seven nonstructural (Npro, p7, NS2/3, NS4A, NS4B, NS5A, and NS5B) proteins, flanked by untranslated regions (UTRs) [13]. Among the structural proteins, C is the core protein of the virus and Erns, E1 and E2 are the glycoproteins of the envelope. Erns stimulates the production of antibodies with limited neutralizing capacity, whereas E2 contains epitopes that are detected by the antibodies crucial for neutralizing viral infectivity, making E2 the principal target of the host immune system, contains major antigenic determinants [14]. However, alterations in such epitopes allow viruses to evade neutralizing immune responses, and, thus, E2 as a complement regulatory protein, is implicated in mechanisms of immune evasion by *P. bovis* [15]. Moreover, it has been proposed that E2 functions as a self-associated molecular pattern (SAMP), allowing the virus to evade detection by host antibodies, being perceived as a self-component [15]. Of the two membrane glycoproteins, E1 and E2, encoded as the *Hepacivirus*, the E2 is required in host cell attachment [16] [17], through binding to cell-surface receptors [14], but not involved for membrane fusion, lacking a class II fusion protein fold [16]. Among the non-structural proteins, the N-terminal protein (Npro) of *P. bovis* encodes a cysteine auto-protease, while NS23 encodes a serine protease. NS23 is the largest viral protein, with a molecular weight of approximately 125 kD, and is crucial for the assembly of infectious viral proteins. Of the two untranslated regions, 5'-UTR is the most highly conserved. It has a palindromic nucleotide sequence and includes the internal ribosome entry site (IRES) [18] [19]. Another characteristic of field strains, retrieved from samples when cultivated for laboratory investigations as virus isolation, it is their ability, or lack thereof, to produce a cytopathic effect. At the moment of their isolation, strains can be distinguished in 2 biotypes, according to their effects on cellular layer in laboratory cultures: cytopathic, able to lyse cells, and non-cytopathic. Cytopathic stains express the NS23 in a single protein and also in 2 separate fragments, NS2 of a molecular weight of 54 kD and NS3 of 80 kD, the second with nucleoside triphosphatase/RNA helicase activity. The non-cytopathic strains are the most prevalent in natural infections and play a significant role in the development of clinical disease [20]. However, this does not characterize a specific functional property of the virus, for example higher virulence, since all the strains possess equally this potential characteristic, even if not expressed. However, at laboratory level, cytopathic strains are easily identifiable in cell culture, while the non-cytopathic ones may go undetected, and resulting, for example, contaminants of bovine fetal serum.

Efforts in taxonomy have been made. The examination of the primary structure of specific genomic segments, including the 5'-UTR and the Npro gene, and more recently reinforced by complete genome sequencing, has commonly been employed for characterization. In addition, analysis of the secondary structure of the 5'-UTR has been conducted, taking into account nucleotide differences within the IRES region through the palindromic nucleotide substitutions (PNS) approach [21] [22]. Based on morphological characteristics, sedimentation coefficient, flotation density and antigenic relationships, BVDV, as well as Border Disease and Classical Swine Fever viruses, were taxonomically clustered in the separate genus *Pestivirus*. Initially the genus was classified in the *Togavirus* family, on the basis of some aspects of the genome organization [23] and then reclassified as *Flaviviridae* [24]. Since the first three species described in the genus, various other species have been identified showing similar genomic characteristics, justifying their clustering in the same genus. Thus, the genus was reorganized and species have been renamed. For example, from the first differentiation of BVDV in 2 separate species, BVDV type I and BVDV type II (the second related to hypervirulent strains) [25], a third BVDV species was described as type 3, also named HoBi like *Pestivirus* [26], currently renamed as *P. bovis*, *P. tauri* and *P. braziliense*, respectively [1].

Also, the genetic categorization of the species on the base of nucleotide sequence variations has been often reconsidered. Various authors analyzed field isolates, belonging to the BVDV species, clustering them into genetic groups. Genotypes a and b were the first described [27], followed by the identification of genotypes c and d [28] [29]. Furthermore, the evaluation of other isolates showed existing additional types [30]-[43], indicating the heterogeneity of the species. Sub genotypes were also identified in the *P. bovis* genotype a (a1 and a2) [44] or in the *P. bovis* genotype b (b1 and b2) [35]. Investigations into antigenic relatedness among genotypes, based on virus neutralization antibody titers of hyperimmunized antisera against the evaluated *P. bovis* genotypes, demonstrated the greatest similarity—reflected by the highest cross-neutralizing antibody titers—between genotypes a and b [23] [44] [45]. This is important since the *P. bovis* homologous pairs of genotypes a and b are commonly incorporated into commercialized vaccines. In addition, *P. bovis* genotype b is probably the most distributed genotype among bovine populations, globally. Therefore, any new observation in the species, and in particular concerning genotype b, deserves careful consideration.

In the present study, the taxonomy of the *P. bovis* species was assessed to determine the clustering pattern of strains classified into genotypes, with focus on genotype b, and originating from various countries, and different hosts, as well as reported as variants possessing specific genomic characteristics, as the recently isolated strain BI-2023 from China [46], showing E2 protein unique mutations and proposed as a new *P. bovis* sub genotype b, genomic evaluations have been performed by the primary and secondary structure analysis.

2. Material and Methods

2.1. Strain Sequences

To investigate genotype variation within the *P. bovis* species, nucleotide sequences from the RNA 5'-UTR and Npro regions of 412 *Pestivirus* strains were analyzed. Sequences were selected on the basis of the presence of the entire IRES in the 5'-UTR (with a minimum length of approximately 240 nucleotides), excluding sequences with incomplete IRES and those showing low quality of nucleotide translation (elevate number of missing nucleotides, indicated by alphabetic letters) and avoiding duplication or even partial overlaps of differently deposited sequences, for example a 5'-UTR deposited alone and another deposited included in a larger portion of the sequence of the same isolate. The complete accession list of the dataset is presented in **Table 1** and **Table S1**. These sequences were subjected to numerical taxonomy considering the primary sequence structure and further using the PNS genotyping method, which evaluates palindromic secondary structure features at 5'-UTR level. The analysis emphasized comparing sequence characteristics of the recently reported contaminant strain BI-2023, identified in a bovine serum batch from China [46], with those of other strains. Viral sequences from diverse geographical regions, host species, and biological product contaminants were retrieved from international databases (DDBJ/EMBL/GenBank). The majority of analyzed strains originated from cattle (*Bos taurus*) (n = 310, 75%). Additional isolates were obtained from other *Bovidae* members, including buffaloes (*Bubalus bubalis*), yaks (*Bos grunniens*), and zebus (*Bos indicus*), as well as sheep (*Ovis aries*) and goats (*Capra hircus*). Further sequences were derived from alpacas (*Vicugna pacos*), Bactrian camels (*Camelus bactrianus*), deer (*Cervus elaphus*), pig (*Sus scrofa domesticus*), mousedeer (*Tragulus javanicus*), mosquitoes (*Culex neavei* and *Mansonia uniformis*) and human (*Homo sapiens*) infections.

Table 1. *Pestivirus bovis* species (BVDV-1) strains (n 126), representative of the different genotypes in the species, considered for sequence primary structure analysis and palindromic secondary structure characteristics at the RNA 5'-UTR (PNS method) or Npro according to primary structure. PNS nomenclature of genotypes is based on divergence in the genus. Nomenclature according to primary sequence analysis as Vilcek *et al.* [31] is indicated under parenthesis. Npro sequence strains accession numbers are indicated under parenthesis.

Genotype	Strain	Origin	Country	Year	Accession	Reference
a	NADL	Bovine	USA	1963	M31182 (M31182)	[155]
a	Singer	Bovine	USA	1993	L32875	[122]
a	SD-1	Bovine	USA		M96751 (M96751)	[12]
a	Weybridge	Sheep	UK		U65024	[156]
a	Oregon C24V	Bovine	USA	1993	L32876 (AF091605)	[122]
b1	Draper	Bovine	USA	1993	L32880	[122]
b1	BI-2023	Contaminant	China	2022	OR753412 (OR753412)	[46]
b1	Sanders	Bovine	USA		L20928	[85]
b2	24-15	Bovine	UK		AF298060	[31]
b2	Osloss	Bovine	USA	1965	M96687 (M96687)	[157]
3 (d)	Europa	Human	Belgium	1989	AB000898	[28]
3 (d)	F	Bovine	Austria		AF298065 (AF287284)	[31]
3 (d)	16 - 111	Bovine	France		AF298056	[31]
3 (d)	10JJ-SKR	Bovine	South Korea		(KC757383)	[158]
4 (b)	438/02	Bovine	Spain	2002	AY159540	[34]
4 (b)	PT42-03	Bovine	Portugal	2003	AY944293	[59]
5 (i)	23 - 13	Bovine	UK		Not deposited	[31]
5 (i)	23 - 15	Bovine	UK		AF298059 (AF287279)	[31]
5 (i)	58 - 1	Bovine	UK		(KF023454)	[88]
5 (i)	2541	Bovine	UK	2009	(JQ920342	[79]
6 (n)	So CP/75	Bovine	Japan	1975	AB359929 (AB105590)	[159]
6 (n)	Shitara-02-06	Bovine	Japan	2006	LC089876	[160]

Continued

7.1 (o)	JS10116	Pig	China	2010	JN248734	[87]
7.1 (o)	AQGN96B15	Bovine	Japan	1996	AB300691	[86]
7.1 (o)	Camel isolate 9	Bactrian camel	China	2010	JX276546	[37]
7.1 (o)	IS25CP/01	Bovine	Japan	2001	AB359931 (AB359931)	[159]
7.1 (o)	IS26/01ncp	Bovine	Japan	2001	(AB359932)	[159]
7.2 (o)	BJ09_24	Bovine	China	2009	HQ116550	[41]
7.2 (o)	S121	Bovine	China	2013	KF006960	[41]
7.2 (o)	S43	Bovine	China	2013	KF006959	[41]
7.3 (o, v)	BJ09-26	Bovine	China	2009	HQ116551	Zhang *et al.*, unpublished
7.3 (o, v)	EN-6	Bovine	China	2017	MN417813	[62]
7.3 (o, v)	EN-19	Bovine	China	2017	MN417826	[62]
7.3 (o, v)	T4-32	Bovine	China	2017	MN417862	[62]
7.3 (o, v)	T4-31-2	Bovine	China	2017	MN417919	[62]
7.3 (o, v)	GA190608	Bovine	China	2019	MT933204	[63]
7.3 (o)	HA2-12	Goat	China	2013	KP749802	[64]
7.3 (o)	JS12/02	Goat	China	2013	KP749794	[64]
7.3 (o)	XH-1	Bovine	China	2016	KY865374	Zhou and Wu, unpublished
7.3 (o)	MF-3	Bovine	China	2016	KY865371	Zhou and Wu, unpublished
7.3 (o)	HY-3	Bovine	China	2016	KY865366	Zhou and Wu, unpublished
7.3 (o, v)	HN1814	Bovine	China	2018	MN442377	[65]
7.3 (o)	HN1626	Bovine	China	2016	MN442366	[65]
7.3 (o)	HN1864	Bovine	China	2018	MN442381	[65]
7.3 (o, v)	HB-03	Bovine	China	2017	ON901785	[161]
8 (c)	Bega	Bovine	Australia		AF049221 (AF049221)	Mackintosh *et al.*, unpublished
8 (c)	CRFK	Contaminant	Japan	1995	D50814	[107]
8 (c)	Manasi	Bovine	China	2006-08	EU159702	[42]
8 (c)	519	Bovine			(AF144464)	[162]
8 (c)	Deer-NZ1	Deer	New Zealand		(U80903)	[106]
9 (h)	G	Bovine	Austria	1998	AF208066 (AF287285)	[31]
9 (h)	KM	Bovine	Slovakia		AF298068	[31]
9 (h)	CH-SM09/20	Bovine	Switzerland		(AY895007)	[163]

Continued

10 (q)	SD0803	Pig	China	2008	JN400273 (JN400273)	[78]
10 (q)	Camel-6	Bactrian camel	China	2010	KC695810 (KC207072)	[164]
11 (e)	3186V6	Bovine	Italy		AF298062 (AF287282)	[31]
11 (e)	10 - 84	Bovine	France	1984	AF298054	[31]
11 (e)	26-V639	Bovine	France		(AF287282)	[31]
11 (e)	CH-Maria	Bovine	Switzerland		(AY895003)	[44]
12.1 (f, s)	22146/81	Bovine	Germany	1981	AJ304376	[35]
12.1 (f, s)	UM136/08	Bovine	Italy	2008	LM994673	[11]
12.1 (f, s)	Mousedeer	Mousedeer	Denmark	2002	AY158154	[165]
12.1 (f, s)	2561	Bovine	UK	2009	JQ920287	[79]
12.2 (f, r)	11207/98	Bovine	Germany	1998	AJ304390	[35]
12.2 (f, r)	4998/89	Bovine	Germany	1989	AJ304385	[35]
12.2 (f, r)	51/06	Bovine	Poland	2006	JN715039	[10]
12.2 (f, r)	CA/181/10	Bovine	Italy	2010	(LM994672)	[11]
12.2 (f, r)	VE/245/12	Bovine	Italy	2012	(LM994671)	[11]
12.2 (f, r)	79/11	Bovine	Italy	2011	KY040384	[97]
13 (j)	17P	Bovine	Argentina	1991	AF244954	[166]
13 (j)	KS86-1ncp	Bovine	Japan	2002	AB042713 (AB078950)	[167]
13 (j)	Deer	Deer	UK	1986	AB040132	[106]
13 (j)	Deer-GB1	Deer	New Zealand		(U80902)	[162]
1.14 (r)	TR70	Bovine	Türkiye	2007	MG670547 (KF154779)	[61]
1.14 (r)	TR73	Bovine	Türkiye		(KF154777)	[104]
1.14 (r)	TR75	Bovine	Türkiye	2007	MG670549 (KF154778)	[60]
15.1 (m)	ZM-95	Pig	China	1995	AF526381 (AF526381)	[168]
15.1 (m)	BJ1305	Bovine	China	2013	KF925505 (KF925522)	[39]
15.1 (m)	TJ0801	Bovine	China	2008	GU120255 (GU120262)	[40]
15.2 (m)	HB-060111	Bovine	China	2011	KJ578822	[36]
1.16 (l)	TR-2007-Gu-175454-4695	Bovine	Türkiye	2007	EU716150	[105]
1.16 (l)	TR16	Bovine	Türkiye	2007	MG670546 (EU163964)	[60]
1.16 (l)	TR27	Bovine	Türkiye		(EU163975)	[169]
1.16 (l)	TR29	Bovine	Türkiye		(EU163977)	[169]
1.16 (l)	TR72	Bovine	Türkiye	2007	MG670548 (KF154776)	[60]
17 (f)	S	Bovine	Austria		Not deposited	[31]
17 (f)	J	Bovine	Austria	1998	AF298067 (AF287286)	[31]
17 (f)	W	Bovine	Austria	1998	AF298073 (AF287290)	[31]
18 (p)	BJ0701	Bovine	China	2007	GU120247 (GU120259)	[40]
18 (p)	BJ0702	Bovine	China	2007	GU120248 (GU120260)	[40]

Continued

18 (p)	BJ0703	Bovine	China	2007	GU120249 (GU120261)	[40]
18 (p)	TJ06	Bovine	China	2006	GU120246	[40]
18 (p)	XY-3	Goat	China	2013	KP749796	[64]
19 (w)	T6-18	Bovine	China	2017	MN417892 (MN417943)	[62]
19 (w)	T6-20	Bovine	China	2017	MN417893 (MN417944)	[62]
20 (g)	A	Bovine	Austria	1998	AF298064 (AF287283)	[31]
20 (g)	L	Bovine	Austria	1998	AF298069 (AF287287)	[31]
21 (v)	TR/Elz-4-2021	Bovine	Türkiye	2020	MZ686434	Abayli, unpublished
21 (v)	TR-Erz-Pst8	Bovine	Türkiye	2016-17	MG973223	[103]
21 (v)	IR-Shiraz-322	Bovine	Iran	2013	LC053995	Seyfi Abad Shapouri *et al.*, unpublished
21 (a, v)	IRTV1	Bovine	Iran	2019	MW431322	Nazeri *et al.*, unpublished
21 (v)	TR-Elz-Pst16	Bovine	Türkiye	2016-17	MG973231	[103]
21 (v)	TY8723	Bovine	Türkiye	2017	MH673456 (MH75872)	[102]
22 (l, x)	71-03	Bovine	France	2005	KF205294 (KF205326)	[170]
22 (l, x)	CH-01-08	Bovine	Switzerland	1995-06	EU180024 (EU180033)	[44]
23.1 (u)	130/15-4215	Bovine	Italy	2015	KY085998	[97]
23.1 (u)	130/15-5364	Bovine	Italy	2015	KY085999	[97]
23.1 (u)	M31182	Yak	China	2010	JQ799141 (JQ799141)	Sun *et al.*, unpublished
23.1 (u)	441/09	Bovine	Italy	2009	KY040367 (KY040435)	[97]
23.2 (u)	GXBH-EB34	Buffalo	China	2013	KJ578813	[36]
23.2 (u)	JS-00108	Bovine	China	2009	KJ578848	[36]
23.2 (u)	QHQL-252	Yak	China	2012	KJ578884	[36]
23.2 (u)	LN309-5	Bovine	China	2012	KJ578803	[36]
23.2 (u)	GXCZ-FB22	Buffalo	China	2013	KJ578807	[36]
23.2 (u)	GXLZ-BB4	Buffalo	China	2013	KJ578814	[36]
23.2 (u)	HB-090166	Bovine	China	2011	KJ578836	[36]
23.2 (u)	JS-03148	Bovine	China	2009	KJ578850	[36]
23.2 (u)	JS-03198	Bovine	China	2009	KJ578851	[36]
23.2 (u)	JS-04138	Bovine	China	2009	KJ578852	[36]
23.2 (u)	NMG311-20	Bovine	China	2012	KJ578866	[36]
24 (k)	Rebe	Bovine	Switzerland		AF299317	[163]
24 (k)	SuwaCp	Bovine	Switzerland	1999	AF117699	[171]
24 (k)	SuwaNcp	Bovine	Switzerland	2000	KC853440	[172]
24 (k)	CH-Bohni	Bovine	Switzerland		(AY894997)	[163]
24 (k)	CH-Suwa	Bovine	Switzerland		(AY894998)	[163]
25 (p)	S153	Bovine	China	2013	KF006964	[41]

In this study, 126 BVDV-1 strains (**Table 1**), representing the reference genotypes of the species and previously evaluated using the PNS method [47]-[49], were included to clarify the clustering within the species. In addition, 286 *Pestivirus* sequences of genotype b of *Pestivirus bovis* (121 of sub genotype b1 and 165 of sub genotype b2) were analyzed at the RNA 5'-UTR level using the PNS method to assess clustering within genotype b. Detailed information on the *P. bovis* genotype b strains are provided in **Table S1**.

2.2. Analysis of the Primary and Secondary Sequence Structure

Nucleotide cDNA sequences at the 5'-UTR and Npro of the 43 *Pestivirus bovis* BVDV-1, representative of 25 genotypes of the species, were aligned using the Clustal X software [50]. The nucleotide sequences were evaluated to identify consensus motifs, relevant to positions from 123 to 890 for 5'-UTR and Npro regions of the *P. bovis* reference strain NADL. Subsequently, phylogenetic trees based on the 5'-UTR and Npro genomic regions were constructed by using the neighbour-joining method [51].

The assessments of divergence among strains, based on qualitative and quantitative variations in palindromic nucleotide pairings, by analyzing key secondary structure regions in the viral RNA 5'-UTR, were used for taxonomic segregation. Specifically, the three variable regions (V1, V2, and V3) were examined according to the PNS genotyping method [21] [22]. For each strain, the complete 5'-UTR sequence was modelled to predict secondary structures, with palindromic IRES variable loci identified and analyzed separately from the remainder of the sequence. Secondary structures were predicted using the Genetyx-Mac version 10.1 software (Software Development Co., Tokyo, Japan), implementing the algorithm of Zuker and Stiegler [52] and computing minimum free energy values following the method of Freier *et al.* [53]. Additionally, the PNS software (version 2.0) [47], developed in C# and designed for *Pestivirus* sequence genotyping, was used to generate secondary structures adapted to perform alignments and evaluate genetic distances. The logic flow applied for test sequence characterization in the PNS software followed a schematic structure through an algorithmic protocol for the identification of the three variable loci in the nucleotide sequence primary structure. The functions responsible for identifying the V1, V2, and V3 variable loci in the nucleotide sequence primary structure and subsequently to construct their secondary structures required great attention and started from the V3 locus. The procedure for constructing the V3 locus intruded a selection logic that prioritizes the most stable structure, characterized by a greater number of strong nucleotide bindings and a smaller number of base pairs. The program specific computational workflow during the identification of the correct V3 locus was implemented considering that, among pestiviruses, the distance between V2 and V3 is typically three nucleotides, and occasionally two nucleotides, whereas in the case of *P. braziliense* the distance may reach five nucleotides. In summary, if *P. braziliense* markers are detected, the program initiates the V3 construction procedure using distances of 2, 3, and 5 nucleotides from V2. If these markers are absent, the construction of V3 is restricted to distances of 2 and 3 nucleotides. In the first scenario, the procedure generates three distinct groups of V3 candidates, each corresponding to a specific distance from V2 and containing nine possible V3 structures. In the second scenario, only two groups of V3 candidates are generated. The most stable V3 structure is then selected among the groups. In cases where structures from different groups show equivalent stability, preference is given to the structure with the smaller number of constituent nucleotides. The procedure involves searching for matches with species markers using the selected V3. If species identification fails, the V3 structure is replaced with another candidate chosen from the remaining V3 structures within the generated groups, and the search for species markers is repeated. If the species is successfully identified, the program proceeds with genotype determination. If species identification remains unsuccessful, the program terminates the analysis and reports a negative result. Genotype determination followed a similar iterative procedure.

Prior performing divergence computing by alignment, as additional exclusion criterion applied to the strain sequences, only secondary structures showing unique base pair combinations were retained for genotyping procedure secondary sequence alignment and computing of divergence values. The other strains showing secondary structure sequence identity or non-relevant base pair changes as G-C pairing instead of a tolerated G * U were excluded. Base-pair divergence between aligned secondary structure sequences was counted for each variation of nucleotides composing the base pairing at each different position in the three loci. The bulges were equally scored as a single bp variation. Only the tolerated pairings G * U or U * G were not considered variations when compared to G-C or C-G, respectively.

The clustering of *P. bovis* strains into genotypes, as well as the relationships among genotypes within the species, was assessed based on nucleotide base-pair variations in the three palindromic loci in the 5'-UTR. Genotypes were defined by specific base-pair (bp) combinations at relatively conserved positions and ranked according to their degree of divergence within the genus, using the most common sequences (reference groups) as a baseline. The most frequent base-pair patterns, representing the predominant sequences, formed a core group, the most homogeneous within the genus. Accordingly, genotype numerical nomenclature reflected the relative level of divergence at species level. Genotype homology was determined by observing shared base-pairings across the three palindromes. Cross-group comparisons were performed by calculating divergence percentages, allowing the identification and quantification of both intra-genotype heterogeneity and inter-genotype genetic distances, applying divergence limit values for genotype and sub genotype determination (9 and 6 base pairs, respectively).

For the verification of the taxonomic clustering at genus level, strains were compared with the secondary structure characteristics of other *Pestivirus* species [47]-[49], reaching a total of 1497 strains analyzed by PNS procedure, a comprehensive number of observations useful for reliable comparison purposes. The genotyping of *P. bovis* strains obtained through PNS was compared with other classifications, as reported by other authors for the same strains, named on the base of primary structure evaluation [31] and its subsequent adaptations. Sequences characteristics have been evaluated to explore links with geographic distribution and animal host preferences. Secondary palindromic structure sequences were further considered for hypothetical nucleotides related to virulence degree in *Pestivirus* strains [54] [55].

3. Results

The primary structure alignment of *P. bovis* 5'-UTR nucleotide sequences from selected strains representative of 25 genotypes of the species allowed to identify consensus nucleotides referred to the positions 123 - 370, corresponding to the 5' untranslated and non-coding region of *P. bovis* reference strain NADL (**Figure 1**). Similar results were obtained through the consideration of the Npro coding genomic region sequence fragments (positions 384 - 890 of *P. bovis* reference strain NADL) (**Figure S1**). For the Npro sequence evaluation, due to the lack of specific sequences, genotypes defined as 4 and 25 were not represented. Phylogenetic trees were constructed from 5'-UTR and Npro alignments of representative strains at the species level, for an estimation of their taxonomic status according to primary sequence structure, allowing a visualization of the identified genogroups within the species (**Figure 2** and **Figure 3**).

The 5'-UTR sequence secondary structures were constructed and considered for further alignment to compute base pairing divergence for the genotyping procedure according to the PNS method. Nucleotide sequences resulted conserved and IRES palindromes were obvious in the region. The 5'-UTR secondary structure of the strain BI-2023 showed a minimum free energy value of −73.99 Kcal/mol. The result of the construction of the variable palindromes of the strain BI-2023 is presented in **Figure 4**. In case of the rare case of difficulties in the construction of palindromes due to sequences showing lower quality of nucleotide translation (few missing nucleotides, indicated by alphabetic letters), the V1, V2 and V3 variable loci in the 5'-UTR RNA were identified in the primary structure of the nucleotide sequence referring to the *P. bovis* species reference strain NADL (**Table 2**).

Strains showing secondary structure sequence identity or non-relevant base pair changes as variations were excluded (56 strains of *P. bovis* genotype b, out of 286) (**Table 3**). For example, the three PNS reference variable loci of the strain BI-2023 as well as PT16-03 [AY944272] and CP7 [U63479] resulted identical to those of strain CD89 [ALIGN_000012] [32]. Similarly, the seven mosquito strains, ARD407607 [PV346708], ARD408029 [PV346715], ARD408157 [PV346707], ARD410730 [PV346698], ARD410734 [PV346699], ARD410758 [PV346716] and ARD410779 [PV346711] were identical to KA91 [AB019684], isolated from cattle in Japan in 1999, showing only a non relevant variation G-C pairing instead of a tolerated G * U in V2/7. These strains were excluded from the selection of relevant base pair combinations for genotyping procedure secondary sequence alignment and computing of divergence values, applying the exclusion criterion of identity (**Table 4**, **Table S2**).

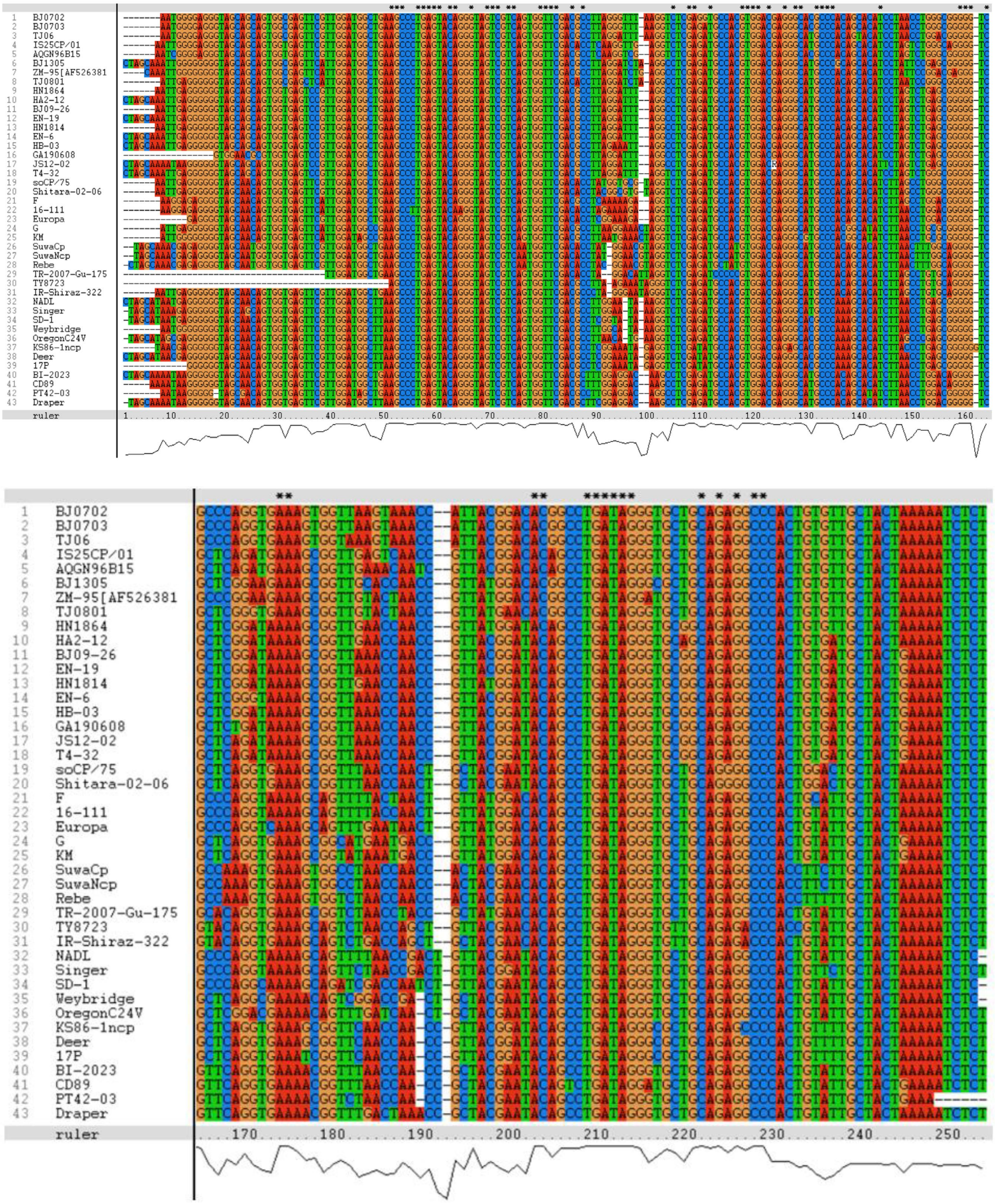

Figure 1. Nucleotide alignment of the 43 *Pestivirus bovis* (BVDV-1) cDNA sequences at the 5'-UTR, representative of 25 genotypes of the species, constructed by Clustal X software [50]. Consensus nucleotides are shown as inverted characters. The nucleotide sequence numbers are given from a consensus alignment, which are relevant to positions 138 - 377 of the *Pestivirus bovis* reference strain NADL. (*) highest consensus motifs among sequences; (-) spaces between adjacent nucleotides introduced for maximum alignment.

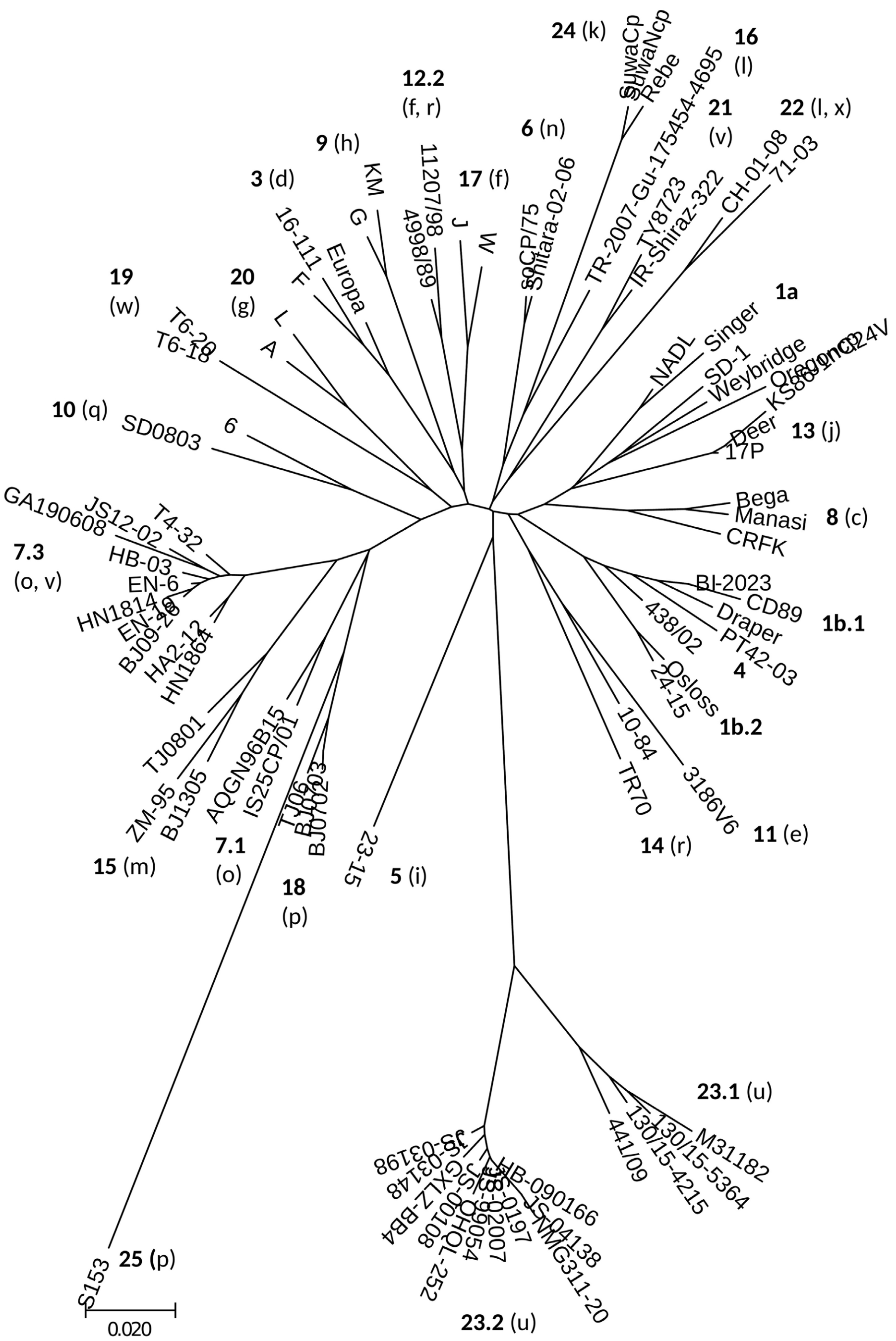

Figure 2. Phylogenetic tree based on the 5'-UTR comparison, suggesting a taxonomic position of the *P. bovis* strains in the genus *Pestivirus*. Strain NADL [M31182] is the reference for *P. bovis* genotype a. Strains Draper [L32880], BI-2023 [OR753412] and CD89 [ALIGN_000012] are references for *P. bovis* genotype b, sub genotype b1. Strain Osloss [M96687] is reference for *P. bovis* genotype b, sub genotype b2. Strains Europa [AB000898], 438/02 [AY159540], 23 - 15 [AF298059], and so CP/75 [AB042661] are references for genotypes 3 to 6. Strains JS10116 [JN248734] and BJ09-26 [HQ116551] are references for the sub genotypes 7.1 and 7.3 of the *P. bovis* genotype 7. CRFK [D50814], KM [AF298068], SD0803 [JN400273], 10 - 84 [AF298054], 11207/98 [AJ304390], 17P [AF244954], TR70 [MG670547], ZM-95 [AF526381], TR-2007-Gu-175454-4695 [EU716150], J [AF298067], and TJ06 [GU120246] are references for genotypes 8 to 18. Strains T6 - 18 [MN417892], A [AF298064] and TY8723 [MH673456] are references for genotypes 19, 20 and 21. Strains CH-01-08 [EU180024], M31182 (Yak) [JQ799141], Rebe [AF299317], and S153 [KF006964] are references for genotypes 22 to 25. Scale bar indicates 10 nucleotide substitutions per 100 nucleotides. Nomenclature of identified genotypes is based on divergence in the genus. Clustering according to primary structure analysis by depositors is indicated under parenthesis.

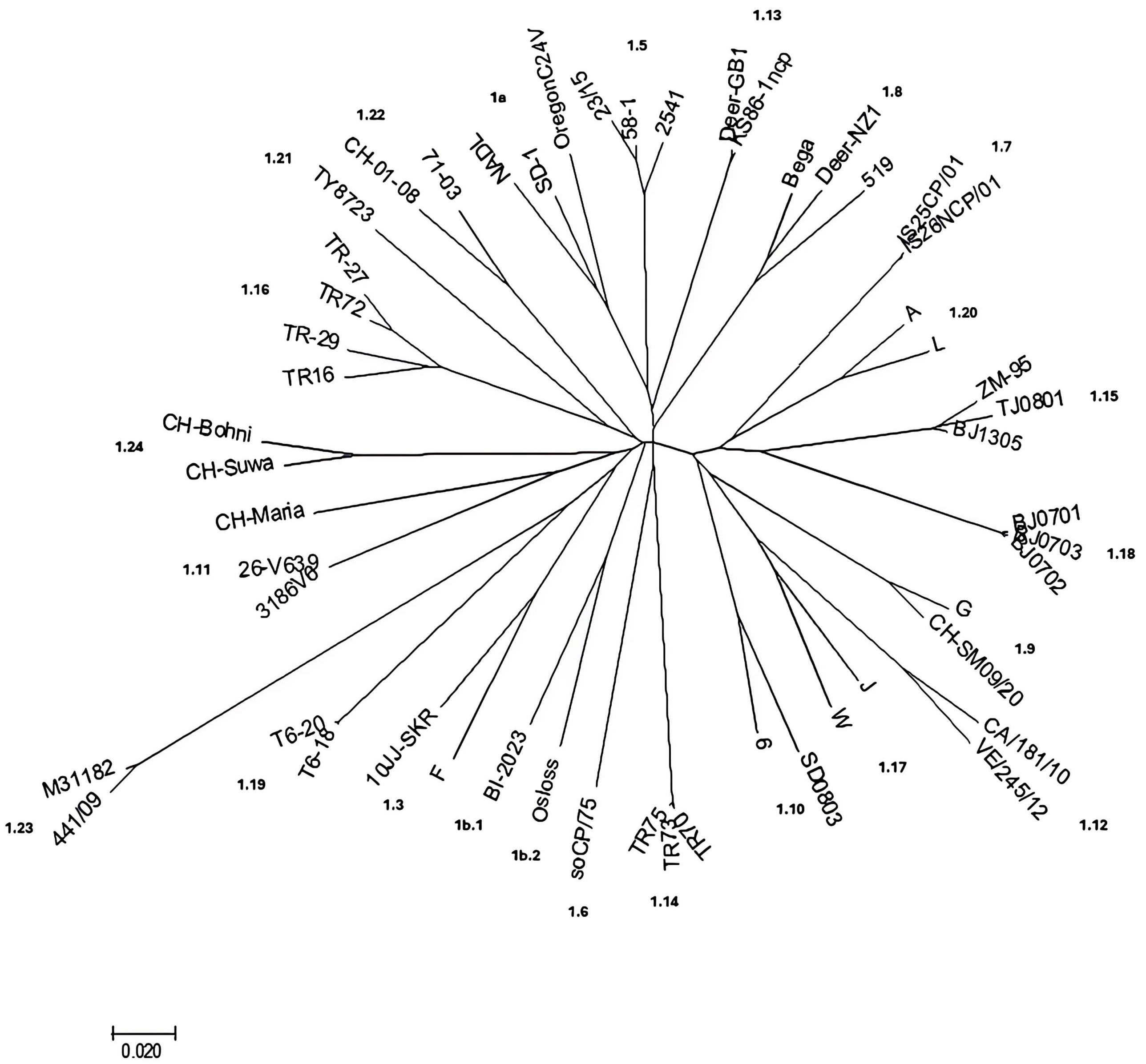

Figure 3. Phylogenetic tree based on the Npro sequence comparisons suggesting a taxonomic position of the *P. bovis* strains in the *Pestivirus* genus. Strains NADL [M31182], Oregon C24 V [AF091605] and SD-1 [M96751] are the references for the P. bovis genotype a. Strains BI-2023 [OR753412] and Osloss [M96687] are the references for the P. bovis genotype b, sub genotypes b1 and b2, respectively. Strains F [AF287284], 10JJ-SKR [KC757383], 23/15 [AF287279], 58-1 [KF023454], 2541 [JQ920342] and so CP/75 [AB105590] are references for genotypes 3, 5 and 6. Strains IS25CP/01 [AB359931], IS26 NCP/01 [AB359932], Bega [AF049221], 519 [AF144464], Deer-NZ1 [U80903], G [AF287285], CH-SM09/20 [AY895007], SD0803 [JN400273], isolate 6 [KC207072], 3186V6 [AF287282], 26-V639 [AF287282] and CH-Maria [AY895003] are references for genotypes 7 to 11. Strains CA/181/10 [LM994672] and VE/245/12 [LM994671] are references for genotype 12. Strains Deer-GB1 [U80902] and KS86-1ncp [AB078950] are references for the genotype 13. Strains TR70 [KF154779], TR73 [KF154777] and TR75 [KF154778] are references for genotype 14. Strains BJ1305 [KF925522], TJ0801 [GU120262] and ZM-95 [AF526381] are references for the genotype 15. Strains TR16 [EU163964], TR27 [EU163975], TR29 [EU163977] and TR72 [KF154776] are references for the genotype 16. Strains J [AF287286], W [AF287290], BJ0701 [GU120259], BJ0702 [GU120260], BJ0703 [GU120261], T6 - 18 [MN417943], T6-20 [MN417944], A [AF287283], L [AF287287], TY8723 [MH75872], CH-01-08 [EU180033], 71-03 [KF205326], M31182 [JQ799141], 441/09 [KY040435], CH-Bohni [AY894997] and CH-Suwa [AY894998] are references for genotypes 17 to 24. Genotypes defined by PNS 1.4 and 1.25 are not represented. Distances were computed using Clustal X [50], version 1.8, using the neighbor-joining method [51]. Scale bar indicates 10 nucleotide substitutions per 100 nucleotides.

V1	V2	V3
19 a g	12 g	10 a
18 g g	11 g g	09 a-c
17 g a	10 g*t	08 t-c
16 t c	09 A c	07 t-a
15 t-a	08 c-g	06 t-a
14 t-a	07 a-t	05 g-c
13 c-g	06 g*t	04 g-c
12 g-c	05 g-c	03 c-g
11 c c	04 t-a	02 a c
10 a-t	03 c-g	01 a-t
09 g-c	02 c-g	
08 c-g	01 a-t	
07 t-ag		
06 . a		
05 t*gt		
04 g-c		
03 g-c		
02 t-a		
01 g-c		

Figure 4. Secondary structure of the V1, V2 and V3 palindromic loci identified in the entire 5'-UTR sequence of *Pestivirus bovis* species genotype b strain BI-2023 [accession OR753412], predicted according to the algorithm of Zuker and Stiegler [52], using the Genetyx-Mac version 10.1 program package (Software Development Co., Ltd., Tokyo, Japan). The minimum free energy was calculated by the method of Freier *et al.* [53]. The three relevant variable regions identified in the sequence were referred to the PNS procedure [21] [22] and displayed as palindromes in the secondary structure by PNS software version 2.0 [47]. Base pairings characteristic to *Pestivirus* genus (PNS genus specific), *P. bovis* species (PNS species specific), genotype b and sub genotype b1 were identified in the sequence. Distance between V2 and V3: 3 nucleotides. The numbering of the base-pairings starts from the bottom of the palindromes in the secondary structure. (-) Watson-Crick nucleotide pairings; (*) tolerated nucleotide pairings.

Table 2. Identification of V1, V2 and V3 variable loci in the 5'-UTR RNA nucleotide sequence primary structure of the *Pestivirus bovis* species (BVDV-1) reference strain NADL. Variable loci are underlined. V1 is the first in the sequence, followed by a conserved 23 nucleotide V2 and a V3 at a distance of three nucleotides. Palindromic base pairings common to *Pestivirus* genus are highlighted in violet. Palindromic base pairings common to *P. bovis* species are highlighted in blue.

Position	Nucleotide sequence
181	gtacagggta gtcgtcagtg gttcgacgcc ttggaataaa ggtctcgaga tgccacgtgg
241	acgagggcat gcccaaagca catcttaacc tgagcggggg tcgcccaggt aaaagcagtt
301	ttaaccgact gttacgaata

Table 3. *Pestivirus bovis* species (BVDV-1), genotype b strains showing sequence identity at the level of the three variable loci.

Sub genotype	Reference strain	Identical strains (non-relevant variations)
b.1	133/02	3310/01; 4092/00.
	1/A/00	2/A/00; 3/A/00.
	2032/01	2708/01.
	Antila	Dorado.
	CD89	BI-2023; CP7; PT16-03.
	Culi4	YVD947.
	Culi6	10A/LC/97; 4325/01; ZVD278.
	F1-4/BR	CH565; Tunisia 294.
	FLK	2110C; 368/02; HeLa; Ind 446; Ind S 1168; Ind S 1171; Ind S 1181; Ind S 1455 (V1/9 G * U); MOLT-4; Q713; WiDr.
	MDBK	U937.
	P	4050/00 (V1/12 G * U).
	S21	76865.
	Vero	CV-1; MDCK.
b.2	3251/01	Lamspringe738.
	4382/01	4629/01 (V2/7 G * U).
	42M	24/15; Buffalo 2; Influenza2 (V1/19 G-C); M346T96 (V1/19 G-C); T; U.
	7546	7548.
	i13	66.1; 66.6; i53.
	i89	i393.
	KA91	ARD407607 (V2/7 GC); ARD408029 (V2/7 GC); ARD408157 (V2/7 GC); ARD410730 (V2/7 GC); ARD410734 (V2/7 GC); ARD410758 (V2/7 GC); ARD410779 (V2/7 GC); E5-158/US; H3-193/US; K2-1/CO.
	TFB	TFB2.
	ZVR711	1248/01.

Table 4. Alignment of *Pestivirus bovis* species (BVDV-1) genotypes variable loci 5'-UTR RNA secondary structure sequences, segregated according to types of base pair combinations. The different types are ordered according to increasing divergence in the genus (*), expressed in number of divergent base pairs, with reference to most common base pairs in the prevalent positions. Highly conserved base pair positions are excluded. Y: G or U. HV: highly variable. Sub genotype b1 strains BI-2023, CP7 and PT16-03, identical to CD89, and sub genotype b2 strains ARD407607, ARD408029, ARD408157, ARD410730, ARD410734, ARD410758 and ARD410779, identical to KA91, were excluded from the selection of relevant base pair combinations for genotyping procedure secondary sequence alignment and computing of divergence values.

Variable locus										**V1**										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	(*)
Prevalent base pairs	**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**	
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**	
Genotype BVDV-1a																				0.83
Singer	.										CG		UA		GU	AA	-	-	-	1
NADL	.										CG		UA		GU	AA	-	-	-	1
BVDV-1b1																				0.34
Draper	.										UA		CC		GG	AG	-	-	-	1
Sanders	.					AA					UA		UU		GG	AG	-	-	-	1
BI-2023											UA		UC		GG	AG	-	-	-	1
CP7											UA		UC		GG	AG	-	-	-	1
CD89											UA		UC		GG	AG	-	-	-	1
PT16-03											UA		UC		GG	AG	-	-	-	1
BVDV-1b2																				0.89
Osloss	.				G						UA		CC		UG	GU	-	-	-	1
KA91	.										UA		UC		GG	AG	-	-	-	0
ARD407607	.										UA		UC		GG	AG	-	-	-	0
ARD408029	.										UA		UC		GG	AG	-	-	-	0
ARD408157	.										UA		UC		GG	AG	-	-	-	0
ARD410730	.										UA		UC		GG	AG	-	-	-	0
ARD410734	.										UA		UC		GG	AG	-	-	-	0
ARD410758	.										UA		UC		GG	AG	-	-	-	0
ARD410779	.										UA		UC		GG	AG	-	-	-	0

Variable locus	**V2**								
Position	1	2	3	4	5	6	7	9	(*)
Prevalent base pairs		**YG**	**YG**	**UA**	**GY**			**GC**	
BVDV1		**CG**	**CG**	**UA**	**GC**			**GC**	
Genotype BVDV-1a									**0.83**
Singer	AU					AC	GC	.	1
NADL	AU					AC	GC	.	1
BVDV-1b1									**0.34**
Draper	AU					GU	AU	.	1
Sanders	AU					GU	AU		1
BI-2023	AU					GU	AU	AC	1
CP7	AU					GU	AU	AC	1
CD89	AU					GU	AU	AC	1
PT16-03	AU					GU	AU	AC	1
BVDV-1b2									**0.89**
Osloss	AU					AU	GU	.	1
KA91	AU					GU	GU	.	0
ARD407607	AU					GU	GU	.	0
ARD408029	AU					GU	GU	.	0
ARD408157	AU					GU	GU	.	0
ARD410730	AU					GU	GU	.	0
ARD410734	AU					GU	GU	.	0
ARD410758	AU					GU	GU	.	0
ARD410779	AU					GU	GU	.	0

Variable locus	V3										
Position	1	2	3	4	5	6	7	8	9	10	(*)
Prevalent base pairs	**AU**		**CG**		**GY**	**UA**				**A**	
BVDV1	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
Genotype BVDV-1a											**0.83**
Singer	.	GU		AU			UG	CC	UC	AA	1
NADL	.	GU		AU			UG	UC	UC	AA	1
BVDV-1b1											**0.34**
Draper	.	AC		GC				UA	GU	AC	1
Sanders	.	AC		GC				UC	AC	.	1
BI-2023		AC		GC				UC	AC		1
CP7		AC		GC				UC	AC		1
CD89		AC		GC				UC	AC		1
PT16-03		AC		GC				UC	AC		1
BVDV-1b2											**0.89**
Osloss	.	GC		GC				UC	AC	.	1
KA91	.	AC		GC				UC	AC	.	0
ARD407607	.	AC		GC				UC	AC	.	0
ARD408029	.	AC		GC				UC	AC	.	0
ARD408157	.	AC		GC				UC	AC	.	0
ARD410730	.	AC		GC				UC	AC	.	0
ARD410734	.	AC		GC				UC	AC	.	0
ARD410758	.	AC		GC				UC	AC	.	0
ARD410779	.	AC		GC				UC	AC	.	0

The nucleotide base pair combinations of the predicted secondary structures of the three variable loci were aligned for comparison, identifying genotypic clusters in the *P. bovis* species (Table 5). The identified *P. bovis* species genotypes were ordered on the basis of their increased divergence in the genus, referring to most common nucleotide pairings. Subsequent analytical classification performed by secondary structure alignment of *P. bovis* species genotype b, based on specific sub type base pairing combinations (b.1: V2/7 AU; b.2: V2/7 GU or GC-GY), revealed the correspondence of the sequence characteristics of strain BI-2023 with others clustered within the sub genotype b1, sharing the common specific base pairing adenine uracil in position 7 of V2 locus. Details of the 5'-UTR secondary structure sequence alignment of *P. bovis* strains belonging to the genotype b, segregated into sub genotypes b1 and b2, respectively, are presented in **Table S2**. At the species and genotype levels, the taxonomical evaluation of the considered strains, determined by secondary structure analysis, matched with the phylogenetic trees constructed from the alignment of primary sequence structure of the 5'-UTR and Npro sequences of representative strains from the identified genogroups, as well partially to the clustering obtained by full genome evaluation. The two virulence related nucleotides in *Pestivirus* were identified on the basis of the *P. bovis* strain Osloss sequence, at position 219 and 278, in relation with primary and secondary palindromic structures. In the variable loci of the BI-2023 strain, N1 and N2 were adenine in V1/19 and uracil in V2/4, respectively.

Table 5. Alignment of *Pestivirus bovis* species (BVDV-1) genotypes variable loci 5'-UTR RNA secondary structure sequences, segregated according to types of base pair combinations. The different types are ordered according to increasing divergence in the genus (*), expressed in number of divergent base pairs, with reference to most common base pairs in the prevalent positions. Highly conserved base pair positions are excluded. Y: G or U. HV: highly variable.

Variable locus	**V1**																		
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22
Prevalent base pairs	**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**
BVDV-1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**
Genotype BVDV-1a																			
Singer	.										CG		UA		GU	AA	-	-	-
NADL	.										CG		UA		GU	AA	-	-	-
BVDV-1b1																			
Draper	.										UA		CC		GG	AG	-	-	-
Sanders	.					AA					UA		UU		GG	AG	-	-	-
BI-2023											UA		UC		GG	AG	-	-	-
BVDV-1b2																			
Osloss	.				G						UA		CC		UG	GU	-	-	-
BVDV-1.3 (D)																			
Europa	.								AU		CG		CA	GG	GA	AA	-	-	-
BVDV-1.4																			
438/02	.										CG		CC		GG	AG	-	-	-
BVDV-1.5 (I)																			
23-13	.										CG		UA		GA	UC	-	-	-
BVDV-1.6 (N)																			

Continued

so CP/75	.								AU		CG		AU	UG	GC	GG	U	-	-
BVDV-1.7 (O)																			
BVDV-1.7.1 (O)																			
JS10116	.										CG		CG	AU	AU	GG	-	-	-
AQGN96BI5	.										CG		CG	AC	AU	GG	-	-	-
Camel isolate 9	.										CG		CG	GU	AU	GG	-	-	-
IS25CP01	.								AU		CG		CG	AU	AU	GG	-	-	-
BVDV-1.7.2 (O)																			
BJ09_24	.										CG		UU	AC	GU	GA	-	-	-
S43	.										CG		UU	AC	GU	GA	-	-	-
S121	.										CG		UU	AC	GU	GA	-	-	-
Genotype BVDV-1.7.3 (O, V)																			
T4-32 (V)	.										CG		UU	AU	GU	GA	-	-	-
T4-31-2 (V)	.										CG		UU	AU	GU	GA	-	-	-
JS12/02 (O)	.										CG		UU	AU	GU	GA	-	-	-
EN-6 (V)	.										CG		UU	AU	GU	GA	-	-	-
GA190608 (V)	.										CG		UU	AU	GU	GA	-	-	-
BJ09_26 (V)	.										CG		UU	AU	GU	GA	-	-	-
HY-3 (O)	.										CG		UU	AC	GU	GA	-	-	-
HB-03 (V)	.										CG		UU	AU	GA	AA	-	-	-
HN1626 (O)	.										CG		UU	AU	GU	GA	-	-	-
XH-1 (O)	.										CG		UU	AU	GU	GA	-	-	-
MF-3 (O) (V1/4 AC)	.										CG		UU	AU	GU	GA	-	-	-
BVDV-1.8 (C)																			
Bega	.								AC		CG		UA	GC	GA	AA	AG	-	-
BVDV-1.9 (H)																			
KM	.										CG		UU	AC	AA	UA	GA	-	-
BVDV-1.10 (Q)																			
SD0803	.										CG	AA	GG	AU	GA	-	-	-	-
BVDV-1.11 (E)																			
26-V639	.										CG		AU		GU	AC	-	-	-

Variable locus	V2								
Position	1	2	3	4	5	6	7	9	(*)
Prevalent base pairs		**YG**	**YG**	**UA**	**GY**			**GC**	
BVDV-1		**CG**	**CG**	**UA**	**GC**			**GC**	
Genotype BVDV-1a									**0.83**
Singer	AU					AC	GC	.	1
NADL	AU					AC	GC	.	1
BVDV-1b1									**0.34**
Draper	AU					GU	AU	.	1
Sanders	AU					GU	AU		1
BI-2023	AU					GU	AU	AC	1
BVDV-1b2									**0.89**
Osloss	AU					AU	GU	.	1
BVDV-1.3 (D)									**0.57**
Europa	AU					GC	AC	.	0
BVDV-1.4									**0**
438/02	AU					AU	AU	.	0
BVDV-1.5 (I)									**0.75**
23-13	GU			CA		GU	GC	.	1
BVDV-1.6 (N)									**0.86**
so CP/75	GU					GU	GC	.	1
BVDV-1.7 (O)									**1.66**
BVDV-1.7.1 (O)									**1.25**
JS10116	GU	UA				GU	GC	.	1
AQGN96BI5	GU	UA				GU	GC	.	1
Camel isolate 9	GU	UA				GU	GC	.	1
IS25CP01	GU	UA				GU	GC	AC	2
BVDV-1.7.2 (O)									**1.33**
BJ09_24	AU	UA				GU	GC	.	1
S43	AU	UA				GU	GC	.	1
S121	AU	UA				GU	GC	AC	2
Genotype BVDV-1.7.3 (O, V)									**1.91**
T4-32 (V)	GU	UA				GU	GC	.	1
T4-31-2 (V)	GU	UA				GU	GC	.	2
JS12/02 (O)	GU	UA				AU	GC	.	1

Continued

EN-6 (V)	GU			UG		AU	GC	.	1
GA190608 (V)	GU	UA		UU		AU	GC	.	2
BJ09_26 (V)	GU	UA		UG		AU	GC	.	2
HY-3 (O)	GU	UA		UG		AU	GC	.	2
HB-03 (V)	GU	UA		UG		AU	GC	.	2
HN1626 (O)	GU	UA		UG		AU	GC	.	2
XH-1 (O)	GU	UA		UG		AU	GC	.	2
MF-3 (O) (V1/4 AC)	GU	UA		UG	GA	AU	GC	.	4
BVDV-1.8 (C)									**1.90**
Bega	AU					GU	GC	.	1
BVDV-1.9 (H)									**1.46**
KM	AU					CU	GC	.	1
BVDV-1.10 (Q)									**1.86**
SD0803	AC	UA				GU	GC	.	1
BVDV-1.11 (E)									**1.58**
26-V639	GU					AU	GC	.	0

Variable locus	**V3**										
Position	1	2	3	4	5	6	7	8	9	10	(*)
Prevalent base pairs	**AU**		**CG**		**GY**	**UA**				**A**	
BVDV1	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
Genotype BVDV-1a											**0.83**
Singer	.	GU		AU			UG	CC	UC	AA	1
NADL	.	GU		AU			UG	UC	UC	AA	1
BVDV-1b1											**0.34**
Draper	.	AC		GC				UA	GU	AC	1
Sanders	.	AC		GC				UC	AC	.	1
BI-2023	.	AC		GC				UC	AC		1
BVDV-1b2											**0.89**
Osloss	.	GC		GC				UC	AC	.	1
BVDV-1.3 (D)											**0.57**
Europa	.	GU		AU				UU	GA	.	0
BVDV-1.4											**0**
438/02	.	GC		GC				UC	AC	.	0
BVDV-1.5 (I)											**0.75**
23-13	.	AC		GC			UG	UU	GU	.	1
BVDV-1.6 (N)											**0.86**
so CP/75	.	GC		GU				UC	AC	.	1
BVDV-1.7 (O)											**1.66**
BVDV-1.7.1 (O)											**1.25**
JS10116	.	GU		GC				GC	AU	.	1
AQGN96BI5	.	GU		GC				GC	AA	.	1
Camel isolate 9	.	GU		GC				GC	AU	.	1
IS25CP01	.	GU		GC				GC	AU	G	2
BVDV-1.7.2 (O)											**1.33**
BJ09_24	.	GU		GC				GC	GC	.	1
S43	.	GU		GC			AU	GC	GC	.	1
S121	.	GU		GC				GC	GC	.	2
Genotype BVDV-1.7.3 (O, V)											**1.91**
T4-32 (V)	.	GU		GC				AC	AC	.	1
T4-31-2 (V)	.	GU		GC				AC	AC	G	2

Continued

JS12/02 (O)	.	GU		GC				AC	AC	.	1
EN-6 (V)	.	GU		GC				AC	AC	.	1
GA190608 (V)	.	GU		GC				AC	AC	.	2
BJ09_26 (V)	.	GU		GC				AC	AC	.	2
HY-3 (O)	.	GU		GC				AC	AC	.	2
HB-03 (V)	.	GU		GC				AC	AC	.	2
HN1626 (O)	.	GU		GC				GC	AC	.	2
XH-1 (O)	.	GU		GC				GC	AA	.	2
MF-3 (O) (V1/4 AC)	.	GU		GC				GC	AA	.	4
BVDV-1.8 (C)											**1.90**
Bega	.	GU		AU			CA	UC	AU	.	1
BVDV-1.9 (H)											**1.46**
KM	.	GU		GC			AG	UU	AA	.	1
BVDV-1.10 (Q)											**1.86**
SD0803	.	GU		GC				GC	UC	.	1
BVDV-1.11 (E)											**1.58**
26-V639	.	GU		GC			CG	GA	AU	.	0

Variable locus	**V1**																		
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22
Prevalent base pairs	**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**
BVDV-1.12 (F, R, S)																			
BVDV-1.12.1 (F, S)																			
22146/81	.										CG		GC	UA	AC	GG	-	-	-
UM136/08	.										CG		GC	UA	AC	GG	-	-	-
Mousedeer	.										CG		GC	UA	GA	GG	-	-	-
2561	.										CG		GC	CG	GC	GG	-	-	-
BVDV-1.12.2 (F, R)																			
11207/98	.										CG		UU	AU	GU	AA	-	-	-
51/06	.										CG		UU	AU	GU	AA	-	-	-
4998/89	.										CG		UU	AU	GU	AG	-	-	-
CA18110	.										UG		AU	GU	AA	-	-	-	-
VE24512	.								AU		CG		UU	AU	GU	AA	-	-	-
79/11	.										CG		UU	AU	GU	GA	-	-	-
BVDV-1.13 (J)																			
Deer	.			UU							CG		CG		GU	AA	A	-	-
BVDV-1.14 (R)																			
TR70	.										CG		AU		GG	AA	-	-	-
TR75	.										CG		AU		GG	AG	-	-	-
BVDV-1.15 (M)																			
BVDV-1.15.1 (M)																			
ZM-95	.										CG		UG	AU	GC	GU	A	-	-
BVDV-1.15.2 (M)																			
HB-060111	.										CG		UA	AU	GC	CU	A	-	-
BVDV-1.16 (L)																			
TR72	.								AU		CG		AU	GU	GA	AC	-	-	-
TR-2007-Gu (V1/5 UC)	.	UC							AU		CG		AU	GU	GA	AC	-	-	-
TR16	GA	UU							AU		CG		AU	GU	GG	AU	-	-	-
BVDV-1.17 (F)																			
S	.										CG		UU	AA	GU	GA	-	-	-
BVDV-1.18 (P)																			
TJ06	.										CG		UA	AU	GU	GU	A	-	-

Continued

BJ0701	.		CG		UA	AU	GU	GU	G	-	-
XY-3	.		CG		UA	AU	GC	GU	A	-	-
BVDV-1.19 (W)											
T6-18 (V1/10 UA)	AU		CG	CG	GC	AU	UU	UG	-	-	-
BVDV-1.20 (G)											
A	.		CG	CG	CC	AG	GU	AA	-	-	-
BVDV-1.21 (V)											
TR-Erz-Pst8 (V1/11 AC)	.		CG		AU		GA	AG	-	-	-
TRElz-4-2021	.		CG	CG	UA	AU	GA	GA	A	-	-
IR-Shiraz-322	.		CG	CG	UA	AU	GA	GA	G	-	-
TY8723	.		CG	UG	UA	AU	AA	GA	A	-	-
IRTV1	.		CG	CG	UA	AU	GA	GA	A	-	-
TR-Elz-Pst16 (V1/11 GC)	.	AU	CG	CG	UA	AU	GA	GA	A	-	-

Variable locus				**V2**					
Position	1	2	3	4	5	6	7	9	(*)
Prevalent base pairs		**YG**	**YG**	**UA**	**GY**			**GC**	
BVDV1		**CG**	**CG**	**UA**	**GC**			**GC**	
BVDV-1.12 (F, R, S)									**2.20**
BVDV-1.12.1 (F, S)									**2**
22146/81	AU					AU	GC	AC	2
UM136/08	AU					AC	GC	AC	2
Mousedeer	AU					AU	GU	AC	2
2561	AU					AU	GU	AC	2
BVDV-1.12.2 (F, R)									**2.30**
11207/98	GU					GC	AU	AC	2
51/06	AU					GC	AU	AC	2
4998/89	AU					GC	GU	AC	2
CA18110	AU					GC	AU	AC	2
VE24512	AU					GC	AU	AC	3
79/11	AU					GC	AU	AC	3
BVDV-1.13 (J)									**1.86**
Deer	AU					AU	GC	.	2
BVDV-1.14 (R)									**2**
TR70	AU				AU	AU	GC	.	2
TR75	AU				AU	AU	GC	.	2
BVDV-1.15 (M)									**3.29**
BVDV-1.15.1 (M)									**3.23**
ZM-95	UA	UA		CG		GC	AC	.	3
BVDV-1.15.2 (M)									**3.50**
HB-060111	AU			CG	AU	GU	GC	.	3
BVDV-1.16 (L)									**3**
TR72	GU					UA	GC	AC	2
TR-2007-Gu (V1/5 UC)	GU					UA	GC	AC	3
TR16	GU					UA	GC	AC	4
BVDV-1.17 (F)									**3**
S	AU					AC	GC	AC	3
BVDV-1.18 (P)									**3**
TJ06	AU					GC	AC	.	2

Continued

BJ0701	AU					GC	GC	.	3
XY-3	AU					GC	AC	.	3
BVDV-1.19 (W)									**3**
T6-18 (V1/10 UA)	AU					CG	GU	.	3
BVDV-1.20 (G)									**3.50**
A	AU				AU	GU	GC	AC	4
BVDV-1.21 (V)									**3.83**
TR-Erz-Pst8 (V1/11 AC)	GU					UA	GU	AC	3
TRElz-4-2021	GU					UA	GU	AC	3
IR-Shiraz-322	GU					UA	GU	AC	4
TY8723	GU					UA	GU	AC	4
IRTV1	GU					UA	GU	AC	4
TR-Elz-Pst16 (V1/11 GC)	GU					UA	GU	AC	5

Variable locus	V3										
Position	1	2	3	4	5	6	7	8	9	10	(*)
Prevalent base pairs	**AU**		**CG**		**GY**	**UA**				**A**	
BVDV1	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
BVDV-1.12 (F, R, S)											**2.20**
BVDV-1.12.1 (F, S)											**2**
22146/81	.	GU		GC		GC		AU	AC	.	2
UM136/08	.	GU		GC		GC	GA	AC	AC	.	2
Mousedeer	.	GU		GC		GC		AU	AC	.	2
2561	.	GU		GC		GC		AU	AC	.	2
BVDV-1.12.2 (F, R)											**2.30**
11207/98	.	GU		GC		GA		AC	AC	.	2
51/06	.	GU		GC		GA		AU	AC	.	2
4998/89	.	GU		GC		GA	AA	AC	AC	.	2
CA18110	.	GU		GC		GA		GC	AU	.	2
VE24512	.	GU		GC		GA	CA	-	-	-	3
79/11	.	GU		GC		GA	CA	-	-	-	3
BVDV-1.13 (J)											**1.86**
Deer	.	GU		GC				CC	AC	.	2
BVDV-1.14 (R)											**2**
TR70	.	GU		AU				UU	UA	-	2
TR75	.	GU		AU				UU	AA	-	2
BVDV-1.15 (M)											**3.29**
BVDV-1.15.1 (M)											**3.23**
ZM-95	.	GU		GC				GU	UC	.	3
BVDV-1.15.2 (M)											**3.50**
HB-060111	.	GU		AU				GU	CC	.	3
BVDV-1.16 (L)											**3**
TR72	.	GC		GC			CU	UC	UC	AA	2
TR-2007-Gu (V1/5 UC)	.	GC		GC			CU	UC	AC	.	3
TR16	.	GC		GC		CA		UU	UC	.	4
BVDV-1.17 (F)											**3**
S	.	GU		GC		AA	UU	AA	AA	-	3
BVDV-1.18 (P)											**3**
TJ06	.	GU	UA	GC			AA	AA	AU	G	2

Continued

BJ0701	.	GU	UA	GC				AA	AU	G	3
XY-3	.	GU	UA	GC				GA	AU	G	3
BVDV-1.19 (W)											**3**
T6-18 (V1/10 UA)	.	GU		GC				AC	AC	.	3
BVDV-1.20 (G)											**3.50**
A	.	GU		GC		GA	AA	GU	AC	.	4
BVDV-1.21 (V)											**3.83**
TR-Erz-Pst8 (V1/11 AC)	.	GU		AU		UG	CA	UC	AC	.	3
TRElz-4-2021	.	AC		AU			CA	UC	AC	.	3
IR-Shiraz-322	.	GC		AU		UG	CA	UC	GC	.	4
TY8723	.	GU		AU		UG	CA	UC	AC	.	4
IRTV1	.	GC		AU		CG	CA	UC	AC	.	4
TR-Elz-Pst16 (V1/11 GC)	.	GU		AU		UG	CA	UC	AC	.	5

Variable locus									**V1**										
Position	1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22
Prevalent base pairs	**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**
BVDV1	**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**
BVDV-1.22 (L, X)																			
BVDV-1.22.1 (L, X)																			
CH-01-08	.		AU								CG	CG	AC	AA	GG	AA	-	-	-
71-15	.		AU								CG	CG	AC	AA	GG	AA	-	-	-
BVDV-1.22.2 (L)																			
PG13a07	.										CG	CA	AC	AA	GG	AA	-	-	-
BVDV-1.23 (U)																			
BVDV-1.23.1 (U)																			
130/15-5364	.								AU		UA	UG	GC	AA	AA	AU	-	-	-
BVDV-1.23.2 (U)																			
M31182	.				G				AU		UA	UG	GC	AA	AA	AU	-	-	-
441/09	.								AU		UA	UG	GA	AA	AA	AU	-	-	-
GXBH-EB34 (V1/10 GA)	.										UA		GC	UA	AU	AC	-	-	-
JS-00108 (V1/10 GG)	.									CA	UA		GC	UA	AU	AC	-	-	-
QHQL-252 (V1/10 GA)	.									CA	UA		GC	UA	AU	AU	-	-	-
LN309-5 (V1/10 GA	.									CA	UA		GC	UA	AU	AC	-	-	-
GXCZ-FB22 (V1/10 GA)	.									CA	UA		GC	UA	AU	AC	-	-	-
BVDV-1.24 (K)																			
BVDV-1.24.1 (K)																			
CH-05-b1	.										CG		AG	UA	GC	GA	A	-	-
BVDV-1.24.2 (K)																			
SuwaCp	AU								AU		CG		AU	UG	GC	GA	A	-	-
BVDV-1.25 (P)																			
S153 (V2/8 CA; V2/10 GG)	GG	CG	GA						UU		CG		UG		UU	GU	A	-	-

Variable locus	**V2**								
Position	1	2	3	4	5	6	7	9	(*)
Prevalent base pairs		**YG**	**YG**	**UA**	**GY**			**GC**	
BVDV1		**CG**	**CG**	**UA**	**GC**			**GC**	
BVDV-1.22 (L, X)									**5.75**
BVDV-1.22.1 (L, X)									**5.66**
CH-01-08	AU		UA	CG	AU	UA	GC	AC	6
71-15	GU		UA	CG	AU	UG	GC	.	5
BVDV-1.22.2 (L)									**6**
PG13a07	AU		UA	CG	AU	AG	GC	AC	6
BVDV-1.23 (U)									**5.37**
BVDV-1.23.1 (U)									**3**
130/15-5364	GC					AC	GC	.	3
BVDV-1.23.2 (U)									**5.71**
M31182	GC					AC	GC	.	7
441/09	AC					AC	GC	.	4
GXBH-EB34 (V1/10 GA)	GC					AC	GC	.	5
JS-00108 (V1/10 GG)	GC					AC	GC	.	6
QHQL-252 (V1/10 GA)	GC					AC	GC	.	6
LN309-5 (V1/10 GA	GC					AC	GC	.	6
GXCZ-FB22 (V1/10 GA)	GC					AC	GC	.	6
BVDV-1.24 (K)									**6**
BVDV-1.24.1 (K)									**3**
CH-05-b1	GU		UA			GC	AC	.	3
BVDV-1.24.2 (K)									**6.75**
SuwaCp	AU		UA		UA	GC	GC	AC	7
BVDV-1.25 (P)									**13**
S153 (V2/8 CA; V2/10 GG)	GU	GG	GG	UG		GC	AC	.	13

Variable locus	V3										
Position	1	2	3	4	5	6	7	8	9	10	(*)
Prevalent base pairs	**AU**		**CG**		**GY**	**UA**				**A**	
BVDV1	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
BVDV-1.22 (L, X)											**5.75**
BVDV-1.22.1 (L, X)											**5.66**
CH-01-08	.	GC		AU			UG	CA	GC	.	6
71-15	.	GC		AU				UA	AC	.	5
BVDV-1.22.2 (L)											**6**
PG13a07	.	GU		GC		GA	GC	UC	AA	.	6
BVDV-1.23 (U)											**5.37**
BVDV-1.23.1 (U)											**3**
130/15-5364	.	AU		GA	CG		UG	UC	UC	CC	3
BVDV-1.23.2 (U)											**5.71**
M31182	UU	GU		UA	CG	UG		CG	CA	CG	7
441/09	.	GU		UA	UG	UG		CG	CA	CG	4
GXBH-EB34 (V1/10 GA)	.	GU		UA	CG	UG		CG	CA	CG	5
JS-00108 (V1/10 GG)	.	GU		UA	CG	UG		CG	CA	CG	6
QHQL-252 (V1/10 GA)	.	GU		UA	CG	UG		CG	CA	CG	6
LN309-5 (V1/10 GA	.	GU		UA	CG	UG		CG	CA	CG	6
GXCZ-FB22 (V1/10 GA)	.	GU		UA	CG	UG	GA	CG	CA	CG	6
BVDV-1.24 (K)											**6**
BVDV-1.24.1 (K)											**3**
CH-05-b1	.	GC	UA	GC			CA	UC	GC	.	3
BVDV-1.24.2 (K)											**6.75**
SuwaCp	.	GC	UA	GC		CA	CA	UC	AC	.	7
BVDV-1.25 (P)											**13**
S153 (V2/8 CA; V2/10 GG)	.	GC	UC	GC	GA	UG		AU	AA	-	13

4. Discussion

4.1. Taxonomy

The genus *Pestivirus* demonstrates to have a particularly active evolution, expressed in the variation of the genomic characteristics of virus isolates. This is directly linked to virus taxonomy and thus requiring frequent updates of classification. Examples are given by new reported atypical sequences. The strains BDV/Burdur/05-TR [KM408491] and BDV/Aydin/04-TR [JX428945] reported in Türkiye from goat in 2005 and sheep in 2004, respectively, were originally described as a new genotype of the Border Disease virus species (BDV-7) [56], but compared to other BDV strains, they represented an outgroup by conventional phylogeny. These atypical Turkish isolates from small ruminants were accordingly suggested as a new genus *Pestivirus* member species (*Pestivirus* I) [57] and recently renamed Aydin-like *Pestivirus* (*Pestivirus aydinense*) [1]. Similarly, the strain NrPV/NYC-D23 [KJ950914], isolated in brown rat (*Rattus norvegicus*) in 2013 in New York, USA [58], has been clustered as a new species in the genus as Rat *Pestivirus* (*Pestivirus ratti*)—Norway rat *Pestivirus* (NRPV). In recent years, thanks to the improvement of molecular techniques and their wider application in the field, many new sequences of *Pestivirus* isolates were deposited in databases and more *P. bovis* genotypes were described. Nevertheless, the characterization of the increasing number of genotype variants was somewhat difficult. Inconsistency in nomenclature was regularly reported during the past decades, generating confusion in the correct determination of *Pestivirus* taxonomy. For example, the genotype d proposed by Baule *et al.* [30], included three genotypes (f, g and h) defined by Vilcek *et al.* [31] [59]. In addition, the genotype c proposed by Baule *et al.* [30], corresponded to genotype j of Vilcek *et al.* [31], but clustered with isolates from *P. bovis* genotype a by Barros *et al.* [59].

The issue remains a source of concern, underscoring the need for standardization. Misuse of nomenclature and homonymy of *P. bovis* genotypes, as l or r, applied to field isolates belonging to genetically divergent groups, was previously described [60] [61]. Similarly, Chinese bovine isolates, reported as *P. bovis* genotype v [62] [63], showed high similarity with other sequences of strains already reported as genotype o [64] [65]. Secondary structure evaluation confirmed their allocation in the *P. bovis* genotype 7 (o) [66]. Another observed problem was the incorrect allocation of strains. Some bovine strains isolated in Italy were reported as *P. bovis* genotype b [67], but further genetic investigations demonstrated their appertaining to genotype a [49]. Similarly, other authors faced to the same difficulty depositing as *P. bovis* genotype a Iranian bovine strains (Nazeri *et al.*, unpublished), which, however, showed atypical sequences, justifying their clustering as *P. bovis* 21 (v) [66]. In both cases, misinterpretation of sequence characteristics was probably due to inadequate selection of reference strains to perform comparison of isolates for genotyping purposes, given that existing software such as Clustal X generates sequence comparison results based on the strains submitted by the user, considered as reference of target species variants. For this reason, it is advisable to accurately choose strains to be used as references and compare results by applying other analytical methods, in order to avoid interpretation difficulties. Moreover, there is a lack of sufficient information on genomic traits of virus population circulating in the studied area. In certain countries, like Iran, investigations on pestiviruses were serological, not followed by virus isolation and characterization. In Iran, since the first report of *P. bovis* in 1970 [68], further studies demonstrated wide circulation of the pathogen, with up to 100% seroprevalence in some cattle herds [69] [70]. However, only few genomic sequences were obtained and deposited [71].

These issues are particularly important. The confusion in nomenclature represents a relevant problem since control and prophylaxis are based on antigen substrate for the production of laboratory tests and vaccines and the selection of the antigen relies on correct taxonomical identification [9]-[11]. Therefore, more attention is required in typing strains, and it is fundamental the choice of the analytical procedure, including target sequences and multiple genotyping methods to be applied, taking into account that *P. bovis* is genetically clustered according to changes in the nucleotide sequence. Characterization of strains by sequencing their entire genome and subsequently comparing simultaneously all translated and non translated regions with other deposited complete sequences was also introduced for genotyping purposes and applied to the *P. bovis* species. Also the entire sequence of strain BI-2023 was compared to other sub genotypes, showing shared sequences with other published full-length *P. bovis* genomes and classified as belonging to the *P. bovis* b [46]. However, not all the variants described in the species have been submitted to full length genome analysis. Thus, it is not possible to obtain an exhaustive comparison. Nevertheless, phylogenetic analysis of trees constructed using the maximum-likelihood method based on the Tamura-Nei model based on the complete genome sequences of established *P. bovis* reference strains [46] corresponded to PNS genotype assignments, demonstrating that 5'-UTR secondary-structure clustering reflects stable lineage relationships within the species. The clustering into *P. bovis* genotype a of strain NADL [AJ133739] was equivalent considering its full genome sequence or only the 5'-UTR. Similarly, the strains BI-2023 [OR753412] or CP7 [U63479] were allocated into *P. bovis* genotype b, either considering their full genome or only the 5'-UTR. This was also the case of strains Bega-like [KF896608] *P. bovis* genotype 8 (c), 10JJ-SKR [KC757383] genotype 3 (d), Carlito [KP313732] genotype 11 (e), KS86-1ncp [AB078950] genotype 13 (j), SuwaNcp [KC853440] genotype 24 (k), IS2601ncp [LC089875] genotype 7 (o) (as complete genome sequencing deposited by Sato and collaborators, while erroneously named genotype m by Pan *et al.*) [46], Shitara [LC089876] genotype 6 (n), SD-15 [KR866116] genotype 15 (m) [43] (erroneously named genotype o by Pan *et al.*) [46] and SD0803 [JN400273] genotype 10 (q). Obviously, the comparison was not complete since full genomes are not available for some strains, representative of genotypes identified by 5'-UTR, such as the genotypes 4 and 25.

Currently, the majority of the sequences (97%) deposited in databases are referred to short portions of the genome, mainly the 5'-UTR [60]. Consequently, *P. bovis* genotypes are predominantly characterized through the evaluation of this untranslated region. The evaluation of other genomic fragments did not reveal additional genomic variants in the species [48]. Results provided by analyzing the Npro genomic region, also used for the genotyping of *P. bovis* isolates, generally correspond to those obtained by 5'-UTR, and those from the E2 region, scarce since rarely applied to *P. bovis*, do not offer additional taxonomical information.

In response to the challenges associated with the classification of genotypes, a more comprehensive assessment approach was adopted. This strategy went beyond simple primary sequence analysis and incorporated the PNS genotyping method, enabling taxonomic differentiation through the specific evaluation of key secondary structure elements, corresponding to the IRES, in the 5'-UTR [21] [22]. The application of the PNS procedure within pestiviruses helped to discriminate easily atypical isolates as Giraffe, Pronghorn or Bungowannah [72]-[74] or contributed to clarify ambiguous classification [61] [66]. The secondary structure analysis was applied in the present study, along with the consideration of sequence primary structure to evaluate the taxonomical features of the *P. bovis* species, with particular concern to the strains' sequence variation characteristics, relevant for genotype and sub genotype determination and linked to epidemiology or virus functionality.

Genotypes and Sub Genotypes

The evaluation of the 5'-UTR of the examined strains enabled the identification of sequence characteristics common to all pestiviruses, as well as species- and genotype-specific PNS corresponding to highly conserved base-pair positions, in comparison with established identification markers for known *Pestivirus* species. Using a divergence threshold of 9 bp for genotype assignment [22], 25 genotypes were identified in the *Pestivirus bovis* species, designated a and b and from 3 through 25. The palindromic structures of the 5'-UTR were easily constructed using both available software, the Genetyx and the PNS [47], revealing a conserved shape within the species. Only in the case of the seven isolates from mosquitoes, it was necessary to correct manually uncoherent nucleotide translation, indicated with alphabetic letters as r or y. Even if minimal in the sequence, this occurrence alters the smooth functioning of the software. The problem was easily solved by comparing the sequences with the reference strain NADL (**Table 2**). Results obtained by primary and secondary structure analyses were comparable. Generally, the genotyping of the strains corresponded to reports from other authors. Only four strains, reported as genotype b, isolated in cattle from Spain, Portugal and Egypt (Egy/Ismailia/2014, 438/02 and PT42-03) [34] [59] [75] have been allocated as *P. bovis* genotype 4, based on secondary structure characteristics. Despite few exceptions, *P. bovis* species strains have been characterized by uracil adenine pairing in V1/15, guanine uracil or guanine cytosine in positions V2/5 and V3/5 and adenine in position V3/10 (characteristic species PNS markers). Within the species, bp roots at low-variable positions (LVP) have been identified for clustering of strains into genotypes. The genotype b was characterized by LVP U-A in V1/14, A-U for b.1 and guanine uracil or guanine cytosine for b.2 in V2/7 and guanine cytosine in V3/4. In this study, the comparison of the BI-2023 5'-UTR primary and secondary structure sequence with reference strains showed its appurtenance to the *P. bovis* species, genotype b, sharing without exception all the consensus motifs common to the *P. bovis* species and the *P. bovis* genotype b identification markers.

Sub genotype determination was possible in various genotypes due to the heterogeneity of nucleotide sequences also observable at primary structure level. The clustering into sub genotypes was even more evident when evaluating the secondary structure (qualitative and quantitative analysis) at the level of root low variable position (LVP) changes or exceptions of species markers and hyper variable bp positions. Segregation was obtained by computing divergence among strains, applying 6 bp of divergence as limit value. Dendrograms constructed in the present study showed the sub genotypes b1 and b2, indicated by clearly separated branches in the genotype b, considering both 5'-UTR and Npro sequences (**Figure 2** and **Figure 3**). This was further confirmed by secondary structure analysis. In particular, the b1 and b2 sub genotypes were divergent at the LVP root V2/7 (**Table 4** and **Table S2**).

The *P. bovis* b sub genotype 1 included the strain BI-2023, which showed identical palindromes with other previously deposited *P. bovis* sub genotype b1 strains, CD89, CP7 and PT16-03. Thus, not confirming the occurrence of a new *Pestivirus bovis* sub genotype b, constituted by the strain BI-2023, as proposed by Pan *et al.* [46]. It is opportune to emphasize that the definition of a sub genotype fundamentally relies on the criteria used to determine evolutionary divergence. In this study, sub genotype classification was grounded in conserved genomic regions, particularly the structurally constrained 5'-UTR, which reflects long-term lineage stability. Taxonomic designations are meant to represent enduring genealogical relationships rather than short-term adaptive changes. Therefore, formal recognition of a new sub genotype should be supported by: 1) consistent genealogical divergence in conserved genomic regions, 2) detection across multiple isolates, and 3) persistence over time. In the absence of such evidence, antigenic variability may be epidemiologically or immunologically meaningful, yet insufficient to warrant taxonomic reassignment within a lineage-based classification system. Within this framework, although strain BI-2023 displays unique amino acid substitutions in the E2 glycoprotein, these variations are interpreted as more likely attributable to antigen-driven adaptive diversification under immune selective pressure rather than stable phylogenetic segregation. While such changes may be biologically significant, they can arise within an otherwise stable evolutionary lineage and do not necessarily signify sustained phylogenetic separation within the species.

In the present study, sub genotypes were also evident in other different *P. bovis* genotypes, showing relevant mutations within their groups: 7 (o), 12 (f, r, s), 15 (m), 22 (l, x), 23 (u) and 24 (k). Specific genomic sequences were characteristic for each group and their sub clusters. For example, the *P. bovis* genotype 7 (o) was subdivided in three sub genotypes, 7.1, 7.2 and 7.3. All *P. bovis* genotype 7 (o) strains shared the LVP root C-G, G:C or U, G-C. While the single sub genotypes were discriminated by different specific base pair combinations, as the A-U base pairing in position 18 in the V1 locus present in the sub genotype 7.1, instead of guanine uracil bp or guanine adenine bulge in the other two genotype 7 sub genotypes (**Table 5**).

Sub genotypes in the *P. bovis* species genotype b have been rarely described by other authors, partially corresponding to the observations reported in the present study. For example, German bovine strains were discriminated in two groups: b1 and b2 [35]. *P. bovis* genotype b strains from alpacas from Canada and USA, were reported as a specific group divergent from other genotype b isolates from cattle by phylogenetic analysis of 5'-UTR and Npro, supposing that unique sub clusters of *P. bovis* b may persist in alpaca populations [76]. By secondary structure analysis, these alpaca strains have been included in *P. bovis* sub genotype b1, being very close to isolates from cattle or contaminants from Belgium and USA [32] [77] with only one or two bp of difference. Furthermore, in general, sub genotypes have been rarely reported despite obvious divergent branches within defined genotypes in the phylogenetic analyses. A genomic analysis, conducted on Portuguese cattle strains [59], resulted in a phylogenetic tree showing the genotype b divided into two different branches, equivalent to b1 and b2 sub genotypes, but not considered by the authors, who even evaluated this genotype as low heterogeneous. More often, terms as genotype and subtype have been misused to define species and genotypes, respectively [78] [79], against international standards, as defined by the International Committee for virus taxonomy, and not related to effective sub clusters of a single genotype. Sub genotypes were also described for *P. bovis* genotype a (a1 and a2) [44]. In this case, the fragment within the 5'-UTR of the strains Pe515, reported as a1 and different from other genotype a strains as the reference SD-1, was related to an unusual longer size of the V1 locus with a variation at the level of the highly variable loop, not useful for *Pestivirus* species characterization. The V1 locus was composed of 21 bp, longer than the typical size of the *P. bovis* species, normally limited to 19 bp. Therefore, the observation was considered an exception in the genotype rather than a new subgroup, lacking supporting elements by secondary structure analysis. In addition, so far, no other similar sequences have been deposited.

4.2. Sequence Variations and Virus Epidemiological Characteristics

The sequence primary and secondary structure analysis demonstrated the utility in epidemiological investigations, allowing the evaluation of sequence traits related to origin and diffusion, animal host species and virulence character of the strains.

4.2.1. Geographic Diffusion

Classical types such as *P. bovis* a and b indicated the cosmopolitan character of the species. Mainly thanks to exchanges of live animals, from the first description in north America, the species gained a large geographic distribution. Genetic investigations helped to retrace the origin of the virus introduced in new countries as well as provided elements suggesting further evolutionary dynamics. For example, the first animals infected by *P. bovis* in China were reported from cows imported from Europe in the northeastern province of Jilin, in 1980 [80]. A virus could be isolated (strain CC-184) and clustered in the *P. bovis* genotype b, by phylogenetic analysis [81]. In the following years (2009-2013), from the same province of Jilin and other Chinese provinces, most of the isolated strains were typed as *P. bovis* genotype b. These strains showed high nucleotide similarity with the *P. bovis* sub genotype b1 American strain CP7 [U63479] [82], reported in 1987 from cattle [83] [84]. Such observations on the sequence characteristics provided evidence in favor of the hypothesis that the strains presently circulating in China originated from Germany, since the strain CP7 was erroneously believed to be isolated in that country [43] [81]. In reality, the CP7 strain was provided to German laboratories by the Cornell University, USA, for research purposes [82]. Therefore, the origine of the infection in China was USA, reaching China indirectly through Europe. Since the first introduction, *P. bovis* b remains one of the two predominant genotypes in the country, along with the genotype 15 (m), present only in China [40] [42]. It is remarkable that the strain BI-2023 shared identical IRES conserved sequences with the strain CP7 as well as with the strain CD89, another isolate from cattle in USA, in 1989 [32]. This indicates the persistence of relevant genomic characteristics among the current viruses circulating in China, unchanged since their introduction from the original American strains, after 36 years. Within the sub genotype b1, the Chinese strain BI-2023, the two American strains CD89 and CP7 and the Portuguese strain PT16-03 possessed a characteristic adenine cytosine bulge in position 9 in the V2 locus, instead of a conserved G-C pairing. This peculiarity was shared with only four other strains: TGAN isolated in USA [85], 3291-97-A isolated in Austria [31] and 2218/01 and 551/02 from Spain [34]. This genetic signature might represent a route of diffusion retracing of strains from their origine in North America, between 1987 and 1993, the further circulation in Europe, between 2001 and 2003 and the current presence in China. Genetic evaluations provide another indication of the introduction of other *P. bovis* genotypes in China from other countries. The *P. bovis* species genotype 7 (o) sub genotype 1 was reported only from China and Japan. However, in Japan, the virus was first isolated in 1996 [86], while, in China only about 10 years later [37] [87], suggesting the origin of a new genotype in China, where at that time it was circulating almost exclusively the genotype b. Furthermore, the other two closely related sub genotypes 7.2 and 7.3 [41] [64], nowadays present only in China, might represent the subsequent development of additional types, supporting the theoretical further genetic evolution of the virus in a new geographically restricted environment. Despite the elements obtained through the evaluation of data from the 5'-UTR or Npro genomic fragments strongly suggest scenarios of specific routes of viral geographic diffusion, such hypotheses consistent with the observed sequence distribution should be supported by further studies to provide formal phylogeographic analyses and reach conclusions on virus circulation dynamics. In fact, 5'-UTR and Npro data are appropriate for taxonomic and comparative lineage assessment, but do not alone allow robust inference of transmission pathways, directionality of spread, or detailed phylogeographic history.

The increased genomic heterogeneity of *P. bovis* in different countries was most likely due to imported new stocks of live animals. For example, in Italy, only 3 genotypes have been described in 2002. Six years later, in 2008, they were 7. The double (n 14) of *P. bovis* genotypes was reported in 2019, with 11 (e), as the prevalent one, which is also the prevalent in France, from where most of the imported animals come from. Also in UK, the increasing diversity of *P. bovis* was ascribed to animal movements [88]. However, also the contamination of biological products for veterinary use was suspected to be responsible of spreading atypical *P. bovis* genotypes, for example, involved with the introduction in Italy of the Asian genotype *P. bovis* 23 (u) [49]. This aspect is recalled also by the strain BI-2023, which was isolated as cattle serum contaminant. Furthermore, other potential ways of *Pestivirus* spread might represent new serious challenges for the control and prevention of such pathogens. Recently, seven full genome sequences obtained from virus isolates from mosquitoes were deposited as *Pestivirus bovis* in GenBank in 2025 by the Institute Pasteur, Dakar, Senegal (Sene, unpublished), and clustered as *P. bovis* genotype b, sub genotype b2 in the present study. The mosquito species, from which the *Pestivirus* were identified, both were known vectors of diseases. The strain ARD410734 [PV346699] was isolated in *Culex neavei*, a known vector competent for the West Nile virus, arthropod-borne pathogen of the genus *Flavivirus*, *Flaviviridae* family [89]. The other mosquito strains were isolated in *Mansonia uniformis*, a zoophilic species able to transmit various diseases in humans, such as lymphatic filariasis, Murray Valley encephalitis, Ross River virus or Kunjin virus [90].

4.2.2. Geographic Segregation-*Pestivirus* Species with Limited Diffusion

Pestiviruses are cosmopolitan, however there is a marked variation in their distribution globally. Some species are restricted in specific areas. Even for certain species more diffused, they are absent in other regions. For example, *Pestivirus ovis* (BDV) remains an exotic disease for South America. Among pestiviruses, genomic characteristics of certain virus strains may be linked to the zones from where they have been isolated. Also, the taxonomic nomenclature reflect this aspect in some cases. At genus level, the BVDV-3 (Bovine diarrhea virus 3-HoBi-like *Pestivirus*) species is defined *Pestivirus braziliense* and the Bungowannah (porcine *Pestivirus*) species *Pestivirus australiense* [1], giving that the first was originally isolated in Brazil [91] and the second was observed in pigs in Australia [92]. However, while *P. australiense* has been reported only from Australia, *Pes-*

tivirus braziliense was also reported in different other countries, such as Thailand [93], China [94], Bangladesh [95], India [96] or Italy [97]. To date, the *Pestivirus* Pronghorn species (*Pestivirus antilocaprae*, pronghorn antelope *Pestivirus*), has only been described in the USA, as the APPV [98] and the Rat *Pestivirus* (*Pestivirus ratti*) [58]. The RaPestV-1 (*Rhinolophus affinis* pestivirus virus 1) of chiropters was reported only in the Southeast Asia and the Aydin-like-pestiviruses (*Pestivirus aydinense*) were reported only in Türkiye [56]. Also in central Italy, unusual strains were observed in sheep and goat [99] [100], characterized according to secondary structure analysis as a new *Pestivirus* species, named BDV-2 [49]. The atypical *Pestivirus* species with limited geographical distribution are schematically presented in **Figure 5**.

Figure 5. Geographic distribution of the atypical *Pestivirus* species.

Compared to the other species within the genus, the *P. bovis* species is the most variable. This characteristic might be explained partially by isolation of virus sub populations in restricted geographic areas. Various *P. bovis* genotypes, with genetic peculiarities, were observed only in few countries. In Europe, the *P. bovis* genotype 9 (h) was common in Switzerland, Italy and Austria, with only few other isolates reported from England, Slovakia, Türkiye and South Africa. *P. bovis* genotype 20 (g) was present only in UK, Austria and South Africa. *P. bovis* genotype 24 (k) was present only in Switzerland and Italy [101]. The presence of European genotypes in South Africa was probably linked to import of live animals and not expression of virus evolution. Also in Asia, some genetic clusters (*P. bovis* genotypes and sub genotypes) showed genetic peculiarities linked to the continent, suggesting geographic isolation [40]. The *P. bovis* genotype 19 (w) represented a separate species cluster, described in China in 2017 [62] and to date not reported elsewhere. Also some *P. bovis* genotypes (10, 15, 18, 23 and 25) have been observed almost exclusively in China. Similarly, *P. bovis* genotypes 6 and 8 were restricted to Asiatic countries or Australia. Sub genotype 7.1 was observed only in China and Japan and sub genotypes 7.2 and 7.3 were exclusive of China. The rare observation of genotypes 6, 8 and 23 in Italy were exceptional events, probably linked to *P. bovis* contaminated bovine fetal serum, given the forbidden import of live animals to Europe from Asian countries. Other genetic clusters have been observed only in Türkiye and Iran. Strains deposited as genotypes a or v, (Abayli, Nazeri *et al.*, Seyfi Abad Shapouri *et al.*, unpublished) [102] [103], were classified by PNS secondary structure analysis as *P. bovis* genotype 21. In addition to the genotype 21, the phylogenetic analysis revealed other rare *P. bovis* clusters specific to Türkiye and expression of geographic segregation: the strains reported as genotype r [60] [104] and the strains reported by as genotype l [105], belonging to the *P. bovis* genotypes 14 and 16, respectively. The genotype 16 (l) resulted to be the predominant *P. bovis* type in Türkiye [103]. As demonstrated by secondary structure analysis, certain genetic traits were related to geographic segregation. All the strains of genotype *P. bovis* 8 (c), isolated in China, Japan and Australia from cattle or contaminants of biological products [42] [106] [107], shared an adenine cytosine enlargement in V1/12, a unique base pairing not observed in any other members in the species. Strains isolated from cattle in China [62] belonging to *P. bovis* genotype 19 (w) showed a unique C-G pairing in V2/6. Similarly, strain S153 [41], *P. bovis* genotype 25, presented a guanine adenine enlargement of the V3 stem in position 5 at the level of the *P. bovis* species marker. These characteristic sequences were expression of geographic segregation and subsequent species genetic evolution, related to viruses present exclusively in Asia.

Geographic segregation was observed at genotype and sub genotype level also in other *Pestivirus* species. In the *Pestivirus tauri* (BVDV type 2 or *Pestivirus B*) species, the genotype b variant 4 (BVDV-2b4) was specific to Asia [108], constituted only by Chinese isolates from cattle and contaminants of bovine fetal serum [38] [41]. The *Pestivirus braziliense* (BVDV type 3, HoBi like or *Pestivirus* H) species showed three genetic groups (3.2, 3.3 and 3.4) associated to cattle and zebu from Bangladesh and India [95] [96]. *Pestivirus ovis* (Border disease virus—BDV, or *Pestivirus D*) species included strains isolated in China and Türkiye, showing high genomic diversity from other clusters in the species [109] [110], suggesting geographic segregation and allocated as genotypes BDV-d, sub genotype d1, BDV-h and BDV-i [111]. Similarly, in the *Pestivirus suis* (Classical swine fever virus—CSFV, or *Pestivirus C*) species, four genogroups were limited to geographic areas: Parambi type (CSFV-A variant 4) was represented by pig and wild boar Indian isolates (Chandramohan *et al.*, Ravishankar *et al.*, Tomar *et al.*, unpublished) [112]; CSFV-B included an ovine isolate from Spain [34]; Okinawa type (CSFV C) included pig strains only described in Japan and the Taiwan region of China (Liu, unpublished) [113]-[115] and CSFV-D referred to a Chinese strain isolated from bovine serum [41], showing atypical sequence characteristics in the species [116].

4.3. Animal Host

Variation in animal hosts and clinical traits of the infection might suggest species definition. In fact, *Pestivirus* species are still generally defined taking into account their most common animal host: *P. suis* (CSFV, or *Pestivirus C*) and *P. scrofae* (Atypical porcine pestivirus virus—APPV) common in pigs; *P. bovis* and *P. tauri* mainly reported in bovines; *P. ovis* (BDV) frequent in sheep. This is also the case for species less frequently described or limited to certain geographic areas as Giraffe (*P. giraffae*), Pronghorn (*P. antilocaprae*), Bungowannah (*P. australiense*), RaPestV-1 (*Rhinolophus affinis* pestivirus virus 1) and Rat *Pestivirus* (*P. ratti*).

However, important cross reactions exist. Pestiviruses may infect various hosts by crossing species barrier. For example, 60 isolates other than *Pestivirus ovis* (BDV) were isolated in small ruminants and characterized as *Pestivirus bovis*, *P. tauri* and *P. braziliensis*, *P. suis* and *P. aydinense* (formerly *Pestivirus* I or BDV-7) [111]. Furthermore, *P. suis* (CSFV) has been reported in cattle (*Bos taurus*) from China, India and Kenya [117]-[119]. Genus *Pestivirus* species officially designed and putative species and their hosts are schematically presented in **Figure 6**. Similarly, in the *Pestivirus bovis* species, the virus is not host species-specific in animals. The mammals currently known to be susceptible include domestic bovines (*Bos taurus*), buffaloes (*Bubalus bubalis*), small ruminants and pork and more than 50 wild species, including *Bovidae*, *Cervidae*, *Antilocapridae*, giraffes, camelids and suides [120] [121] and experimentally rabbits [23]. Also, at *P. bovis* genotype level, host species are heterogeneous. In the *P. bovis* genotype b the following hosts have been described: alpaca (*Vicugna pacos*), two hump camels (*Camelus bactrianus*), buffaloes, bovines, humans (*Homo sapiens*), mosquitoes (*Culex neavei* and *Mansonia uniformis*), sheep (*Ovis aries*), yaks (*Bos grunniens*) and zebu (*Bos indicus*). At sub genotype level, cattle is the predominant host in both b1 and b2 sub genotypes. In addition, in b1 alpaca, buffalo, sheep and human and in b2 buffalo, zebu, Bactrian camel, yak and mosquitoes are other less frequently described hosts. However, in general, sequences of strains belonging to the same sub genotype were similar and no nucleotide peculiarities were related to specific host species. For example, in the sub genotypes of genotype 7, cattle, the predominant host species, included in all three sub genotypes, showed genomic correlation with the strains isolated from pig and Bactrian camel (7.1) or from goats (7.3).

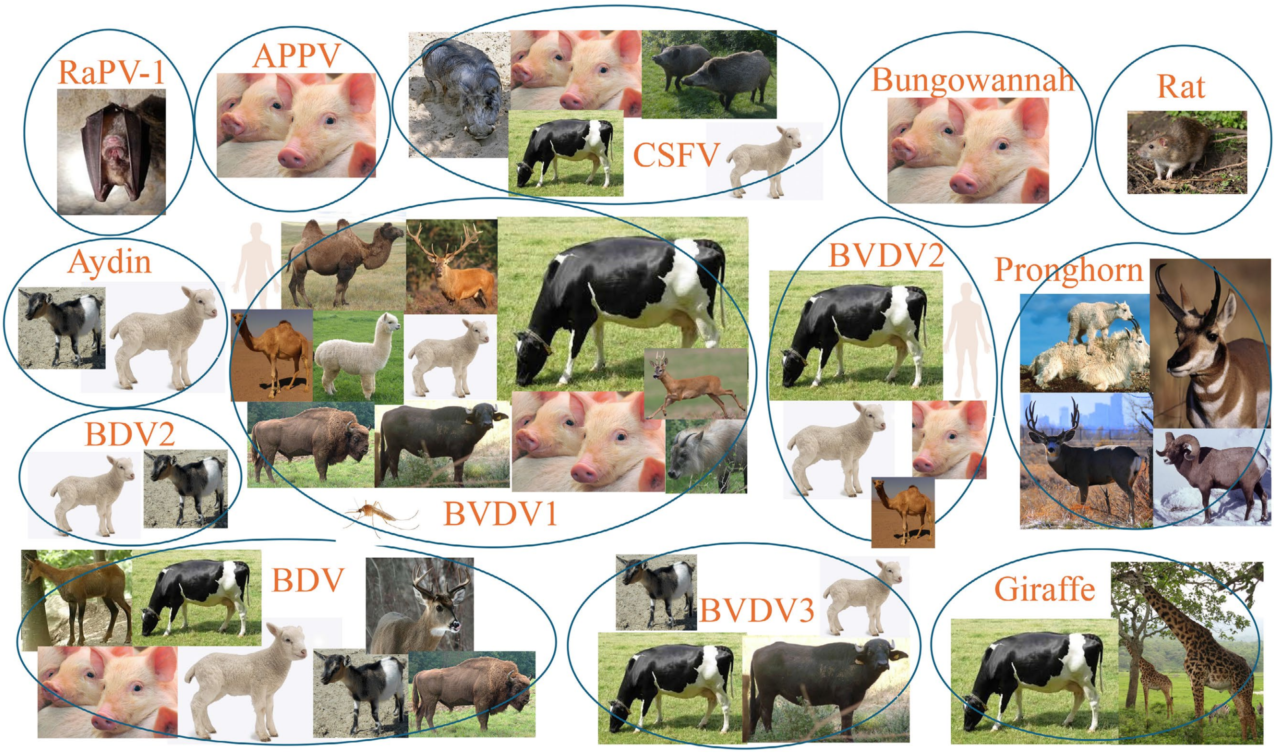

Figure 6. Genus *Pestivirus* species officially designed and putative species and their hosts. BVDV-1 (Bovine diarrhea virus 1—*Pestivirus bovis*); BVDV-2 (Bovine diarrhea virus 2—*Pestivirus tauri*); BVDV-3 (Bovine diarrhea virus 3—*Pestivirus braziliense*, HoBi-like *Pestivirus*); BDV (Border Disease virus—*Pestivirus ovis*); BDV-2 (Border Disease virus 2); CSFV (Classical swine fever virus—*Pestivirus suis*); Giraffe (*Pestivirus giraffae*); Pronghorn (*Pestivirus antilocaprae*, pronghorn antelope *Pestivirus*); Bungowannah (*Pestivirus australiense*, porcine *Pestivirus*); RaPestV-1 (*Rhinolophus affinis* pestivirus virus 1); APPV (Atypical porcine pestivirus virus—*Pestivirus scrofae*); Rat (*Pestivirus ratti* , rat *Pestivirus*); Aydin (*Pestivirus aydinense*, Aydin-like *Pestivirus*).

4.4. Virulence

Hemorrhagic syndrome, the most dangerous clinical form of *P. bovis*, was first reported in serious clinical disease among cattle from USA and Canada, due to highly virulent strains of *P. tauri* species [122] Later, strains from the same species but showing low virulence were also identified in North America and Europe [123]. In the *Pestivirus bovis* species, the European strains (Culi 1, 4, 6, L256 and Marloie), isolated in cattle, in Belgium and France [32], were also associated to high virulence, causing hemorrhagic syndrome. Experimental trials using *Pestivirus tauri* strains resulted in fatal disease in susceptible animals, whereas *Pestivirus bovis* strains were unable to produce disease under similar conditions [124]. This finding suggests that the development of the occasional course of the hemorrhagic syndrome associated with *Pestivirus bovis* requires additional unidentified co-factors, while the virus may acts as primary etiological agent in the occurrence of the epidemic form.

In terms of nucleotide sequence, based on the observations of sequence characteristics of *Pestivirus tauri* hypervirulent strains [54], two nucleotides associated with virulence in *Pestivirus* strains, designated N1 and N2 and corresponding to nucleotides 219 and 278 of the *Pestivirus bovis* reference Osloss strain, respectively, were identified within palindromic structures of the IRES. Specifically, nucleotide 219 (N1) was located in the variable V1 loop region, occupying position 19 (left part of the stem) from the base of the palindrome and positioned eight nucleotides into the V1 loop downstream of the first cytosine of the genus *Pestivirus*-characteristic C C palindrome enlargement. The nucleotide 278 (N2) was situated in the V2 stem region at position 4 (left part of the stem), one nucleotide downstream of the cytosine in the first genus *Pestivirus*-characteristic cytosine guanine bp, and five nucleotides upstream of the first guanine of the conserved V2 loop. The evaluation of N1 and N2 nucleotide variations in *P. tauri* showed that the combination UC was associated to high virulence, CU was related to both high and low virulence (inconclusive) and AU to low virulence [55]. In the *P. bovis* species, the high virulent strains sub genotype b1 Culi 4, Culi 6, sub genotype b2 Marloie and genotype 11 (e) Culi 1 and L256, all showed a virulence marker AU, associated at the contrary to low virulence in *P. tauri*.

Also in the variable loci of the *P. bovis* sub genotype b1 BI-2023 strain, N1 and N2 were adenine in V1/19 and uracil in V2/4, respectively. The strain BI-2023 was classified as *P. bovis* genotype b [46], and clustered in the sub genotype b1 in the present study. A comparison with other genotype b strain sequences showed that the BI-2023 E2 and Erns genomic regions presented unique mutations (several aa residues). Considering that E2 is the main component exposed on the outer surface of the virus envelope, inducing the production of neutralizing antibodies during infections with *Pestivirus bovis* [125] and Erns is another envelope protein involved in antibody binding and virus inactivation [125], it was supposed that the modified protein encoded by the BI-2023 E2 might have a weaker antibody-binding ability and thus facilitating the virus evasion from the host immune system, suggesting virus functionality potentially related to virulence. The fact that PNS analysis did not show structural segregation of BI-2023, despite unique mutations in the E2 protein, induces conceptual reflection between taxonomy based on phylogenetic stability and functional/antigenic relevance. But these aspects are only apparently conflictual, since taxonomy does not prevail over functional/antigenic criteria. Only functional characteristics linked to conserved genomic regions will be stable features of the virus and may find correspondence into defined taxonomy. This is not the case of functions related to hypervariable regions as E2. At this level, such properties are expression of virus defense mechanisms against host immunity. This capacity even if possibly enhanced in the BI-2023 strain, it is potentially common to all other genetic group members of the species, and not distinctive. Furthermore, this observation remains theoretical and linked to variable segments of the genome. Since the strain BI-2023 was identified from a batch of cattle serum commercialized in China, no clinical data were available. However, by comparing the secondary structure, BI-2023 resulted identical to the sub genotype b1 bovine strains PT16-03 [59], CP7 [82] and CD89 [32]. Exception made for the strain PT16-03, lacking also of information on pathogenicity, both the American strains CP7 and CD89 were associated to hemorrhagic syndrome—mucosal disease, a lethal clinical course of *Pestivirus bovis* [126] [127]. While the determination of virulence patterns in these *Pestivirus bovis* strains remains unclear, they were associated only to genotypes b and 11. Further studies are necessary for the comprehension of virus properties, in particular virulence.

4.5. Genomic Characteristics and Potential Implications

RNA viruses can undergo rapid mutation as a result of replication errors introduced by the low-fidelity RNA-dependent RNA polymerase. Also the replication of *Pestivirus bovis* is characterized by highly mutation rates. Point mutations are continuous and occur randomly in all parts of the genome, each replicational cycle, at a rate of 10^{-3} - 10^{-4} per incorporated nucleotide. The high level of mutation activity, protecting from host antibody pressure [123], can drive the virus to produce high heterogeneity in the species [128] [129]. Due to this, the nucleotide usage variation of *Pestivirus bovis* is regarded as a key evolutionary mechanism driving the diversification of coding regions and thereby influencing the evolutionary trajectory of the species [130], with the potential to generate different virus genotypes and even new species.

The growing number of reports describing the heterogeneity of *Pestivirus bovis* highlights a major concern regarding the emergence and dissemination of atypical genetic variants of the species, which may have important negative impacts for welfare and health of animals and thereby hindering successful disease control [11]. In addition, within the *Pestivirus* genus members, there is a certain degree of genetic correspondence in antigens targeted for their diagnosis [131]-[133]. This similarity complicates molecular diagnosis and vaccine-induced protection, prompting research toward the development of more specific diagnostic methods and broadly cross-protective vaccines [134]. Moreover, the genetic heterogeneity among circulating strains may further hinder the obtention of effective immunological products and reliable testing tools [14]. Atypical and highly divergent types of strains may hamper the effectiveness of commercialized vaccinal products or negatively influence diagnostic analysis through the variability of the virus antigens, which may reduce or impede the cross-protection provided by antibodies, at the end too specific, among and even within the *Pestivirus* species. These issues were reported for *P. braziliensis* [135]. Some reports concerned also *P. bovis*. Studies on antigenic similarity among genotypes, measuring virus cross neutralizing antibody titers of hyperimmunized antiserums against studied *P. bovis* genotypes, showed very weak antigenic similarity among strains of genotypes a and b compared with viruses from other different genotypes, particularly 17 (f) and 24 (k) [23] [44] [45]. But even between a and b some reports showed low level of antibody response to the genotype b by vaccines prepared with genotype a [136], and some insufficient responses to protect against infections caused by genotype b [137] [138].

The importance of gene analysis highlights the relevance of the correct selection of target genomic sequences and the applied analytical methods. The 5'-UTR, considered in the present study, demonstrated reliability for virus evaluation, also allowing the application of secondary structure analysis, not possible in other regions. Even if untranslated regions, as per definition, do not correspond to produced antigens, nucleotide changes in the 5'-UTR sequences are linked to the molecular variations in parts of the *Pestivirus bovis* genome, related to antigenic traits, expressing structural and non structural proteins, also strongly immunogenic such as E2 and NS3 [139]-[143], which are important for diagnostic serology and production of vaccines [144] [145]. The nucleotide sequence of the 5'-UTR is highly conserved across all pestiviruses, making it a valuable target for species and genotype determination. Its secondary structure is organized into four domains, from A to D. The domain D is the largest, comprising approximately two-thirds of the 3′ portion of the 5'-UTR. This region is predicted to form a complex palindromic stem-loop structure [18] [19] and represents a critical segment of the 5'-UTR, as it contains the IRES, which is essential for translation, transcription, and replication in pestiviruses [18]. Consequently, mutations within the 5'-UTR are likely to compromise viral viability. Stable nucleotide variations in this region, therefore, are of particular significance for understanding viral evolutionary history. Furthermore, the IRES is a binding site for antiviral drugs, as demonstrated in studies conducted on *Pestivirus* species [146], including human chronic hepatitis C virus (HCV) [147], CSFV [148] as well as on *P. bovis* [149]-[151]. Therefore, from a clinical application standpoint, further analyses might identify more virulent strains, or pinpoint strain’s IRES more sensitive to known antiviral drugs, highly pertinent to genetic investigation aspects.

The consideration of other genomic regions might be problematic if changes due to mutation rate do not relate to generated stable virus population clusters. In particular, while E2 is regarded as important for precise typing of CSFV [152], in the *Pestivirus bovis* species the coding region for E2 is the most variable and least conserved portion within the genomic nucleotide sequence [14]. Analyzing the E2 gene of *Pestivirus bovis*, also considered conserved D7E residues of isolates resulted mutated, leading to vaccination failure, increased range of animal hosts and ineffectiveness of diagnostic kits [153]. The variability of E2 was observed in different countries, as Poland [14] or Indonesia [153], and as well as in other *Pestivirus* species such as *Pestivirus tauri* [123], APPV and NRPV [154]. High heterogeneity was observed among the infecting viral strains of the *Pestivirus tauri* species, suggesting its contribution to the viral pathogenesis [123]. Also in this species, analysis of variable sequence fragments across the genome showed that nucleotide variations were predominantly clustered at the level of the first half, particularly within the region encompassing the E2 and NS2 - 3 genes [123]. Both APPV and NRPV species showed low E2 sequence conservation [154]. Furthermore, APPV and a bat *Pestivirus* showed a reduction in the size of their E2 surface glycoprotein, with about 130 residues less, and 19% - 20% decreased sequence conservation [154]. In the case of strain BI-2023, supposed virus characteristics were referred to atypical E2 sequences. Taking into account the high variability of this region and the absence of any link with variations at the level of conserved fragments of the genome, the stability of the observed sequences it is unlikely, as well as the maintaining of eventual virus related properties.

5. Conclusions

The study demonstrated the useful application of combined analytical molecular techniques relying on both primary and secondary structure evaluation of the virus genome for the determination of genotypes and sub genotypes within the *P. bovis* species. Sequence variations in the 5'-UTR were relevant for taxonomical classification, and in some cases resulted in specific genomic traits of virus populations circulating only in restricted areas, expression of the species evolutionary history, possibly also due to geographic segregation. While described in other pestiviral species, variations related to the adaptation to specific animal hosts, were not observed in the *P. bovis*, indicating the high potential of this species to cross species barrier. Other sequence variations at the level of other genomic regions as E2, even if supposed to be linked to important virus functions as the enhanced capacity to escape host defense mechanisms, could not be correlated to variations in the conserved 5'-UTR, thus considered to be replication events related to high mutation rate, not stable and therefore not useful for control and prevention strategies.

Identifying the genomic characteristics of virus isolates is essential for designing effective control or eradication programs, guiding vaccine development, and supporting targeted efforts such as tracing the sources of infection during outbreaks [10] [88]. Taking into account that laboratory testing tools and vaccines available on the market rely on antigenic substrate from selected viruses [9] [11], the genetic heterogeneity of the *P. bovis* species may lead to challenges in diagnosis and prevention efforts. This indicates the need to apply simultaneously various genotyping methods, based only on conserved genomic regions, which are representative for all the rest of the virus genome, to minimize misinterpretation and ensure precise genetic analysis, particularly considering the critical role of epidemiological data, to protect the welfare and the health of animals from the diffusion of dangerous molecular variants.

Authors' Contributions

M. Giangaspero and S. Zhang contributed equally to the present study.

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Conflicts of Interest

Authors declare that there is no conflict of interest.

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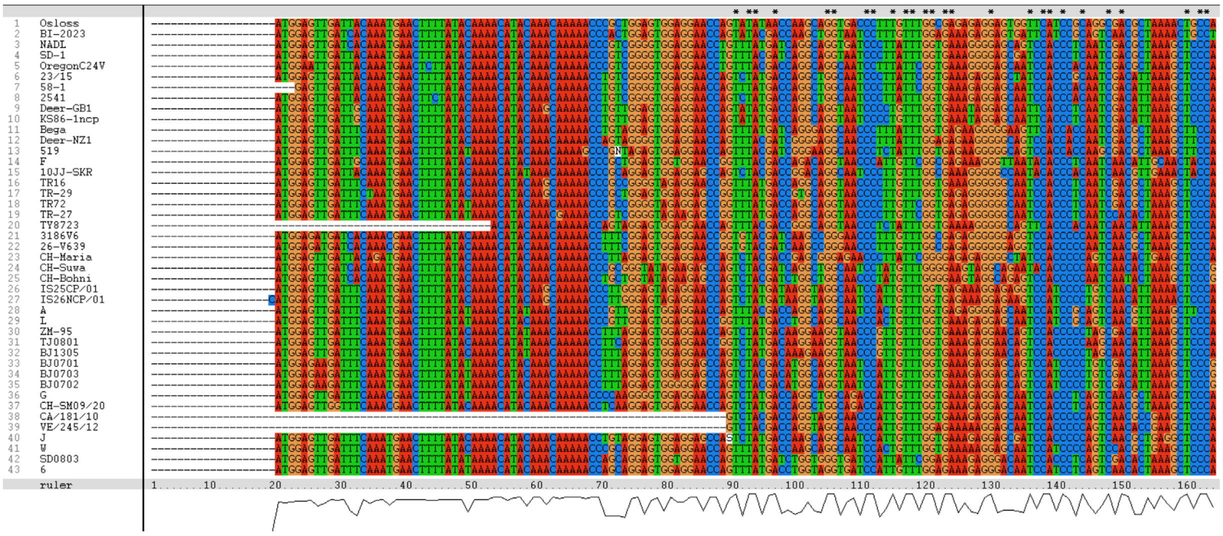
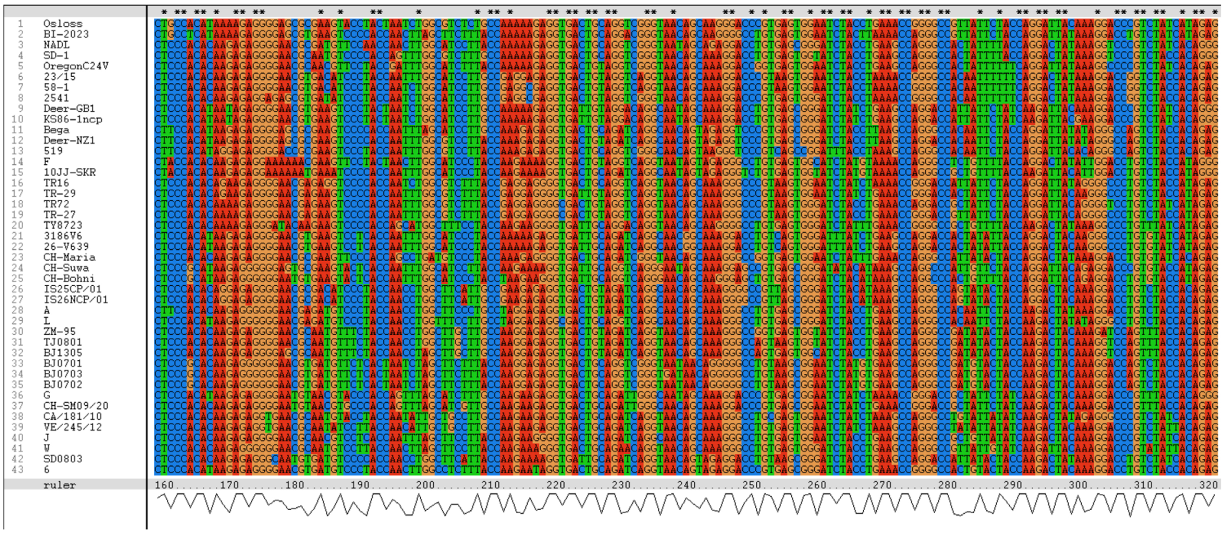
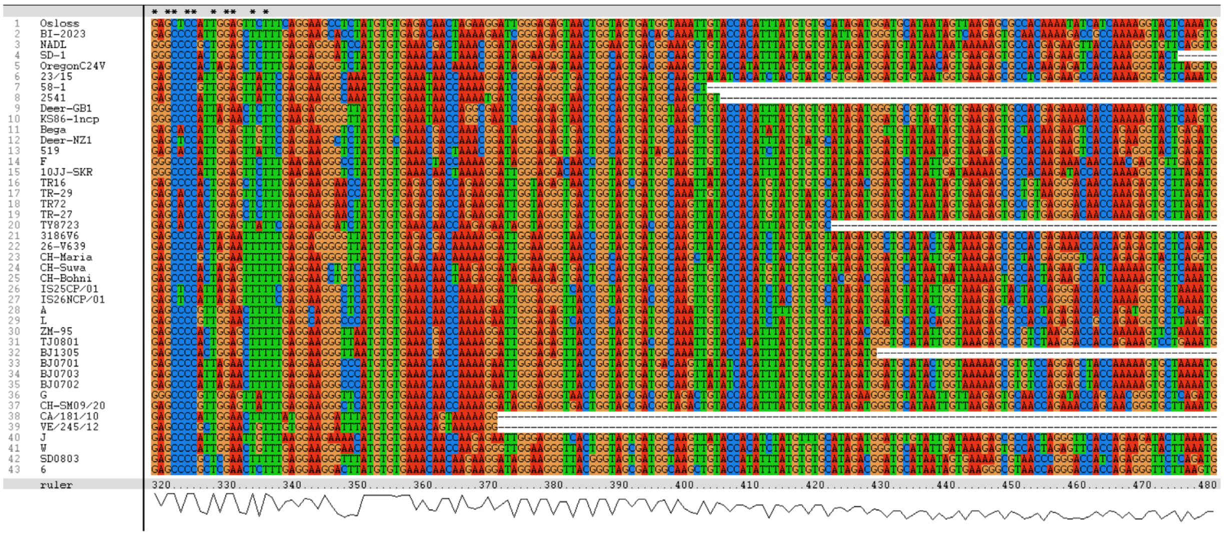
Supplementary Material (SM)







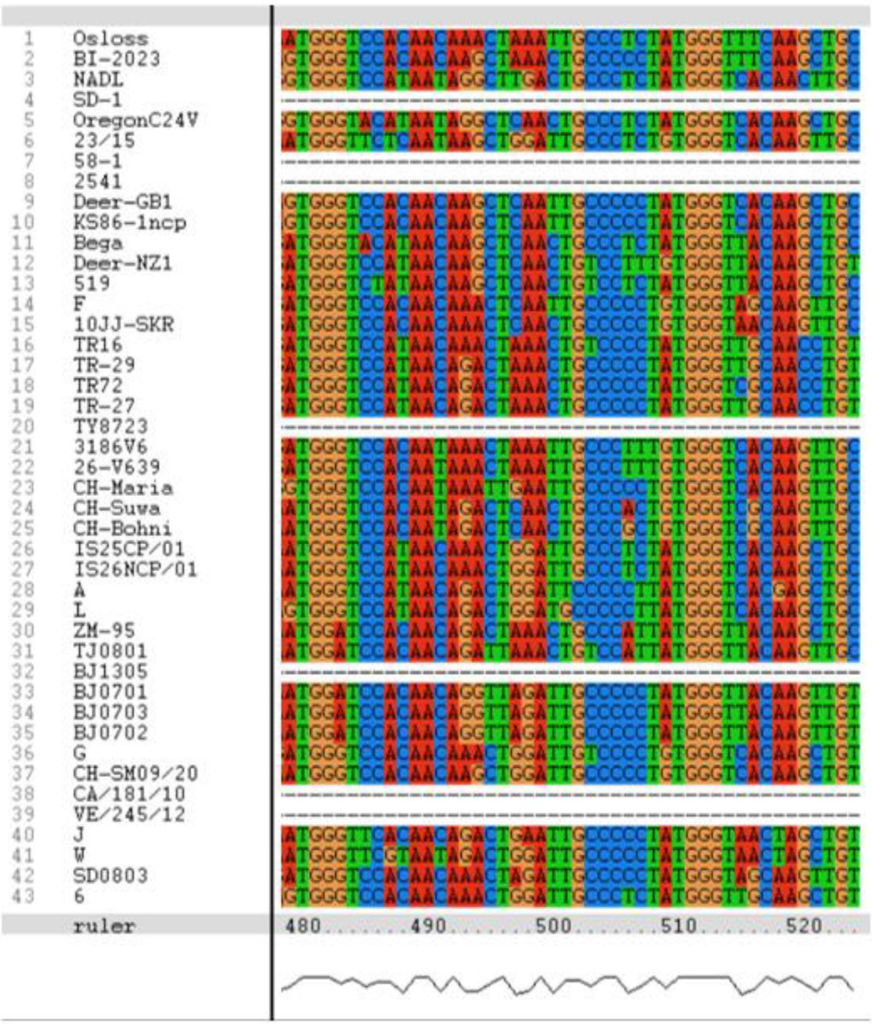


Figure S1. Npro alignment.

Supplementary Material (SM) Table S1

https://drive.google.com/file/d/1dryRs5zcNMW5gBovsqahgtJWe-ME9r6J/view?pli=1

Supplementary Material (SM) Table S2

https://drive.google.com/file/d/14y-Kf1HvAc60jNIrel3aYMug1Zq1ynhs/view