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Pestivirus A Bovine viral diarrhea virus type 1 species genotypes circulating in China and Turkey

Massimo Giangaspero[1*] and Shuquin Zhang[2]

[1]*Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy*
[2]*Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, People's Republic of China*

Abstract

Background: *Pestivirus* A Bovine viral diarrhea virus type 1 (BVDV-1) is a heterogeneous species within the genus, affecting cattle and other ruminants, with economic impact on livestock production.

Aim: The study aimed to update the taxonomy of the *Pestivirus* A, BVDV-1 species and to verify the clustering of the strains reported as genotype 1v, originating from different countries.

Methods: Recently deposited strains from China, Turkey, and Iran have been evaluated by the palindromic nucleotide substitutions (PNS) genotyping method.

Results: Based on secondary structure analysis of the 5′-UTR sequences, strains reported as 1v from China were clustered as sub genotype 1.7.3 (1o). Genotype 1.19 (1w) was restricted to China and genotype 1.21 (1v) was present only in Turkey and Iran.

Conclusion: The application of the PNS method clarified the taxonomical status of strains, revealing the homonymy of genetically different clusters. Furthermore, these observations indicated geographic segregation in the *Pestivirus* A species, and confirmed the occurrence of new atypical genetic variants, with potential implications on control and prophylaxis.

Keywords: Asia, Bovine viral diarrhea virus type 1, Geographic segregation, *Pestivirus*, Taxonomy.

Introduction

Pestivirus A Bovine viral diarrhea virus type 1 (BVDV-1) is an established species of the genus *Pestivirus* of the family Flaviviridae, responsible for a disease with widespread distribution, affecting cattle and other ruminants, presenting a wide range of clinical manifestations, with a substantial economic impact on livestock production. Currently, it is difficult to obtain an exhaustive comparison of all variants in the BVDV-1 species by full-length genome analysis, giving that about 97% of deposited sequences are restricted to genome short portions (5′-UTR mainly) (Yesilbag *et al.*, 2017). Therefore, genotypes are characterized predominantly by the analysis of the 5′-UTR. While the E2 gene sequence is considered relevant for accurate typing of classical swine fever virus (CSFV) (Postel *et al.*, 2012), this region is rarely applied to BVDV-1 strains and does not provide additional information in terms of taxonomy. Also, Npro genomic region is used to characterize BVDV isolates and results generally correspond to the clustering based on 5′-UTR and no additional variants in the species were detected with other analytic techniques evaluating other genomic regions (Giangaspero *et al.*, 2018). BVDV is categorized genetically on the base of nucleotide sequence variations. Strains (field isolates) have been segregated into genotypes by different authors. Initially, genotypes BVDV-1a and BVDV-1b have been reported (Harasawa, 1996). Furthermore, genotypes BVDV-1c and BVDV-1d have been described (Giangaspero *et al.*, 1997, 2001). The evaluation of other isolates, by sequence alignment and construction of phylogenetic trees, demonstrated the presence of other genotypes within the BVDV-1 species (Baule *et al.*, 1997; Tajima *et al.*, 2001; Vilček *et al.*, 2001; Couvreur *et al.*, 2002; Ciulli *et al.*, 2003; Hurtado *et al.*, 2003). Also sub genotypes have been defined, for example, genotype BVDV-1a was subdivided into a1 and a2 (Bachofen *et al.*, 2008) or BVDV-1b into b1 and b2 (Tajima *et al.*, 2001). According to secondary structure, limit values of a number of divergent base pairs (bps) have been identified to determine genotypes or sub-genotypes (Giangaspero and Harasawa, 2007). Another characteristic of the field isolates is their capacity to induce or not cytopathic effect on cellular substrate (disruption of cells) when grown in tissue culture for laboratory investigations. Thus, strains are distinguished into two biotypes: non-cytopathic and

***Corresponding Author:** Massimo Giangaspero. Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy. Email: *giangasp@gmail.com*

cytopathic. The non-cytopathic strains are predominant in nature and important for clinical disease (Rajput *et al.*, 2017).

In recent years, a relatively large number of new sequences of isolates, mainly from domestic animals, have been deposited in genetic sequence databases, in particular from Asian countries, and additional BVDV-1 genotypes were reported describing atypical variants within the species (Xue *et al.*, 2010; Zhong *et al.*, 2011; Gao *et al.*, 2013; Wang *et al.*, 2014; Zhang *et al.*, 2014b; Deng *et al.*, 2015; Weng *et al.*, 2015; Zhu *et al.*, 2016). A certain confusion in the nomenclature of the increasing number of genotypes was evident and it is still a matter of concern, indicating the need for harmonization. Homonymy of BVDV-1 genotypes, as 1l or 1r applied to strains from different genetic clusters, was previously reported (Yesilbag *et al.*, 2017; Giangaspero *et al.*, 2018). More recently, strains collected from cattle in China in 2017 were reported as belonging to two unknown groups, tentatively typed as "BVDV-1v" and "BVDV-1w" (Deng *et al.*, 2020). Deng clustered as 1v also the two goat strains JS12/02 and HA2-12 from China (Deng *et al.*, 2020). However, these stains have been previously reported as 1o (Mao *et al.*, 2016). In 2019, a non-cytopathic strain of BVDV was isolated from cattle and named GA190608 (Tian *et al.*, 2021). Phylogenetic analysis based on the 5′-UTR sequence revealed that the BVDV isolate GA190608 clustered with strains HN1814, EN-19, and BJ09_26 in a separate branch, which has tentatively been classified as a new genetic subtype, "1v." The strain EN-19 was reported as 1v (Deng *et al.*, 2020), the strain HN1814 was reported as 1o (Shi *et al.*, 2020) and the strain BJ09_26 was deposited without a defined genotype. Furthermore, during 2016 and 2020, virus strain sequences were deposited as 1l or 1v from cattle isolates from Turkey (Oguzoglu *et al.*, 2019; Timurkan and Aydin, 2019; In 2013 and 2019, bovine strains were reported from Iran, also allocated as BVDV-1a, but showing atypical sequences.

Based on the issues arising from genotype classification, a wider evaluation procedure, not limited to primary sequence analysis, but extended to the application of the palindromic nucleotide substitutions (PNS) genotyping method was considered for the taxonomical segregation through the exclusive consideration of strategic genomic secondary structure sequences corresponding to the internal ribosome entry site (IRES), in the 5′-UTR, responsible for translational, transcriptional, and replication events in pestiviruses (Harasawa and Giangaspero, 1998; Giangaspero and Harasawa, 2007). Within pestiviruses, PNS sequence characterization easily discriminated officially defined species as well as atypical species as Giraffe, Pronghorn, or Bungowannah (Harasawa *et al.*, 2000; Giangaspero and Harasawa, 2008, 2011) or helped to clarify erroneous classification of strains into genotypes (Giangaspero *et al.*, 2018). In the present study, in order to revise the classification of the BVDV-1 species and determine genotypic variations in the species, the 5′-UTR genomic region of the BVDV-1 strains reported as 1l, 1v, and 1w have been analyzed with the PNS procedure.

Material and Methods

Strain sequences

In order to determine genotypic variations in the BVDV-1 species, the 5′-UTR genomic region nucleotide sequences (derived from Sanger dideoxy sequencing technology) of 149 *Pestivirus* strains, have been analyzed for numerical taxonomy, considering the 3 variable loci at the level of the 5′-UTR, according to the PNS genotyping method, and focusing comparison of sequence characteristics between bovine strains recently reported from China and Turkey with the others. The virus nucleotide sequences, with different geographical origin from different host species or contaminants of biological products, were obtained from the DDBL/EMBL/GenBank DNA database (Table 1). The majority of the tested virus sequences originated from strains isolated from cattle (*Bos taurus*) from China (n = 29) (Deng *et al.*, 2020; Shi *et al.*, 2020; Tian *et al.*, 2021; Zhu *et al.*, 2022; from Turkey (n = 25) (Oguzoglu *et al.*, 2019; Timurkan and Aydin, 2019; and from Iran (n = 5). In addition, two strains were isolated in small ruminants (*Capra hircus*) from China (Mao *et al.*, 2016). BVDV-1 species strains (n = 88), reference for the different genotypes reported in the species, previously analyzed by the PNS method (Giangaspero and Apicella, 2014; Giangaspero *et al.*, 2018, 2019b) have also been evaluated in the present study, in order to verify the allocation in the BVDV-1 species.

Secondary structure analysis

Qualitative and quantitative evaluation of genomic sequence divergence, in terms of palindromic nucleotide base pairings variations, has been applied for taxonomical segregation, through the evaluation of relevant secondary structure regions in the 5′-UTR of the viral RNA, the three variable regions, V1, V2, and V3 genomic sequences, according to the genotyping based on the PNS method (Harasawa and Giangaspero, 1998; Giangaspero and Harasawa, 2007). Secondary structures were obtained for the entire 5′-UTR sequence of each strain. Palindromic sequences corresponding to the IRES three variable loci were identified in the predicted secondary structure and considered out of the rest of the nucleotide sequence. Nucleotide sequence secondary structures were predicted according to the algorithm of Zuker and Stiegler (1981) using the Genetyx-Mac version 10.1 program package (Software Development Co., Ltd., Tokyo, Japan). The minimum free energy was calculated by the

Table 1. List of BVDV-1 species genotypes strains (n 149) evaluated according to palindromic secondary structure characteristics at the RNA 5′-UTR (PNS method).

Genotype	Strain	Origin	Country	Year	Accession	Reference
1a	NADL	Cattle	USA	1963	M31182	Collett *et al.*, 1988
1a	Singer	Cattle	USA	1993	L32875	Pellerin *et al.*, 1994
1a	SD-1	Cattle	USA		M96751	Deng and Brock, 1992
1a	Weybridge	Sheep	UK		U65024	Vilček *et al.*, 1997
1a	Oregon C24V	Cattle	USA	1993	L32876	Pellerin *et al.*, 1994
1b1	Draper	Cattle	USA	1993	L32880	Pellerin *et al.*, 1994
1b1	NY-1	Cattle	USA	1993	L32879	Pellerin *et al.*, 1994
1b1	Sanders	Cattle	USA		L20928	Qi *et al.*, 1993
1b2	24-15	Cattle	UK		AF298060	Vilček *et al.*, 2001
1b2	Osloss	Cattle	USA	1965	M96687	De Moerlooze *et al.*, 1993
1b2	QL1903	Cattle	China	2019	MN849041	Tian *et al*., 2021
3 (d)	Europa	Human	Belgium	1989	AB000898	Giangaspero *et al.*, 1997
3 (d)	F	Cattle	Austria		AF298065	Vilček *et al.*, 2001
3 (d)	16-111	Cattle	France		AF298056	Vilček *et al.*, 2001
4 (b)	438/02	Cattle	Spain	2002	AY159540	Hurtado *et al.*, 2003
4 (b)	PT42-03	Cattle	Portugal	2003	AY944293	Barros *et al.*, 2006
5 (i)	23-13	Cattle	UK		Not deposited	Vilček *et al.*, 2001
5 (i)	23-15	Cattle	UK		AF298059	Vilček *et al.*, 2001
6 (n)	So CP/75	Cattle	Japan	1975	AB359929	Nagai *et al.*, 2008
6 (n)	Shitara-02-06	Cattle	Japan	2006	LC089876	Sato *et al.*, 2016
7.1 (o)	JS10116	Pig	China	2010	JN248734	Deng *et al.*, 2012
7.1 (o)	AQGN96B15	Cattle	Japan	1996	AB300691	Yamamoto *et al.*, 2008
7.1 (o)	Camel isolate 9	Bactrian camel	China	2010	JX276546	Gao *et al.*, 2013
7.1 (o)	IS25CP/01	Cattle	Japan	2001	AB359931	Nagai *et al.*, 2008
7.1 (o)	IS26/01ncp	Cattle	Japan	2001	AB359932	Nagai *et al.*, 2008
7.2 (o)	BJ09_24	Cattle	China	2009	HQ116550	Zhang *et al.*, 2014b
7.2 (o)	S121	Cattle	China	2013	KF006960	Zhang *et al.*, 2014b
7.2 (o)	S43	Cattle	China	2013	KF006959	Zhang *et al.*, 2014b
7.3 (o, v)	BJ09-26	Cattle	China	2009	HQ116551	–
7.3 (o, v)	EN-6	Cattle	China	2017	MN417813	Deng *et al.*, 2020
7.3 (o, v)	EN-7	Cattle	China	2017	MN417814	Deng *et al.*, 2020
7.3 (o, v)	EN-8	Cattle	China	2017	MN417815	Deng *et al.*, 2020
7.3 (o, v)	EN-9	Cattle	China	2017	MN417816	Deng *et al.*, 2020
7.3 (o, v)	EN-19	Cattle	China	2017	MN417826	Deng *et al.*, 2020
7.3 (o, v)	T4-32	Cattle	China	2017	MN417862	Deng *et al.*, 2020
7.3 (o, v)	T4-31-2	Cattle	China	2017	MN417919	Deng *et al.*, 2020
7.3 (o, v)	GA190608	Cattle	China	2019	MT933204	Tian *et al.*, 2021
7.3 (o)	HA2-12	Goat	China	2013	KP749802	Mao *et al.*, 2016
7.3 (o)	JS12/02	Goat	China	2013	KP749794	Mao *et al.*, 2016
7.3 (o)	XH-1	Cattle	China	2016	KY865374	–
7.3 (o)	XH-2	Cattle	China	2016	KY865375	–

Continued

Genotype	Strain	Origin	Country	Year	Accession	Reference
7.3 (o)	XH-5	Cattle	China	2016	KY865376	–
7.3 (o)	XH-6	Cattle	China	2016	KY865377	–
7.3 (o)	MF-3	Cattle	China	2016	KY865371	–
7.3 (o)	HY-3	Cattle	China	2016	KY865366	–
7.3 (o, v)	HN1814	Cattle	China	2018	MN442377	Shi *et al*., 2020
7.3 (o)	HN1626	Cattle	China	2016	MN442366	Shi *et al.*, 2020
7.3 (o)	HN1641	Cattle	China	2016	MN442367	Shi *et al.*, 2020
7.3 (o)	HN1732	Cattle	China	2017	MN442373	Shi *et al.*, 2020
7.3 (o)	HN1736	Cattle	China	2017	MN442374	Shi *et al.*, 2020
7.3 (o)	HN1852	Cattle	China	2018	MN442379	Shi *et al.*, 2020
7.3 (o)	HN1859	Cattle	China	2018	MN442380	Shi *et al.*, 2020
7.3 (o)	HN1864	Cattle	China	2018	MN442381	Shi *et al.*, 2020
7.3 (o)	HN1918	Cattle	China	2019	MN442383	Shi *et al.*, 2020
7.3 (o, v)	HB-03	Cattle	China	2017	ON901785	Zhu *et al*., 2022
8 (c)	Bega	Cattle	Australia		AF049221	–
8 (c)	CRFK	Contaminant	Japan	1995	D50814	Harasawa and Mizusawa, 1995
8 (c)	Manasi	Cattle	China	2006–2008	EU159702	Zhong *et al.*, 2011
9 (h)	G	Cattle	Austria	1998	AF208066	Vilček *et al.*, 2001
9 (h)	KM	Cattle	Slovakia		AF298068	Vilček *et al.*, 2001
10 (q)	SD0803	Pig	China	2008	JN400273	Deng *et al.*, 2014
10 (q)	Camel-6	Bactrian camel	China	2010	KC695810	Gao *et al.*, 2014
11 (e)	3186V6	Cattle	Italy		AF298062	Vilček *et al.*, 2001
11 (e)	10-84	Cattle	France	1984	AF298054	Vilček *et al.*, 2001
12.1 (f, s)	22146/81	Cattle	Germany	1981	AJ304376	Tajima *et al.*, 2001
12.1 (f, s)	UM136/08	Cattle	Italy	2008	LM994673	Giammarioli *et al.*, 2015
12.1 (f, s)	Mousedeer	Mousedeer	Denmark	2002	AY158154	Grondahl *et al.*, 2003
12.1 (f, s)	2561	Cattle	UK	2009	JQ920287	Strong *et al.*, 2013
12.2 (f, r)	11207/98	Cattle	Germany	1998	AJ304390	Tajima *et al.*, 2001
12.2 (f, r)	4998/89	Cattle	Germany	1989	AJ304385	Tajima *et al.*, 2001
12.2 (f, r)	51/06	Cattle	Poland	2006	JN715039	Kuta *et al.*, 2013
12.2 (f, r)	CA/181/10	Cattle	Italy	2010	LM994672	Giammarioli *et al.*, 2015
12.2 (f, r)	VE/245/12	Cattle	Italy	2012	LM994671	Giammarioli *et al.*, 2015
12.2 (f, r)	79/11	Cattle	Italy	2011	KY040384	Lanave *et al.*, 2017
13 (j)	17P	Cattle	Argentina	1991	AF244954	Jones *et al.*, 2001
13 (j)	KS86-1ncp	Cattle	Japan	2002	AB042713	Nagai *et al.*, 2001
13 (j)	Deer	Deer	UK	1986	AB040132	Becher *et al.*, 1997
1.14 (r)	TR70	Cattle	Turkey	2007	MG670547	Giangaspero *et al.*, 2018
1.14 (r)	TR75	Cattle	Turkey	2007	MG670549	Yesilbag *et al.*, 2017
15.1 (m)	ZM-95	Pig	China	1995	AF526381	Xu *et al.*, 2006
15.1 (m)	BJ1305	Cattle	China	2013	KF925505	Weng *et al.*, 2015
15.1 (m)	TJ0801	Cattle	China	2008	GU120255	Xue *et al.*, 2010
15.2 (m)	HB-060111	Cattle	China	2011	KJ578822	Deng *et al.*, 2015
1.16 (l)	TR-2007-Gu-175454-4695	Cattle	Turkey	2007	EU716150	Oguzoglu *et al.*, 2012

Continued

Genotype	Strain	Origin	Country	Year	Accession	Reference
1.16 (l)	TR16	Cattle	Turkey	2007	MG670548	Yesilbag *et al.*, 2017
1.16 (l)	TR72	Cattle	Turkey	2007	MG670546	Yesilbag *et al.*, 2017
1.16 (l)	TR72	Cattle	Turkey	2007	MG670548	Giangaspero *et al.*, 2018
17 (f)	J	Cattle	Austria	1998	AF298067	Vilček *et al.*, 2001
17 (f)	W	Cattle	Austria	1998	AF298073	Vilček *et al.*, 2001
18 (p)	BJ0701	Cattle	China	2007	GU120247	Xue *et al.*, 2010
18 (p)	BJ0702	Cattle	China	2007	GU120248	Xue *et al.*, 2010
18 (p)	BJ0703	Cattle	China	2007	GU120249	Xue *et al.*, 2010
18 (p)	TJ06	Cattle	China	2006	GU120246	Xue *et al.*, 2010
18 (p)	XY-3	Goat	China	2013	KP749796	Mao *et al.*, 2016
19 (w)	T6-18	Cattle	China	2017	MN417892	Deng *et al.*, 2020
19 (w)	T6-20	Cattle	China	2017	MN417893	Deng *et al.*, 2020
20 (g)	A	Cattle	Austria	1998	AF298064	Vilček *et al.*, 2001
20 (g)	L	Cattle	Austria	1998	AF298069	Vilček *et al.*, 2001
21 (v)	TR-Erz-BV8	Cattle	Turkey	2016–2017	MG913800	Timurkan and Aydin, 2019
21 (v)	TR-Erz-BV7	Cattle	Turkey	2016–2017	MG913799	Timurkan and Aydin, 2019
21 (v)	TR/Elz-6-2021	Cattle	Turkey	2020	MZ686435	–
21 (v)	TR/Elz-4-2021	Cattle	Turkey	2020	MZ686434	–
21 (v)	TR-Erz-Pst8	Cattle	Turkey	2016–2017	MG973223	Timurkan and Aydin, 2019
21 (v)	IR-Shiraz-317	Cattle	Iran	2013	LC053994	–
21 (v)	IR-Shiraz-322	Cattle	Iran	2013	LC053995	–
21 (v, 1a)	IRTV2	Cattle	Iran	2019	MW431323	–
21 (v, 1a)	IRTV1	Cattle	Iran	2019	MW431322	–
21 (v, 1a)	IREE1	Cattle	Iran	2019	MW431320	–
21 (v)	TR-Elz-Pst16	Cattle	Turkey	2016–2017	MG973231	Timurkan and Aydin, 2019
21 (v)	TY8723	Cattle	Turkey	2017	MH673456	Oguzoglu *et al.*, 2019
21 (v)	TY8856	Cattle	Turkey	2017	MH673457	Oguzoglu *et al.*, 2019
21 (v)	TY6943	Cattle	Turkey	2017	MH673458	Oguzoglu *et al.*, 2019
21 (v)	TY7414	Cattle	Turkey	2017	MH673459	Oguzoglu *et al.*, 2019
21 (v)	TY8761	Cattle	Turkey	2017	MH673460	Oguzoglu *et al.*, 2019
21 (v)	TY5670	Cattle	Turkey	2017	MH673461	Oguzoglu *et al.*, 2019
21 (v)	TY5968	Cattle	Turkey	2017	MH673462	Oguzoglu *et al.*, 2019
21 (v)	TY8815	Cattle	Turkey	2017	MH673463	Oguzoglu *et al.*, 2019
21 (v)	TY5295	Cattle	Turkey	2017	MH673464	Oguzoglu *et al.*, 2019
21 (v)	TY4263	Cattle	Turkey	2017	MH673465	Oguzoglu *et al.*, 2019
21 (v)	TY5267	Cattle	Turkey	2017	MH673466	Oguzoglu *et al.*, 2019
21 (v)	TY4145	Cattle	Turkey	2017	MH673467	Oguzoglu *et al.*, 2019
21 (v)	TY3661	Cattle	Turkey	2017	MH673468	Oguzoglu *et al.*, 2019
21 (v)	TY3686	Cattle	Turkey	2017	MH673469	Oguzoglu *et al.*, 2019
21 (v)	TY3002	Cattle	Turkey	2017	MH673470	Oguzoglu *et al.*, 2019

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Genotype	Strain	Origin	Country	Year	Accession	Reference
21 (v)	TY3716	Cattle	Turkey	2017	MH673471	Oguzoglu *et al.*, 2019
21 (v)	TY3659	Cattle	Turkey	2017	MH673472	Oguzoglu *et al.*, 2019
21 (v)	TY10	Cattle	Turkey	2017	MH673473	Oguzoglu *et al.*, 2019
21 (v)	TY1000	Cattle	Turkey	2017	MH673474	Oguzoglu *et al.*, 2019
22 (l, x)	71-03	Cattle	France	2005	KF205294	Jackova *et al.*, 2008
22 (l, x)	CH-01-08	Cattle	Switzerland	1995–2006	EU180024	Bachofen *et al.*, 2008
23.1 (u)	130/15-4215	Cattle	Italy	2015	KY085998	Lanave *et al.*, 2017
23.1 (u)	130/15-5364	Cattle	Italy	2015	KY085999	Lanave *et al.*, 2017
23.2 (u)	M31182	Yak	China	2010	JQ799141	–
23.2 (u)	441/09	Cattle	Italy	2009	KY040367	Lanave *et al.*, 2017
23.2 (u)	GXBH-EB34	Buffalo	China	2013	KJ578813	Deng *et al.*, 2015
23.2 (u)	JS-00108	Cattle	China	2009	KJ578848	Deng *et al.*, 2015
23.2 (u)	QHQL-252	Yak	China	2012	KJ578884	Deng *et al.*, 2015
23.2 (u)	LN309-5	Cattle	China	2012	KJ578803	Deng *et al.*, 2015
23.2 (u)	GXCZ-FB22	Buffalo	China	2013	KJ578807	Deng *et al.*, 2015
23.2 (u)	GXLZ-BB4	Buffalo	China	2013	KJ578814	Deng *et al.*, 2015
23.2 (u)	HB-090166	Cattle	China	2011	KJ578836	Deng *et al.*, 2015
23.2 (u)	JS-03148	Cattle	China	2009	KJ578850	Deng *et al.*, 2015
23.2 (u)	JS-03198	Cattle	China	2009	KJ578851	Deng *et al.*, 2015
23.2 (u)	JS-04138	Cattle	China	2009	KJ578852	Deng *et al.*, 2015
23.2 (u)	NMG311-20	Cattle	China	2012	KJ578866	Deng *et al.*, 2015
24 (k)	Rebe	Cattle	Switzerland		AF299317	Stalder *et al.*, 2005
24 (k)	SuwaCp	Cattle	Switzerland	1999	AF117699	Schweizer and Peterhans, 1999
24 (k)	SuwaNcp	Cattle	Switzerland	2000	KC853440	Marques Antunes de de Oliveira *et al.*, 2013
25 (p)	S153	Cattle	China	2013	KF006964	Zhang *et al.*, 2014b

Nomenclature of identified genotypes is based on divergence in the genus. Clustering according to primary sequence analysis as Vilček *et al.* (2001) is indicated under parenthesis.

method of Freier *et al.* (1986). The PNS software version 2.0 (Giangaspero and Apicella, 2014), prepared for the application on the genotyping procedures with the keys for *Pestivirus* identification of genomic sequences, using the C# programming language, was also applied for the construction of secondary structure sequence alignment, in order to compute genetic distance among strains. Segregation of BVDV-1 species strains into genotypes and relatedness among genotypes within the species was evaluated according to changes in nucleotide bps at the level of the secondary palindromic structure of the three variable loci. Genotypes were identified according to bp combinations at the level of low-variable positions (LVPs), and ranked according to increasing divergence in the genus (group reference strain value), with reference to prevalent bps in prevalent positions, the most common sequences observed, constituting the homogeneous core group within the genus. Thus, the numeric order of the nomenclature reflected genotypes' level of divergence within the species. Among genotypes, homology was evaluated in terms of shared bps in the three variable loci in the 5′-UTR. Cross comparison between groups within the genus has been evaluated by computing the divergence percentage, identifying strains showing multiple relation (sequences sharing base pairings specific to different genomic groups, and scoring low divergence values) or borderlines (sequences showing qualitative similarities with a genomic group, but with high divergence values, candidates for reallocation as separate groups in the genus), and indicating divergence within groups and

among groups quantifying the heterogeneity of a genotype and the genetic distance between groups.

Primary sequence analysis

A phylogenetic tree based on the 5′-UTR was constructed following sequence alignment with Clustal X (Thompson *et al.*, 1997) by using the neighbor-joining method (Saitou and Nei, 1987). In addition, a basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov) web-based sequence analysis tool with default values was used to find homologous hits for the 5′-UTR sequence analysis of the considered strains, reported as genotypes v and w. The classification among BVDV-1 strains according to the PNS analysis based on changes in the secondary structure was compared with those based on primary structure of the 5′-UTR performed through sequence alignment and construction of phylogenetic trees by all other authors reporters of the strains considered in the present study and named according the nomenclature proposed by different authors as Vilček *et al.* (2001), with 11 genotypes, and subsequent further application by others authors.

Ethical approval

The local ethics committee was consulted and deemed full ethical approval unnecessary.

Results

The observation made on the nucleotide sequences allowed the identification of consensus motifs shared by all the *Pestivirus* species, genus-specific base-pairings (10 PNS positioned in the V1 and V2 loci), and characteristic species and genotype-specific PNS. Based on the divergence limit value of 9 bp for genotype determination (Giangaspero and Harasawa, 2007), 25 genotypes within the BVDV-1 species have been identified, from 1a to 1.25. The geographical distribution of the newly described atypical genotypes BVDV-1.19 and BVDV-1.21 is presented in Figure 1, with the list of all other genotypes reported in the concerned countries (including Italy and Japan for comparison), and the year of collection. Secondary sequence construction, efficiently obtained by both available software, Genetyx and PNS (Giangaspero and Apicella, 2014), revealed a conserved palindromic structure in the species. Different base pairing combinations were

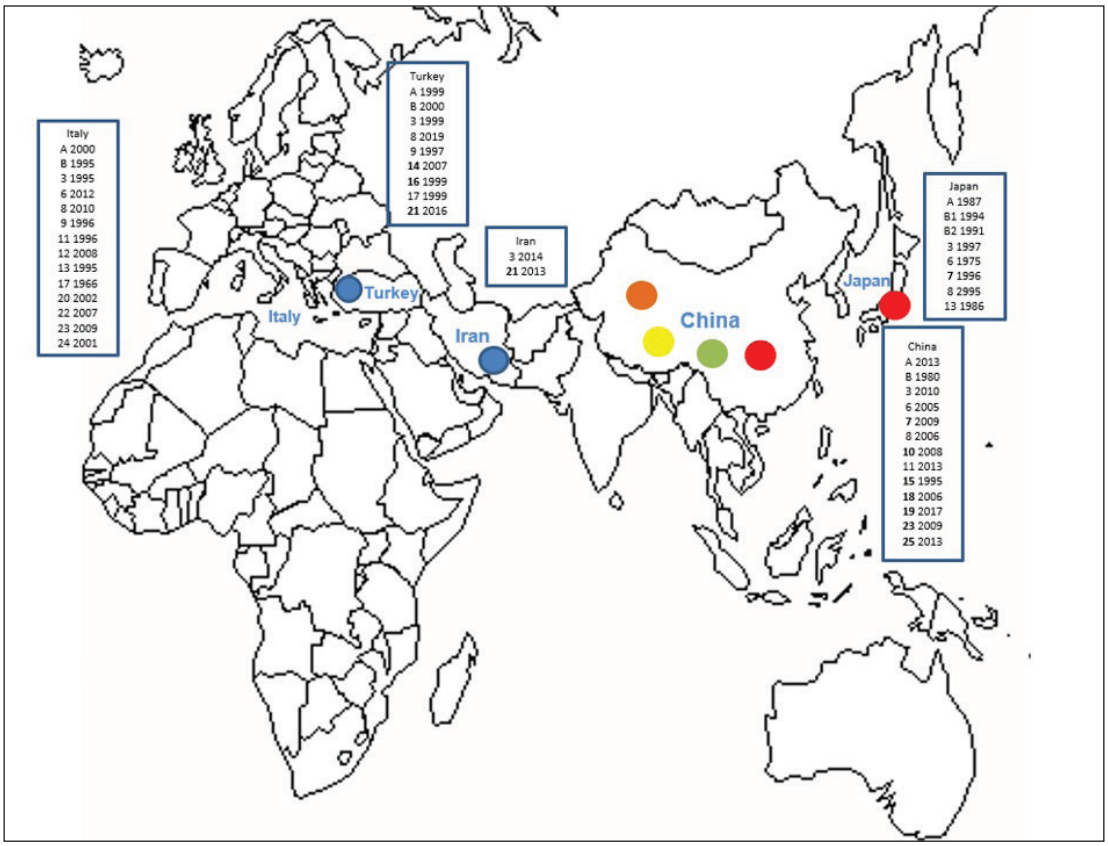

Fig. 1. Geographic distribution of the atypical genotypes of the BVDV-1 species 1.19 (1w) (brown circle), 1.21 (1v) (blue circle), and 1.7 sub genotypes 7.1, 7.2, and 7.3 (red, green, and yellow circles, respectively). The other genotypes reported from Turkey, Iran, and China are listed with the year of first isolation. Studies on BVDV in Iran were mainly based on seroprevalence, not completed by genetic characterization of isolates. Data were compared to the occurrence of the different genotypes reported in Italy and Japan. BVDV-1 genotypes 1.4 and 1.5 were reported as 1b and 1i only in UK, Spain, Portugal, and Egypt.

Table 2. Alignment of BVDV-1 species genotypes variable loci 5'-UTR RNA secondary structure sequences, segregated according to types of bp combinations.

Variable locus		V1																	V2								V3								
Position		1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)	
Prevalent base pairs		**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**		**YG**	**YG**	**UA**	**GY**			**GC**	**AU**		**CG**		**GY**	**UA**				**A**		
BVDV1		**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**		
Genotype BVDV-1a																																								**0.83**
BVDV-1	Singer	.									CG		UA			GU	AA	-	-	-	AU					AC	GC	.	.	GU		AU			UG	CC	UC	AA	1	
BVDV-1	NADL	.									CG		UA			GU	AA	-	-	-	AU					AC	GC	.	.	GU		AU			UG	UC	UC	AA	1	
BVDV-1b1																																								**0.34**
BVDV-1	Draper	.									UA		CC			GG	AG	-	-	-	AU					GU	AU	.	.	AC		GC				UA	GU	AC	1	
BVDV-1	Sanders	.					AA				UA		UU			GG	AG	-	-	-	AU					GU	AU		.	AC		GC				UC	AC	.	1	
BVDV-1b2																																								**0.89**
BVDV-1	Osloss	.				G					UA		CC			UG	GU	-	-	-	AU					AU	GU	.	.	GC		GC				UC	AC	.	1	
BVDV-1.3	**(D)**																																							**0.57**
BVDV-1	Europa	.								AU	CG		CA	GG	GA	AA		-	-	-	AU					GC	AC	.	.	GU		AU				UU	GA	.	0	
BVDV-1.4																																								**0**
BVDV-1	438/02	.									CG		CC			GG	AG	-	-	-	AU					AU	AU	.	.	GC		GC				UC	AC	.	0	
BVDV-1.5	**(I)**																																							**0.75**
BVDV-1	23-13	.									CG		UA			GA	UC	-	-	-	GU			CA		GU	GC	.	.	AC		GC			UG	UU	GU	.	1	
BVDV-1.6	**(N)**																																							**0.86**
BVDV-1	so CP/75	.								AU	CG		AU	UG	GC	GG	U	**-**	**-**		GU					GU	GC	.	.	GC		GU				UC	AC	.	1	
BVDV-1.7	**(O)**																																							**1.66**
BVDV-1.7.1	**(O)**																																							**1.25**
BVDV-1	JS10116	.									CG		CG	AU	AU	GG		-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AU	.	1	
BVDV-1	AQGN96BI5	.									CG		CG	AC	AU	GG		-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AA	.	1	
BVDV-1	Camel isolate 9	.									CG		CG	GU	AU	GG		-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AU	.	1	
BVDV-1	IS25CP01	.								AU	CG		CG	AU	AU	GG		-	-	-	GU	UA				GU	GC	AC	.	GU		GC				GC	AU	G	2	
BVDV-1.7.2	**(O)**																																							**1.33**
BVDV-1	BJ09_24	.									CG		UU	AC	GU	GA		-	-	-	AU	UA				GU	GC	.	.	GU		GC				GC	GC	.	1	
BVDV-1	S43	.									CG		UU	AC	GU	GA		-	-	-	AU	UA				GU	GC	.	.	GU		GC			AU	GC	GC	.	1	
BVDV-1	S121	.									CG		UU	AC	GU	GA		-	-	-	AU	UA				GU	GC	AC	.	GU		GC				GC	GC	.	2	
BVDV-1.7.3	**(O, V)**																																							**1.91**
BVDV-1 (v)	T4-32	.									CG		UU	AU	GU	GA		-	-	-	GU	UA				GU	GC	.	.	GU		GC				AC	AC	.	1	
BVDV-1 (v)	T4-31-2	.									CG		UU	AU	GU	GA		-	-	-	GU	UA				GU	GC	.	.	GU		GC				AC	AC	G	2	
BVDV-1 (o)	JS12/02	.									CG		UU	AU	GU	GA		-	**-**	**-**	GU	UA				AU	GC	.	.	GU		GC				AC	AC	.	1	
BVDV-1 (v)	EN-6	.									CG		UU	AU	GU	GA		-	-	-	GU			UG		AU	GC	.	.	GU		GC				AC	AC	.	1	
BVDV-1 (v)	GA190608	.									CG		UU	AU	GU	GA		-	-	-	GU	UA		UU		AU	GC	.	.	GU		GC				AC	AC	.	2	
BVDV-1 (v)	BJ09_26	.									CG		UU	AU	GU	GA		-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2	
BVDV-1 (o)	HY-3	.									CG		UU	AC	GU	GA		-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2	
BVDV-1 (v)	HB-03	.									CG		UU	AU	GA	AA		-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2	
BVDV-1 (o)	HN1626	.									CG		UU	AU	GU	GA		-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				GC	AC	.	2	
BVDV-1 (o)	XH-1	.									CG		UU	AU	GU	GA		-	-	-	GU	UA		UG		AU	GC	.		GU		GC				GC	AA	.	2	
BVDV-1 (o)	MF-3 (V1/4 AC)	.									CG		UU	AU	GU	GA		-	-	-	GU	UA		UG	GA	AU	GC	.	.	GU		GC				GC	AA	.	4	

Continued

Variable locus											V1													V2								V3											
Position		1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22		1	2	3	4	5	6	7	9		1	2	3	4	5	6	7	8	9	10		(*)	
Prevalent base pairs		**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**			**YG**	**YG**	**UA**	**GY**			**GC**		**AU**		**CG**		**GY**	**UA**				**A**			
BVDV1		**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**			**CG**	**CG**	**UA**	**GC**			**GC**		**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**			
BVDV-1.8	**(C)**																																										**1.90**
BVDV-1	Bega	.								AC		CG		UA	GC	GA	AA	AG	-	-		AU					GU	GC	.		.	GU		AU			CA	UC	AU	.		1	
BVDV-1.9	**(H)**																																										**1.46**
BVDV-1	KM	.										CG		UU	AC	AA	UA	GA	-	-		AU					CU	GC	.		.	GU		GC			AG	UU	AA	.		1	
BVDV-1.10	**(Q)**																																										**1.86**
BVDV-1	SD0803	.										CG	AA	GG	AU	GA	-	-	-	-		AC	UA				GU	GC	.		.	GU		GC				GC	UC	.		1	
BVDV-1.11	**(E)**																																										**1.58**
BVDV-1	26-V639	.										CG		AU		GU	AC	-	-	-		GU					AU	GC	.		.	GU		GC			CG	GA	AU	.		0	
BVDV-1.12	**(F, R, S)**																																										**2.20**
BVDV-1.12.1	**(F, S)**																																										**2**
BVDV-1	22146/81	.										CG		GC	UA	AC	GG	-	-	-		AU					AU	GC	AC		.	GU		GC		GC		AU	AC	.		2	
BVDV-1	UM136/08	.										CG		GC	UA	AC	GG	-	-	-		AU					AC	GC	AC		.	GU		GC		GC	GA	AC	AC	.		2	
BVDV-1	mousedeer	.										CG		GC	UA	GA	GG	-	-	-		AU					AU	GU	AC		.	GU		GC		GC		AU	AC	.		2	
BVDV-1	2561	.										CG		GC	CG	GC	GG	-	-	-		AU					AU	GU	AC		.	GU		GC		GC		AU	AC	.		2	
BVDV-1.12.2	**(F, R)**																																										**2.30**
BVDV-1	11207/98	.										CG		UU	AU	GU	AA	-	-	-		GU					GC	AU	AC		.	GU		GC		GA		AC	AC	.		2	
BVDV-1	51/06	.										CG		UU	AU	GU	AA	-	-	-		AU					GC	AU	AC		.	GU		GC		GA		AU	AC	.		2	
BVDV-1	4998/89	.										CG		UU	AU	GU	AG	-	-	-		AU					GC	GU	AC		.	GU		GC		GA	AA	AC	AC	.		2	
BVDV-1	CA18110	.										UG		AU	GU	AA	-	-	-	-		AU					GC	AU	AC		.	GU		GC		GA		GC	AU	.		2	
BVDV-1	VE24512	.								AU		CG		UU	AU	GU	AA	-	-	-		AU					GC	AU	AC		.	GU		GC		GA	CA	-	-	-		3	
BVDV-1	79/11	.										CG		UU	AU	GU	GA	-	-	-		AU					GC	AU	AC		.	GU		GC		GA	CA	-	-	-		3	
BVDV-1.13	**(J)**																																										**1.86**
BVDV-1	Deer	.			UU							CG		CG		GU	AA	A	-	-		AU					AU	GC	.		.	GU		GC				CC	AC	.		2	
BVDV-1.14	**(R)**																																										**2**
BVDV-1	TR70	.										CG		AU		GG	AA	-	-	-		AU				AU	AU	GC	.		.	GU		AU				UU	UA	-		2	
BVDV-1	TR75	.										CG		AU		GG	AG	-	-	-		AU				AU	AU	GC	.		.	GU		AU				UU	AA	-		2	
BVDV-1.15	**(M)**																																										**3.29**
BVDV-1.15.1	**(M)**																																										**3.23**
BVDV-1	ZM-95	.										CG		UG	AU	GC	GU	A	-	-		UA	UA		CG		GC	AC	.		.	GU		GC				GU	UC	.		3	
BVDV-1.15.2	**(M)**																																										**3.50**
BVDV-1	HB-060111	.										CG		UA	AU	GC	CU	A	-	-		AU			CG	AU	GU	GC	.		.	GU		AU				GU	CC	.		3	
BVDV-1.16	**(L)**																																										**3**
BVDV-1	TR72	.								AU		CG		AU	GU	GA	AC	-	-	-		GU					UA	GC	AC		.	GC		GC			CU	UC	UC	AA		2	
BVDV-1	TR-2007-Gu (V1/5 UC)	.	UC							AU		CG		AU	GU	GA	AC	-	-	-		GU					UA	GC	AC		.	GC		GC			CU	UC	AC	.		3	
BVDV-1	TR16	GA	UU							AU		CG		AU	GU	GG	AU	-	-	-		GU					UA	GC	AC		.	GC		GC		CA		UU	UC	.		4	
BVDV-1.17	**(F)**																																										**3**
BVDV-1	S	.										CG		UU	AA	GU	GA	-	-	-		AU					AC	GC	AC		.	GU		GC		AA	UU	AA	AA	-		3	
BVDV-1.18	**(P)**																																										**3**
BVDV-1	**TJ06**	.										CG		UA	AU	GU	GU	A	-	-		AU					GC	AC	.		.	GU	UA	GC			AA	AA	AU	G		2	
BVDV-1	BJ0701	.										CG		UA	AU	GU	GU	G	-	-		AU					GC	GC	.		.	GU	UA	GC				AA	AU	G		3	
BVDV-1 (p)	XY-3	.										CG		UA	AU	GC	GU	A	-	-		AU					GC	AC	.		.	GU	UA	GC				GA	AU	G		3	

Continued

Variable locus											V1														V2										V3							
Position		1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10		(*)	
Prevalent base pairs		**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**		**YG**	**YG**	**UA**	**GY**			**GC**	**AU**		**CG**		**GY**	**UA**				**A**			
BVDV1		**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**			
BVDV-1.19	**(W)**																																								3
BVDV1 (w)	T6-18 (V1/10 UA)	AU										CG	CG	GC	AU	UU	UG	-	-	-	AU					CG	GU	.	.	GU		GC				AC	AC	.		3	
BVDV-1.20	**(G)**																																								**3.50**
BVDV-1	A	.										CG	CG	CC	AG	GU	AA	-	-	-	AU				AU	GU	GC	AC	.	GU		GC		GA	AA	GU	AC	.		4	
BVDV-1.21	**(V)**																																								**3.83**
Prevalent base pairs		**GY**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GY**		**CG**		**UA**					**-**	**-**	**-**		**YG**	**YG**	**UA**	**GY**			**GC**	**AU**		**CG**		**GY**	**UA**				**A**			
BVDV-1	TR-Erz-Pst8 (V1/11 AC)	.										CG		AU		GA	AG	-	-	-	GU					UA	GU	AC	.	GU		AU		UG	CA	UC	AC	.		3	
BVDV-1 (v)	TRElz-4-2021	.										CG	CG	UA	AU	GA	GA	A	-	-	GU					UA	GU	AC	.	AC		AU			CA	UC	AC	.		3	
BVDV-1 (v)	IR-Shiraz-322	.										CG	CG	UA	AU	GA	GA	G	-	-	GU					UA	GU	AC	.	GC		AU		UG	CA	UC	GC	.		4	
BVDV-1 (v)	TY8723	.										CG	UG	UA	AU	AA	GA	A	-	-	GU					UA	GU	AC	.	GU		AU		UG	CA	UC	AC	.		4	
BVDV-1 (a)	IRTV1	.										CG	CG	UA	AU	GA	GA	A	-	-	GU					UA	GU	AC	.	GC		AU		CG	CA	UC	AC	.		4	
BVDV-1	TR-Elz-Pst16 (V1/11 GC)	.								AU		CG	CG	UA	AU	GA	GA	A	-	-	GU					UA	GU	AC	.	GU		AU		UG	CA	UC	AC	.		5	
BVDV-1.22	**(L, X)**																																								**5.75**
BVDV-1.22.1	**(L, X)**																																								**5.66**
BVDV-1	**CH-01-08**	.		AU								CG	CG	AC	AA	GG	AA	-	-	-	AU		UA	CG	AU	UA	GC	AC	.	GC		AU			UG	CA	GC	.		6	
BVDV-1	71-15	.		AU								CG	CG	AC	AA	GG	AA	-	-	-	GU		UA	CG	AU	UG	GC	.	.	GC		AU				UA	AC	.		5	
BVDV-1.22.2	**(L)**																																								**6**
BVDV-1	PG13a07	.										CG	CA	AC	AA	GG	AA	-	-	-	AU		UA	CG	AU	AG	GC	AC	.	GU		GC		GA	GC	UC	AA	.		6	
BVDV-1.23	**(U)**																																								**5.37**
BVDV-1.23.1	**(U)**																																								**3**
BVDV-1	130/15-5364	.								AU		UA	UG	GC	AA	AA	AU	-	-	-	GC					AC	GC	.	.	AU		GA	CG		UG	UC	UC	CC		3	
BVDV-1.23.2	**(U)**																																								**5.71**
BVDV-1	**m31182**	.				G				AU		UA	UG	GC	AA	AA	AU	-	-	-	GC					AC	GC	.	UU	GU		UA	CG	UG		CG	CA	CG	U	7	
BVDV-1	441/09	.								AU		UA	UG	GA	AA	AA	AU	-	-	-	AC					AC	GC	.	.	GU		UA	UG	UG		CG	CA	CG	U	4	
BVDV-1	GXBH-EB34 (V1/10 GA)	.										UA		GC	UA	AU	AC	-	-	-	GC					AC	GC	.	.	GU		UA	CG	UG		CG	CA	CG	U	5	
BVDV-1	JS-00108 (V1/10 GG)	.									CA	UA		GC	UA	AU	AC	-	-	-	GC					AC	GC	.	.	GU		UA	CG	UG		CG	CA	CG	U	6	
BVDV-1	QHQL-252 (V1/10 GA)	.									CA	UA		GC	UA	AU	AU	-	-	-	GC					AC	GC	.	.	GU		UA	CG	UG		CG	CA	CG	U	6	
BVDV-1	LN309-5 (V1/10 GA)	.									CA	UA		GC	UA	AU	AC	-	-	-	GC					AC	GC	.	.	GU		UA	CG	UG		CG	CA	CG	U	6	
BVDV-1	GXCZ-FB22 (V1/10 GA)	.									CA	UA		GC	UA	AU	AC	-	-	-	GC					AC	GC	.	.	GU		UA	CG	UG	GA	CG	CA	CG	U	6	
BVDV-1.24	**(K)**																																								**6**
BVDV-1.24.1	**(K)**																																								**3**
BVDV-1	CH-05-b1	.										CG		AG	UA	GC	GA	A	-	-	GU		UA			GC	AC	.	.	GC	UA	GC			CA	UC	GC	.		3	
BVDV-1.24.2	**(K)**																																								**6.75**
BVDV-1	**SuwaCp**	AU								AU		CG		AU	UG	GC	GA	A	-	-	AU		UA		UA	GC	GC	AC	.	GC	UA	GC		CA	CA	UC	AC	.		7	
BVDV-1.25	**(P)**																																								**13**
BVDV-1	S153 (V2/8 CA; V2/10 GG)	GG	CG	GA						UU		CG		UG		UU	GU	A	-	-	GU	GG	GG	UG		GC	AC	.	.	GC	UC	GC	GA	UG		AU	AA	-		13	

The different types are ordered according to increasing divergence in the genus (*), expressed in number of divergent bps, 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.

Table 3. BVDV-1 species strains showing sequence identity (A) and showing very low bp variations (B) at the level of the three variable loci.

A		
Genotype	**Reference strain**	**Identical strains (non relevant variations)**
BVDV-1.7 (1o)	BJ09_26	EN-19.
BVDV-1.7 (1o)	EN-6	EN-8; EN-9.
BVDV-1.7 (1o)	HN1626	HA2-12; HN1641; HN1732; HN1736, HN1814; HN1852; HN1859; HN1864; HN1918.
BVDV-1.7 (1o)	XH-1	XH-2; XH-5; XH-6.
BVDV-1.19 (1w)	T6-18	T6-20.
BVDV-1.21 (1v)	TY8723	TY1000; TY3002; TY3659; TY3661; TY3686; TY3716; TY4145; TY4263; TY5267; TY5295; TY5968; TY5670; TY6943; TY7414; TY8761; TY8815; TY10; TY8856.
BVDV-1.21 (1v)	IR-Shiraz-322	IR-Shiraz-317.
BVDV-1.21 (1v)	IRTV1	IRTV2; IREE1; TR-Erz-BV7 (V1/15 UG; V3/6 UG); TR-Erz-BV8 (V1/15 UG; V3/6 UG).
BVDV-1.21 (1v)	TRElz-4-2021	TRElz-6-2021.
B		
Sub genotype	**Strain**	**Related strains showing very low bp variations**
BVDV-1.7.1 (1o)	JS10116	1 bp: Camel isolate 9; 2 bp: AQGN96BI5; 3 bp: IS25CP01.
BVDV-1.7.1 (1o)	Camel isolate 9	1 bp: JS10116; 2 bp: AQGN96BI5; 4 bp: IS25CP01.
BVDV-1.7.1 (1o)	AQGN96BI5	2 bp: JS10116, Camel isolate 9.
BVDV-1.7.1 (1o)	IS25CP01	3 bp: JS10116; 4 bp: Camel isolate 9.
BVDV-1.7.2 (1o)	BJ09_24	1 bp: S43, S121.
BVDV-1.7.2 (1o)	S43	1 bp: BJ09_24; 2 bp: S121.
BVDV-1.7.2 (1o)	S121	1 bp: BJ09_24; 2 bp: S43.
BVDV-1.7.3 (1o)	T4-32	1 bp: T4-31-2, JS12/02; 2 bp: GA190608, BJ09_26; 3 bp: EN-6, HY-3, HN1626; 4 bp: HB-03, XH-1.
BVDV-1.7.3 (1o)	T4-31-2	1 bp: T4-32; 2 bp: JS12/02; 3 bp: GA190608, BJ09_26; 4 bp: EN-6, HY-3, HN1626.
BVDV-1.7.3 (1o)	JS12/02	1 bp: T4-32, EN-6, GA190608, BJ09_26; 2 bp: T4-31-2, HY-3, HN1626; 3 bp: HB-03, XH-1.
BVDV-1.7.3 (1o)	EN-6	1 bp: JS12/02, BJ09_26, HN1626; 2 bp: GA190608, HY-3; 3 bp: T4-32, HB-03, XH-1; 4 bp: T4-31-2.
BVDV-1.7.3 (1o)	GA190608	1 bp: JS12/02, BJ09_26; 2 bp: T4-32, EN-6, HY-3, HN1626; 3 bp: T4-31-2, HB-03, XH-1.
BVDV-1.7.3 (1o)	BJ09_26	1 bp: JS12/02, EN-6, GA190608, HY-3, HN1626; 2 bp: T4-32, HB-03, XH-1; 3 bp: T4-31-2; 4 bp: MF-3.
BVDV-1.7.3 (1o)	HY-3	1 bp: BJ09_26; 2 bp: JS12/02, EN-6, GA190608, HN1626; 3 bp: T4-32, HB-03, XH-1; 4 bp: T4-31-2.
BVDV-1.7.3 (1o)	HN1626	1 bp: EN-6, BJ09_26; 2 bp: JS12/02, GA190608, HY-3; 3 bp: T4-32, HB-03, MF-3; 4 bp: T4-31-2.
BVDV-1.7.3 (1o)	HB-03	1 bp: XH-1; 2 bp: BJ09_26; 3 bp: JS12/02, EN-6, GA190608, HY-3, HN1626; 4 bp: T4-32, XH-1.
BVDV-1.7.3 (1o)	XH-1	1 bp: HN1626; 2 bp: BJ09_26, MF-3; 3 bp: JS12/02, EN-6, GA190608, HY-3; 4 bp: T4-32, HB-03.
BVDV-1.7.3 (1o)	MF-3	2 bp: XH-1; 3 bp: HN1626; 4 bp: BJ09_26.
BVDV-1.21 (1v)	TY8723	1 bp: IRTV1; 3 bp: TRElz-4-2021, IR-Shiraz-322, TR-Elz-Pst16.
BVDV-1.21 (1v)	IR-Shiraz-322	2 bp: IRTV1; 3 bp: TY8723; 4 bp: TRElz-4-2021, TR-Elz-Pst16.
BVDV-1.21 (1v)	IRTV1	1 bp: TY8723; 2 bp: TRElz-4-2021, IR-Shiraz-322, TR-Elz-Pst16.
BVDV-1.21 (1v)	TRElz-4-2021	2 bp: IRTV1; 3 bp: TY8723; 4 bp: IR-Shiraz-322, TR-Elz-Pst16.
BVDV-1.21 (1v)	TR-Elz-Pst16	2 bp: IRTV1; 3 bp: TY8723; 4 bp: TRElz-4-2021, IR-Shiraz-322.

Table 4. Reorganization of genotype BVDV-1.7 (1o): the determinative LVP genotype markers (V1/14 C-G, V2/7 G:Y and V3/4 G-C), positions with shared common bp (V2/2 U-A and V3/2 G*U) and exceptions in V1/4 and 12, V2/5 and 9, and V3/7 and 10 were excluded in order to consider only specific qualitative differences to characterize sub-groups and verify homogeneity.

Variable locus		V1																		V2								V3										
Position		1	2	3	5r	6	7	8	9	12	13	14	15	16	17	18	19	20	21	22	1	2	3	4	5	6	7	9	1	2	3	4	5	6	7	8	9	10	(*)
BVDV1		**GC**	**UA**	**GC**	**UG**	**A**	**UA**	**CG**	**GC**	**GY**	**CG**		**UA**	**HV**	**GA**	**HV**	**HV**		**-**	**-**		**CG**	**CG**	**UA**	**GC**			**GC**	**AU**		**CG**		**GY**	**UA**	**UA**	**HV**	**HV**	**A**	
BVDV-1.7.1	JS10116	.										CG		CG	AU	AU	GG	-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AU	.	1
BVDV-1.7.1	AQGN96BI5	.										CG		CG	AC	AU	GG	-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AA	.	1
BVDV-1.7.1	Camel isolate 9	.										CG		CG	GU	AU	GG	-	-	-	GU	UA				GU	GC	.	.	GU		GC				GC	AU	.	1
BVDV-1.7.1	IS25CP01	.								AU		CG		CG	AU	AU	GG	-	-	-	GU	UA				GU	GC	AC	.	GU		GC				GC	AU	G	2
BVDV-1.7.2	BJ09_24	.										CG		UU	AC	GU	GA	-	-	-	AU	UA				GU	GC	.	.	GU		GC				GC	GC	.	1
BVDV-1.7.2	S43	.										CG		UU	AC	GU	GA	-	-	-	AU	UA				GU	GC	.	.	GU		GC			AU	GC	GC	.	1
BVDV-1.7.2	S121	.										CG		UU	AC	GU	GA	-	-	-	AU	UA				GU	GC	AC	.	GU		GC				GC	GC	.	2
BVDV-1.7.3 (v)	T4-32	.										CG		UU	AU	GU	GA	-	-	-	GU	UA				GU	GC	.	.	GU		GC				AC	AC	.	1
BVDV-1.7.3 (v)	T4-31-2	.										CG		UU	AU	GU	GA	-	-	-	GU	UA				GU	GC	.	.	GU		GC				AC	AC	G	2
BVDV-1.7.3 (o)	JS12/02	.										CG		UU	AU	GU	GA	-	-	-	GU	UA				AU	GC	.	.	GU		GC				AC	AC	.	1
BVDV-1.7.3 (v)	EN-6	.										CG		UU	AU	GU	GA	-	-	-	GU			UG		AU	GC	.	.	GU		GC				AC	AC	.	1
BVDV-1.7.3 (v)	GA190608	.										CG		UU	AU	GU	GA	-	-	-	GU	UA		UU		AU	GC	.	.	GU		GC				AC	AC	.	2
BVDV-1.7.3 (v)	BJ09_26	.										CG		UU	AU	GU	GA	-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2
BVDV-1.7.3 (v)	HB-03	.										CG		UU	AU	GA	AA	-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2
BVDV-1.7.3 (o)	HY-3	.										CG		UU	AC	GU	GA	-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				AC	AC	.	2
BVDV-1.7.3 (o)	HN1626	.										CG		UU	AU	GU	GA	-	-	-	GU	UA		UG		AU	GC	.	.	GU		GC				GC	AC	.	2
BVDV-1.7.3 (o)	XH-1	.										CG		UU	AU	GU	GA	-	-	-	GU	UA		UG		AU	GC	.		GU		GC				GC	AA	.	2
BVDV-1.7.3 (o)	MF-3 (V1/4 AC)	.										CG		UU	AU	GU	GA	-	-	-	GU	UA		UG	GA	AU	GC	.	.	GU		GC				GC	AA	.	4

Continued

Camel isolate 9	1	-															
AQGN96BI5	2	2	-														
IS25CP01	3	4	5	-													
BJ09_24	6	6	5	9	-												
S43	7	7	6	10	1	-											
S121	7	7	6	8	1	2	-										
T4-32	5	6	6	8	4	5	5	-									
T4-31-2	6	7	7	7	5	6	6	1	-								
JS12/02	6	7	7	9	5	6	6	1	2	-							
EN-6	8	9	9	11	7	8	8	3	4	1	-						
GA190608	7	8	8	10	6	7	7	2	3	1	2	-					
BJ09_26	7	8	8	10	6	7	7	2	3	1	1	1	-				
HY-3	8	8	7	11	5	6	6	3	4	2	2	2	1	-			
HN1626	6	7	7	9	5	6	6	3	4	2	1	2	1	2	-		
HB-03	7	8	8	10	8	9	9	4	5	3	3	3	2	3	3	-	
XH-1	6	7	6	9	5	6	6	4	5	3	3	3	2	3	1	4	-
MF-3	8	9	8	11	7	8	8	6	7	5	5	5	4	5	3	6	2
	JS10116	Camel isolate 9	AQGN96BI5	IS25CP01	BJ09_24	S43	S121	T4-32	T4-31-2	JS12/02	EN-6	GA190608	BJ09_26	HY-3	HN1626	HB-03	XH-1

Values exceeding limit of sub-genotype determination (six divergent base pairs) indicated divergence among groups. Based on base pairing characteristics and divergence values (qualitative and quantitative analysis), the genotype BVDV-1.7 is clustered into three different sub-genotypes. Base pairings characteristic of sub types 7.2 and 7.3 are highlighted in green and blue.

identified for genotype characterization and considered for identification marker definition. The predicted secondary structures of the three variable loci were aligned for comparison of base pairings in the different positions (Table 2). Strains showing sequence identity at the level of the three variable loci or sharing non-relevant variations as G*U or G-C (G:Y) were excluded (Table 3). Some strains showed very low bp variations, indicating close genetic relatedness (Table 3). At the species level, the observed taxonomic status of the examined strains corresponded to the estimation obtained by phylogenetic trees constructed from the alignment with the representative strains from the identified genogroups (Fig. 2).

The secondary structure bps combinations of the 11 Chinese strains EN-6, T4-32, T4-31-2, GA190608, BJ09_26, HB-03 (reported as 1v), and JS12/02, HN1626, HY-3, XH-1, and MF-3 (reported as 1o) constituted a homogeneous group (divergence values ranging from 1 to 7, mean 2.89). Similarly, the Turkish strains TR-Erz-Pst8, TRElz-4-2021, TY8723, and TR-Elz-Pst16, reported as 1v, and the Iranian strains IR-Shiraz-322 and IRTV1, also reported as 1a, constituted a homogeneous group (divergence values from 1 to 8; mean 4.2). The Chinese strains T6-18 and T6-20 (reported as 1w) were divergent from all other BVDV-1 sequence clusters. No relationship among these three groups could be observed. The considered strains reported as 1v or 1o originated from China were divergent from the Turkish and Iranian 1v strains (deposited as 1a or 1l), with divergence values from 11 to 17, and divergent from the Chinese strains reported as 1w, with values from 9 to 14. Similarly, the 1w Chinese strains were divergent from the Turkish and Iranian strains with divergence values from 13 to 15.

Comparison of Chinese strains reported as 1o or 1v (EN-6, T4-32, T4-31-2, GA190608, BJ09_26, HB-03, JS12/02, HN1626, XH-1, HY-3, and MF-3) with other representatives of the BVDV-1 species showed divergence from the majority of the genotypes. Distance from classical BVDV-1 genotypes 1a and 1b exceeded the limit value, ranging from 10 up to 16 divergent bp. In particular, the Chinese strains were divergent also from other Asian genotypes 1.8 (1c), 1.18 (1p), 1.23 (1u), and 1.25. Divergence from strain Bega of genotype BVDV-1.8 ranged from 12 to 16 bp, from strains of genotype 1.18 from 8 to 15, and from strains of genotype 1.23 from 15 to 20 bp. High divergence was also obtained with strain S153 (genotype 1.25) with values from 19 to 21 bp. However, they were related to the Asian genotypes 1.10 (1q) (divergence 27.27%, values from 7 to 10, mean 8.63), genotype 1.6 (1n) (divergence 27.27%, values from 7 to 12, mean 9), genotype 1.15 (1m) (divergence 40.91%, value from 7 to 11, mean 9.32), and with the highest relatedness with genotype 1.7 (1o) (divergence 7.79%, only 6 divergences out of 77 comparisons, values from 5 to 11, mean 7.21). The group was also partially related with sub genotype 1.12.2 (1f or 1r) (divergence 59.09%, values from 5 to 15, mean 10.15). The partial relation was particularly evident with the bovine German strain 11207/98, reference in the sub genotype 1.12.2, showing very low divergence values, from 5 to 8. But the group was also divergent from sub genotype 1.12.1 (1f or 1s) (divergence 72.73%, values from 8 to 15, mean 10.29). The genotype 1.7 (1o) was subdivided into three sub-genotypes (divergence limit value of 6 bp for sub-genotype determination): 7.1, 7.2, and 7.3 numbered according to their divergence in the species, 1.25, 1.33, and 2.82 divergence value mean, respectively. In subgenotype BVDV-1.7.1 were allocated four strains: JS10116, AQGN96BI5, Camel isolate 9, IS26/01ncp, and IS25CP01. In subgenotype 1.7.2 were clustered strains BJ09_24, S43, and S121. Subgenotype BVDV-1.7.3 included the strains JS12/02, HN1626, XH-1, HY-3, and MF-3, reported as 1o. Strain MF-3 showed an adenine cytosine bulge at the level of V1/4, a particular exception of the genus marker guanine cytosine or uracil (G:Y) base pairing (Giangaspero and Apicella, 2018). The strains T4-32, T4-31-2, EN-6, GA190608, HB-03, and BJ09_26, reported as 1v, were also included in subgenotype 1.7.3. All BVDV-1.7 (1o) strains shared the determinative LVP root C-G, G:Y, G-C in V1/14, V2/7, and V3/4, respectively. At the level of additional markers (V2/2 U-A; V3/9 A-U/A A/G-C), in V2/2, an exception was made for strain EN-6 which showed a typical BVDV-1 C-G, all other strains showed the characteristic 1.7 (1o) U-A base pairing (shared only with Chinese genotypes 1.10 and 1.15—1q and 1m). In V3/9, 9 strains, out of the 11 new considered strains, showed an A C bulge, different from previously described A-U/A A/G-C bp. New differences were at the level of positions V2/6 (A-U in nine strains, instead of G*U) and V3/8 (A C bulge in eight strains, instead of G-C). The allocation of the strains in the genotype BVDV-1.7 (1o) was reorganized. The determinative LVP genotype markers (V1/14 C-G, V2/7 G:Y, and V3/4 G-C), positions with shared common bp (V2/2 U-A and V3/2 G*U), and exceptions in V1/4 and 12, V2/5 and 9, and V3/7 and 10 were excluded in order to consider only specific qualitative differences to characterize sub groups and verify homogeneity. Base pairing divergence values exceeding the limit of sub-genotype determination (six divergent bps) were considered for computing clustering into sub -genotypes. Based on base pairing characteristics and divergence values (qualitative and quantitative analysis), the genotype BVDV-1.7 (1o) was clustered into three different homogeneous sub genotypes (Table 4). Within the sub-genotypes, there were no differences in terms of sequence peculiarities among cattle, the predominant host species, the strain isolated from a batracian camel of 1.7.1 or the two strains isolated from goats, belonging to 1.7.3.

A comparison of Chinese strains reported as 1w (T6-18 and T6-20) showed divergence from the all the other genotypes in the BVDV-1species. Distance from classical BVDV-1 genotypes 1a and 1b ranged from 11 to 14 divergent bp. Also, with other Asian genotypes the

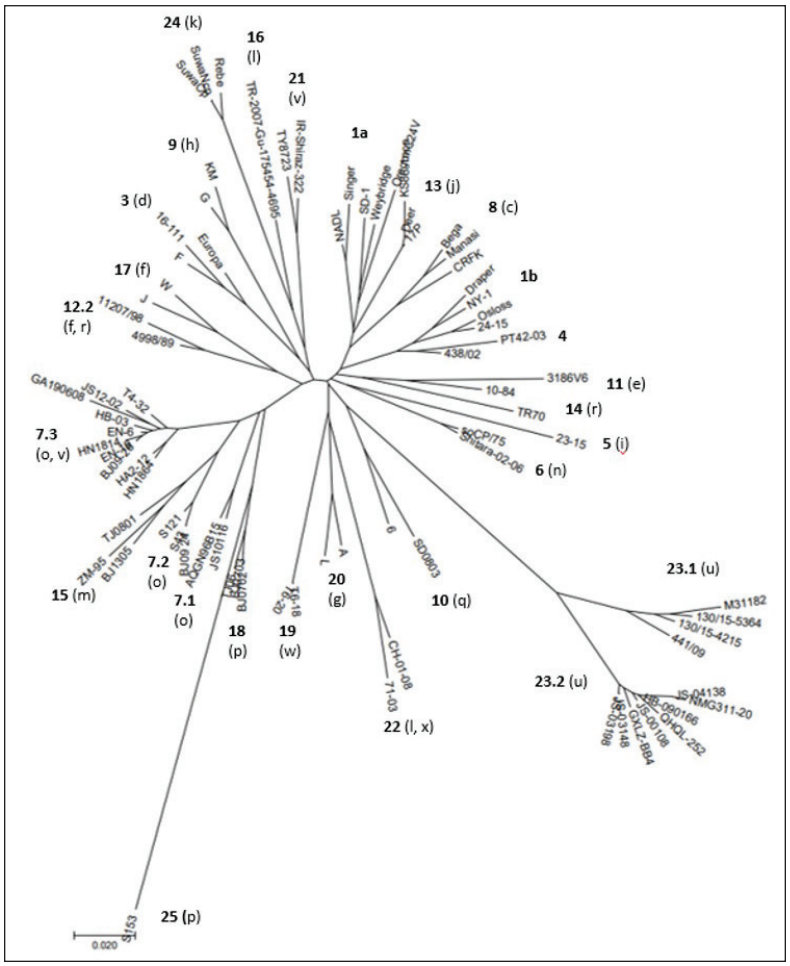

Fig. 2. Phylogenetic tree based on the 5′-UTR comparison, suggesting a taxonomic position of the BVDV-1 strains in the genus *Pestivirus*. Strain NADL [M31182] is the reference for the BVDV-1a genotype, strains Draper [L32880] and Osloss [M96687] are the references for the BVDV-1b genotype, sub genotypes 1b1 and 1b2. Strains Europa [AB000898], 438/02 [AY159540], 23-15 [AF298059], and so CP/75 [AB042661] are references for the BVDV-1.3 to BVDV-1.6 genotypes. Strains JS10116 [JN248734], BJ09_24 [HQ116550] and BJ09-26 [HQ116551] are reference for the three sub genotypes of the BVDV-1.7 genotype, circulating exclusively in China. 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 the BVDV-1.8 to BVDV-1.18 genotypes, and strain A [AF298064] is reference for the genotype BVDV-1.20. Strains T6-18 [MN417892] and TY8723 [MH673456] are references for the BVDV-1.19 and BVDV-1.21 genotypes, restricted to China and Turkey, and Iran, respectively. Strains CH-01-08 [EU180024], M31182 (Yak) [JQ799141], Rebe [AF299317], and S153 [KF006964] are references for the BVDV-1.22 to BVDV-1.25 genotypes. 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.

divergence was constantly high, from values of 11 with genotypes 1.7 (1o) and 1.10 (1q), 12 with genotype 1.6 (1n), 12–14 with genotype 1.18 (1p), 14 with genotype 1.8 (1c), 13–14 with genotype 1.15 (1m), and highest values of 16–18 with genotype 1.23 (1u) and 23 divergent pb with genotype 1.25. The considered Turkish and Iranian strains were also divergent from all the other clusters in the BVDV-1 species. The divergence with reference strains of genotype 1a was 12–13 bp and with 1b ranged from 13 to 18. When compared with other Asian

genotypes, the divergence was 10–16 with genotypes 1.6 (1n), 1.7 (1o), 1.8 (1c), 1.10 (1q) 1.15 (1m), and 1.18 (1p) and up to 18–24 with genotypes 1.23 (1u) and 1.25. The comparison of the Turkish and Iranian strains with atypical Turkish strains previously reported as 1.14 (1r) and 1.16 (1l) also showed divergence. With the strains TR70 and TR75 (1.14) divergence ranged from 11 to 17, mean 14.58, and with strains TR16, TR72, and TR-2007-Gu-175454-4695 (1.16) from 9 to 15, mean 11.83. At the level of PNS consensus motifs shared by all species in the genus, the strain T6-18 showed an exception in V1/10: U-A pairing instead of A-U. Similarly, in the V1 stem position 11, the strains TR-Erz-Pst8 and TR-Elz-Pst16 were characterized by an A C bulge and a G-C pairing, respectively, instead of the conserved bulge formed by two cytosine nucleotides.

The evaluation of the sequences by BLAST showed a similar order of genetic distance observed by the alignment of a sequence secondary structure. A low percentage of nucleotide identity was obtained when comparing the Chinese strains reported as 1v with BVDV-1b2 strains (89.52%). Higher identity was observed by comparing the Chinese strains reported as 1v with other Chinese strains reported as 1o. The strain HY-3 (1o) shared 98% identities with T4-31-2 (1v). The strain EN-6 (1v) was 99.59% identical with strain BJ09_26 (1v) and 98.78% with strains HN1732, HN1736, HN1814, and HN1859, all reported as 1o, 98.59% with T4-32 (1v) and the strains 1o HA2-12, HY-3, and XH-6, 97.88% with JS12/02 (1o) and 97.52% with strains XH-1, XH-2, XH-5, and MF-3 (1o). Similarly, the BVDV-1 strain IR-Shiraz-322 [LC053995], reported from Iran, showed 97% identities with the Turkish strain TY8723, deposited as 1l and reported as 1v.

Taking into account the mean divergence values in the *Pestivirus* genus of the characterized groups (3 and 3.83 for the strains 1w from China and 1v, 1l, or 1a from Turkey and Iran, respectively) and that the PNS nomenclature is based on the increasing divergence in the genus, the nomenclature was modified accordingly. The group of the Chinese strains T6-18 and T16-20 (1w) was clustered in the species as BVDV-1.19 and the group from Turkey and Iran (1v, 1l, or 1a) of strains TR-Erz-Pst8, TY8723, IR-Shiraz-322, TRElz-4-2021, IRTV1, and TR-Elz-Pst16 as BVDV-1.21. Subsequently, the genotype BVDV-1.19 (1g), with a mean divergence value in the genus of 3.5 bp, was renamed 1.20, and the genotypes BVDV-1.20 (1l or x), BVDV-1.21 (1u), BVDV-1.22 (1k), and BVDV-1.23, with a divergence in the genus higher than 5 bp, were renamed as 1.22, 1.23, 1.24, and 1.25. Details of the computing of divergence values (exceeding limit value 9 for genotype determination) based on 5′-UTR secondary structure sequence alignment of BVDV-1 bovine strains EN-6, T4-32, T4-31-2, GA190608, BJ09_26, HY-3, and HN1626, HB-03, XH-1, MF-3 and one goat strain JS12/02, reported as genotype 1o or 1v isolated from China (Mao *et al.*, 2016; Deng *et al.*, 2020; Shi *et al.*, 2020; Tian *et al.*, 2021; Zhu *et al.*, 2022; strain T6-18, reported as genotype 1w isolated from China (Deng *et al.*, 2020), strains TY8723, TR-Erz-Pst8, TRElz-4-2021, TR-Elz-Pst16, IRTV1, and IR-Shiraz-322, reported as genotype 1a, 1l, or 1v, isolated from Turkey and Iran (Oguzoglu *et al.*, 2019; Timurkan and Aydin, 2019; clustered according to PNS method as 1.7.3, 1.19, and 1.21, respectively are presented in Supplementary Material 1.

Discussion

Taxonomy

The taxonomy of the *Pestivirus* A, BVDV-1, species was updated and clarified, in particular with concern to the strains of Chinese and Turkish origin, both reported as genotype 1v. In order to verify their allocation in the genus, sequences have been compared with other *Pestivirus* species strains previously analyzed by the PNS method (Giangaspero and Apicella, 2014; Giangaspero *et al.*, 2018, 2019b), reaching a total number of 1,487 observations obtained with PNS procedure. Divergence values were used as reference for genotype and sub genotype allocation of the considered strains (divergence limit value of 9 and 6 bps, respectively). For example, the goat strain XY-3, reported as BVDV-1p (Mao *et al.*, 2016), diverged only 3 bp from the strain BJ0701, reference strain of the genotype 1.18 (1p) or the Chinese camel isolate 9, clustered as 1m (Gao *et al.*, 2013), diverged of only 2 bp from the strain AQGN96BI5 (Yamamoto *et al.*, 2008) of genotype 1.7 (1o), and 4 bp from the 1.7 reference strain IS25CP/01 (Nagai *et al.*, 2008), thus justifying the reallocation in this BVDV group.

The Turkish strains considered in the present study were reported as 1l (Timurkan and Aydın, 2019) or 1v (Oguzoglu *et al.*, 2019; The strains reported as 1v by Oguzoglu *et al.* (2019), as the bovine strain TY8856, first they have been deposited as genotype BVDV-1l, a genotype considered as the predominant sub-type of BVDV-1 in Eastern Anatolia, Turkey, based on the phylogenetic analysis of 5′-UTR, Npro, and E2 genomic regions of isolates from cattle (Timurkan and Aydın, 2019). The 5′-UTR, complete E2 gene and Npro gene region sequences of these strains have been deposited under accession numbers from MH673456 to MH673474 for 5′-UTR, from MH673439 to MH67344, and from MH67346 to MH673455 for E2, and from MH758720 to MH758737 for Npro, respectively. Erroneously, accession numbers of deposited sequences in GenBank of the Turkish BVDV-1v strains were indicated by Oguzoglu *et al.* (2019) for 5′-UTR MH673439-MH673455 and for E2 gene MH673456-MH673474. In reality, accession numbers were inverted and MH67345 was not found. The fact that authors deposited these strains as 1l indicates the difficulty to cluster atypical sequences within a heterogeneous species like BVDV-1. The divergence from other reported genetic groups induced first to refer to previous types characteristic of Turkey, like the strain TR-2007-Gu-175454-4695 (Oguzoglu *et al.*, 2012). The same difficulty was encountered by the

authors that deposited as BVDV-1a the Iranian strains IRTV1, IRTV2, and IREE1 since available software as Clustal X provides results of sequence comparison depending on considered reference strains loaded by the users. To avoid interpretation difficulties, it is, therefore, useful to accurately select reference strains and possibly apply other evaluation methods. BLAST could easily reveal a high homology of these Iranian strains with the Turkish atypical strains deposited as 1l. On the other side, the erroneous allocation of the strains was also probably due to the determinative LVP (V1/14; V2/7; V3/4) root Y:G, G:Y, and A-U shared with genotype 1a. However, observing the secondary structure by the PNS method, both group of strains from Turkey and Iran were well correlated and formed a single BVDV-1 genetic group, while clearly distant from the Chinese strains, despite the 1v homonymy. These strains have been defined as genotype BVDV-1.21, according to the PNS nomenclature, based on increasing divergence in the genus *Pestivirus* and thus, indicating a consistent genetic distance from the classical types as BVDV-1a and BVDV-1b, characterized by most common sequence traits, and expression of genetic evolution within the species and geographic segregation, circulating only in Anatolia and Iran, and to date not reported elsewhere.

While genotype 1.19 (1w) constituted a separate group in the species, reported in China more recently in 2017 (Deng *et al.*, 2020), the Chinese group of strains reported as 1v (Deng *et al.*, 2020; Tian *et al.*, 2021; Zhu *et al.*, 2022) and those reported as 1o and considered as 1v (Shi *et al.*, 2020; was related to genotype 1.7 (1o) and constituting a new sub genotype (1.7.3). This corresponded to the observation made by Shi *et al.* (2020), who reported nine *Pestivirus* A isolates as putative new 1o sub genotype, based on phylogenetic analysis of partial 5′-UTR and Npro sequences. The sub genotype 1.7.1 included one Chinese pig strain JS10116, reported as genotype 1o by Deng *et al*. (2012), the Chinese camel isolate 9, clustered as 1m (Gao *et al.*, 2013) and three cattle isolates from Japan, reported as 1o, AQGN96BI5 (Yamamoto *et al.*, 2008), IS26/01ncp and IS25CP/01 (Nagai *et al.*, 2008). Three Chinese strains, all reported as genotype 1m, from cattle, S43, S121, and BJ09_24 (Zhang *et al.*, 2014b) were clustered in the sub-genotype BVDV-1.7.2. Sub genotypes 1.7.2 and 1.7.3 diverged from 1.7.1 at the level of V1 locus in positions 16, 18, and 19 with U U bulge, G*U or G A and G A or A A bulges, respectively (Table 4). In addition, 1.7.2 showed characteristic bp A-U in V2/1 and G-C in V3/9.

Turkey and Iran

Since the first report of BVDV in Turkey in the 1990', in addition to genotype BVDV-1.21, other rare BVDV-1 strains were characteristic of the country and expression of geographic segregation: the BVDV-1.16 bovine strains reported as genotype 1l (Oguzoglu *et al.*, 2012), and strains TR70, TR73 and TR75 belonging to the BVDV-1.14, reported as genotype 1r, restricted to Turkey (Yesilbag *et al.*, 2014, 2017; Giangaspero *et al.*, 2018). Until now, nine genotypes were described in Turkey. In addition to the atypical genetic clusters, other classical genotypes have been reported: 1a and 1b, 1.3 (d), 8 (c), 9 (h), and 17 (f). In Iran, BVDV was first described by Mirshamsy *et al.* (1970). Studies were mainly based on serology showing a high prevalence of BVDV antibodies in cattle in some areas of the country, up to 100% (Kargar Moakhar *et al.*, 1995; Khezri, 2015). However, studies were generally not completed by sequencing and characterization of isolates, and only a few reports and deposited sequences are available (Khodakaram-Tafti *et al.*, 2016).

China

The first report of BVDV in China was in the north eastern province of Jilin in 1980 related to 1b infected cattle imported from Europe (Li *et al.*, 1983). The phylogenetic analysis clustered the first BVDV strain CC-184, which was isolated in China from the cows imported from Europe, to BVDV-1b (Li *et al.*, 1983; Wang *et al.*, 1996). The majority of BVDV strains isolated later from Jilin and other regions in China, including isolates JL-1 [KF501393] in 2009 and BVDV-CC13B [KF772785] in 2013, were of genotype BVDV-1b. These strains shared the highest sequence identity with the CP7 strain [U63479], originally isolated in Germany in 1987 (Zhang *et al.*, 2014a; Zhu *et al.*, 2014). Those results supported the hypothesis that the strains of the predominant genotype BVDV-1b currently spread in China were originally from European countries (Shi and Zhang, 1987; Wang *et al.*, 1996; Zhu *et al.*, 2016), and probably from Germany. Similarly, in the present study, genetic characteristics suggested the evolution of the virus population in China from strains introduced from Germany. The sub-genotype 1.7.3, including exclusively Chinese strains reported as 1v or 1o, was partially related to sub-genotype 1.12.2, showing an overall divergence of 59.09% (39 divergences from 5 to 15, with mean divergence value 10.15, out of 66 comparisons and 8 limit values—9 bp). The Chinese strain BJ09-26 was the first isolated of the sub genotype 1.7.3, in 2009. The other 1.7.3 strains were reported in following years from 2013 to 2019. The strains belonging to 1.12.2, reported as 1f or 1r, included the strains 11207/98 and 4998/89, isolated in Germany in 1989. The other members of the sub-genotype were strain 51/06 isolated in Poland in 2006, and the strains CA18110, 79/11, and VE24512, isolated in Italy later (from 2010 to 2012). The relationship between the two sub genotypes was particularly evident with strain 11207/98, distant only 5 bp from strain T4-32 or 6 bp from strains EN-6, T4-31-2, and JS12/02. 11207/98 was divergent of 9 bp from both strains JS10116 and BJ09_24, isolated in China in 2010 and 2009, belonging to sub genotypes 1.7.1 and 1.7.2, respectively. Taking into account the dates and places of isolation, it is likely that the origin of sub-genotype 1.7.3 is Germany, where the sub-genotype 1.12.2 appeared and diffused later in other European countries and China. Since the first introduction, BVD

outbreaks were not often reported until the later 1990s and early 2000s. Further, BVD outbreaks were reported in many regions across China and over the half of the 25 genotypes described in the species, 13 could be identified: BVDV-1a, 1b, 1.3, 1.6, 1.7, 1.8, 1.10, 1.11, 1.15, 1.18, 1.19, 1.23, and 1.25. The predominant genotypes were BVDV-1b and BVDV-1.15 (1m) (Xue *et al.*, 2010, Zhong *et al.*, 2011).

Italy and Japan

The comparison of the epidemiological situation of BVDV in China to the occurrence of the different genotypes reported in Italy showed a similar heterogeneity of the species, with specific differences at the level of circulating types. Fourteen genotypes were observed in Italy: BVDV-1a, 1b1, and 1b2, 1.3, 1.6, 1.8, 1.9, 1.11, 1.12, 1.13, 1.17, 1.20, 1.22, 1.23, and 1.24. The BVDV-1b and 1.11 (1e) genotypes were predominant. The occurrence of genotype 1a was also demonstrated and reported for the first time in north, central, and island (Sardinia) regions (Giammarioli *et al.*, 2008) and then in southern regions (Giangaspero *et al.*, 2019a). Three uncommon genotypes were reported in north and central Italy, 1.13 (j), 1.17 (f), and 1.22 (l, x) (Falcone *et al.*, 2003; Luzzago *et al.*, 2014; Cerutti *et al.*, 2016). Other strains belonged to genotypes circulating in a few countries. For example, genotype 1.9 (h) was spread only in Switzerland, Austria, and Italy, and rarely reported also in UK, Slovakia, and South Africa. Genotype 1.20 (g) was rare and reported only in Austria, UK, and South Africa. The first evidence for the circulation of this genotype in Italy was reported by Giammarioli *et al.* (2008) and Luzzago *et al.* (2014), and further rarely detected also in southern Italy. Only two strains were reported, one from Basilicata (Luzzago *et al.*, 2014) and another from Calabria (Decaro *et al.*, 2016). Similarly, genotype 1.24 (k) was rare and reported only in Switzerland, and detected for the first time in northern and southern Italy (Luzzago *et al.*, 2014). The few BVDV-1.6 (n), 1.8 (c), and 1.23 (u) strains detected in Italy, represented an exception in genotypes characteristic from Asia. The progressive increasing of genetic variability of BVDV-1 in Italy (3 genotypes in 2002, 7 in 2008, 12 in 2014, and 14 reported in 2019) was probably mainly due to import of live animals. More than 1,300,000 bovines are imported yearly, mainly from France and largely destined to the northern regions of Veneto, Lombardia, and Piedmont. The fact that in France BVDV-1.11 (e) is prevalent (Jackova *et al.*, 2008), probably explains the high prevalence of this genotype in northern regions, in particular Piedmont (Luzzago *et al.*, 2014). The introduction of BVDV-1.23 in Italy was suspected to be due to contamination of biological products for veterinary use (Giangaspero *et al.*, 2019a).

In Japan, the vaccine against BVDV was introduced since 1973, but the use was infrequent, due to the accidental production of persistently infected animals occurred following the vaccination of pregnant cows with the modified live virus. The number of BVD cases increased in recent years, with a mean of 198 cases per year (Tajima, 2021). There is currently no national eradication program for BVD in Japan, but regional voluntary eradication trials have been performed since the 2000's, along with regional surveillance to identify persistently infected cattle (Abe *et al.*, 2016, Akagami *et al.*, 2020). In terms of genetic diversity, BVDV may not vary to the same extent as in European countries (Nagai *et al.*, 2001; Tajima, 2006; Abe *et al.*, 2016). BVDV-1 species was far less heterogeneous than in China, showing seven genotypes: 1a, 1b (b1 and b2), 1.3, 1.6, 1.7, 1.8, and 1.13. However, Japan shared with China three genotypes (1.6, 1.7, 1.8) restricted to Far East Asia and Austral Asia (1.6 present also in South Korea and 1.8 also in Australia). In particular, the sub genotype 7.1 was present exclusively in Japan and China. Taking into account the earlier reporting of the variant in Japan (1996) and more than a decade later in China in 2009, this may suggest the origin of the introduction in China of a new genotype and a subsequent evolution in two additional sub types (7.2 and 7.3).

Geographic segregation

The BVDV-1 species was the most heterogeneous within the genus *Pestivirus*, and this may be due to geographic segregation. Different BVDV-1 species genotypes, showing particular genetic characteristics, were restricted to specific geographic areas also in Asia. Some genetic variants appeared to be restricted in certain areas, as certain genotypes circulating only in Turkey or China, suggesting geographic isolation (Xue *et al.*, 2010). Some sub genotypes and genotypes were characteristic from Asia. Sub genotypes 1.7.2 and 1.7.3 and genotypes 1.10, 1.15, 1.18, and 1.25 were circulating exclusively in China, and also genotype 1.23 has been reported almost exclusively in China. Other genotypes (1.6, 1.7.1, and 1.8) appear to be restricted to Asian or Austral Asian countries. In rare occasions, strains belonging to Asian genotypes 1.6, 1.8, and 1.23 were also reported in Italy, representing exceptions. Observing the secondary structure, the geographic segregation was associated to specific sequence characteristics. For example, Chinese strains of the genotype BVDV-1.10 showed divergence in V1/15 with A A or C A bulges, instead of BVDV-1 species marker U-A pairing. In sub genotype 1.15.2, all strains showed species marker exception in V2/5, with A-U instead of G-C. Similarly, strain S153 (Zhang *et al.*, 2014b), genotype 1.25, showed an atypical G A bulge at the level of BVDV-1 species marker position in V3/5. The Asian clusters 1.8 and 1.15.2 shared a root characteristic of genotype BVDV-1a (V1/14 C-G, V2/7 G-C, and V3/4 A-U). All the strains belonging to group 1.18 (bovine or contaminant strains from Australia, China, and Japan) showed an A C bulge (enlargement in the RNA secondary structure) in position 12 in V1 locus, a base pairing not present in any member in

the genotype 1a. Among Chinese strains of the sub-genotype 15.2, characterized also by an exception at the level of species marker in V2/5 (A-U instead of G-C), root A was present in the majority of the strains. A peculiar U-A pairing in V2/2 was present only among certain Asian genotypes (1.7, 1.10, and 1.15.1). Genotype 1.23 showed a new and atypical V1/14 U-A, V2/7 G-C, and V3/4 U-A root associated with genotype b (sub genotype 2). The V1/14 U-A was shared exclusively with BVDV-1b and BVDV-1.21 which was characteristic, and in all the BVDV-2 species strains.

With reference to Asia, also in other *Pestivirus* species geographic segregation was observed. In the *Pestivirus* B (Bovine viral diarrhea virus type 2, BVDV-2) species, only one group appeared specific to Asia. The genotype b variant 4 (BVDV-2b4) included only Chinese isolates: the bovine strain SD-1301 (Wang *et al.*, 2014) and the contaminants S143, S172, and S51 (Zhang *et al.*, 2014b). *Pestivirus* H (Bovine viral diarrhea virus type 3, BVDV-3) species genotypes BVDV-3.2, BVDV-3.3, and BVDV-3.4 were specific to zebu and bovine isolates from India and Bangladesh, respectively (Haider *et al.*, 2014; Mishra *et al.*, 2014). *Pestivirus* D (Border disease virus, BDV) species sequence characteristics of Chinese and Turkish strains were highly divergent from other genogroups, indicating geographic segregation. Chinese strains, reported as genotype BDV-3 (Ghiforn type—PNS BDV-j), AH12-01, AH12-02, and AHHX15 (Li *et al.*, 2013) have been clustered as genotype BDV-d, sub genotype d1. These strains showed high homology with strain 297 (Leskova *et al.*, 2013), clustered in the same genotype, but as separate sub genotype BDV-d2. Similarly, other Chinese strains JS12/04, JSLS12-01, and JSYZ15 (Li *et al.*, 2013) have been clustered as genotype BDV-h. Turkish strains TR-13 and TR-14, reported as a distinct group in the BDV species (Toplu *et al.*, 2012), have been clustered as genotype BDV-i. The ovine Turkish strains BDV/Aydin/04-TR and BDV/Burdur/05-TR (Oguzoglu *et al.*, 2009) represented the *Pestivirus* I species. In the *Pestivirus* C (CSFV) species, three genetic clusters referred specifically to Asian countries. The CSFV genotype a variant 4 (type Parambi) included only pig and wild boar strains from India (Bhaskar *et al.*, 2015; CSFV pig strains clustered into genotype C (type Okinawa) were reported only from Japan and Taiwan (Harasawa and Giangaspero, 1999; Sakoda *et al.*, 1999; Lin *et al.*, 2007; The Chinese strain S171 (Zhang *et al.*, 2014b), isolated from bovine serum, was clustered as CSFV-d.

Potential implications

Despite the untranslated region, by definition, it is not translated into antigens, the molecular variations at the level of 5′-UTR are correlated to parts of the BVDV genome which contribute to antigenic variation, expressing structural and nonstructural strongly immunogenic proteins as E2 and NS3 (Lazear *et al.*, 2013; Lanyon *et al.*, 2014; Yitagesu *et al.*, 2021; Zheng *et al.*, 2021; Chi *et al.*, 2022), and thus important also for serological diagnosis and vaccine development (Mahony *et al.*, 2015; Al-Kubati *et al.*, 2021). Studies on antigenic similarity among genotypes, measuring virus neutralization antibody titers of hyperimmunized antiserums against studied BVDV-1 genotypes, showed the highest similarity (highest cross neutralizing antibody titers) between genotypes 1a and 1b (homologous pairs which are widely used in commercial vaccines) compared to the very weak antigenic similarity among 1a and 1b with the viruses from other different genotypes, particularly BVDV-1.17 (1f) and BVDV-1.24 (1k) (Castrucci, 1978; Bachofen *et al.*, 2008; Alpay and Yesilbag, 2015).

The growing number of reports on BVDV-1 heterogeneity raises significant concerns about the emergence and spread of new BVDV variants, with possible implications on animal health and disease control (Giammarioli *et al.*, 2015). Although there is no clear indication that atypical strains within the species may negatively influence diagnostic analysis or impair the efficacy of the available vaccines, the antigenic differences limit the cross-protection among the highly divergent types of bovine pestiviruses. Such problems are evident with the *Pestivirus* H (Bauermann *et al.*, 2013). Also, some reports concerned BVDV-1, showing a low level of antibody response to the BVDV-1b by BVDV-1a vaccines (Fulton *et al.*, 2003) and some insufficient responses to protect against BVDV-1b infections (Grooms *et al.*, 2007; Ridpath *et al.*, 2010). Therefore, it is important to constantly monitor the evolution of the virus population for early detection of new variants, through the testing of field isolates, applying adequate genotyping methods. Furthermore, cross-protection studies should be carried out taking into account reported atypical strains and the regional epidemiological situation to determine whether future vaccines should be produced using several BVDV genotypes.

Conclusion

The study demonstrated the potential utility of the PNS technique to resolve some of the BVDV genetic classification difficulties. The evaluation of secondary structure by PNS method of strains clustered as new BVDV-1 genotypes 1.19 (1w) and 1.21 (1v), characteristic of China, Turkey, and Iran, respectively. Confusion in nomenclature was clarified, allocating as sub genotype 1.7.3 (1o) the Chinese strains reported as 1v.

The heterogeneity in pestiviruses has the potential to cause diagnostic and prophylactic difficulties because commonly available tests and vaccines are based on viral antigenic substrate (Bolin *et al.*, 1985; Giammarioli *et al.*, 2015). Recognition of the molecular characteristics of field strains is important for the control or eradication programs design, vaccine development or for specific purposes as the retracing of infection sources in case of outbreaks (Booth *et al.*, 2013; Kuta *et al.*, 2013). Increasing genetic heterogeneity in *Pestivirus* species, also due to geographic

segregation, indicates a need for the application of different analytical procedures to avoid interpretation difficulties and obtain accurate genetic analysis, given the importance of epidemiological data, to preserve animal health and welfare against the spread of potentially virulent genetic clusters in naïve animal populations.

Conflict of interest

Authors declare that there is no conflict of interest.

Authors' contribution

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

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Data availability

Data available on request from the authors.

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Supplementary Material 1. Computing of divergence values based on 5′-UTR secondary structure sequence alignment of BVDV-1 bovine strains EN-6, T4-32, T4-31-2, GA190608, BJ09_26, HY-3, HN1626, HB-03, XH-1, MF-3 and one goat strain JS12/02, reported as genotype 1o or 1v, isolated from China (Mao *et al.*, 2016; Deng *et al.*, 2020; Shi *et al.*, 2020; Tian *et al.*, 2021; Zhu *et al.*, 2022; strain T6-18, reported as genotype 1w isolated from China (Deng *et al.*, 2020), strains TY8723 TR-Erz-Pst8, TRElz-4-2021, TR-Elz-Pst16, IRTV1, and IR-Shiraz-322, reported as genotype 1a, 1l, or 1v, isolated from Turkey and Iran (Oguzoglu *et al.*, 2019; Timurkan and Aydin, 2019; clustered according to PNS method as 1.7.3, 1.19, and 1.21,
respectively.

	EN-6	T4-32	T4-31-2	GA190608	BJ09_26	HY-3	JS12/02	HN1626	HB-03	XH-1	MF-3	
1a Singer	11	11	11	12	12	12	11	12	12	12	14	Divergent
1a NADL	11	11	11	12	12	12	11	12	12	12	14	
1b1 Draper	13	12	12	14	14	14	13	14	14	14	16	Divergent
1b1 Sanders	12	11	12	13	13	13	11	12	12	13	15	
1b2 Osloss	10	11	12	11	11	11	9	10	10	11	13	
1.6 (N) so CP/75	8	7	8	9	9	9	8	9	10	10	12	Related (3 divergences out of 11, 27.27%, 4 limit values) (From 7 to 12 mean 9)
1.7 (O) JS10116	8	5	6	7	7	8	6	6	7	6	8	Related (From 5 to 11 mean 7.21)
1.7 (O) AQGN96BI5	9	6	7	8	8	7	7	7	8	6	8	
1.7 (O) Camel isolate 9	9	6	7	8	8	8	7	7	8	7	9	
1.7 (O) IS25CP01	11	8	7	10	10	11	9	9	10	9	11	
1.7 (O) BJ09_24	7	4	5	6	6	5	5	5	8	5	7	
1.7 (O) S43	8	5	6	7	7	6	6	6	9	6	8	
1.7 (O) S121	8	5	6	7	7	6	6	6	9	6	8	
1.8 (C) Bega	13	12	13	14	14	14	13	14	12	14	16	Divergent
1.10 (Q) SD0803	10	7	8	9	9	10	8	8	8	8	10	Related (3 divergences out of 11, 27.27%, 2 limit values) (From 7 to 10 mean 8.63)

Continued

	EN-6	T4-32	T4-31-2	GA190608	BJ09_26	HY-3	JS12/02	HN1626	HB-03	XH-1	MF-3	
1.12.1 (F, S) 22146/81	9	10	11	10	10	10	9	10	10	11	13	Divergent
1.12.1 (F, S) UM136/08	10	10	11	11	11	11	10	12	11	13	15	
1.12.1 (F, S) mousedeer	9	10	11	10	10	10	9	10	9	11	13	
1.12.1 (F, S) 2561	8	9	10	9	9	9	8	9	10	10	12	
1.12.2 (F, R) 11207/98	6	5	6	7	7	8	6	8	7	9	11	Partially related (39 divergences out of 66, 59.09%, 8 limit values) (From 5 to 15 mean 10.15)
1.12.2 (F, R) 51/06	9	8	9	10	10	11	8	9	8	10	12	
1.12.2 (F, R) 4998/89	7	6	7	8	8	9	7	9	8	10	12	
1.12.2 (F, R) CA18110	12	11	12	13	13	13	12	12	13	12	14	
1.12.2 (F, R) VE24512	12	11	11	13	13	14	12	13	12	13	15	
1.12.2 (F, R) 79/11	10	9	9	11	11	12	10	11	12	11	13	
1.15.1 (M) ZM-95	9	8	9	9	8	9	9	7	9	7	10	Related (9 divergences out of 22, 40.91%, 8 limit values) (From 7 to 11 mean 9.32)
1.15.2 (M) HB-060111	9	10	11	11	10	11	11	8	11	9	10	
1.18 (P) TJ06	12	11	10	13	13	14	12	13	14	13	15	Divergent
1.18 (P) BJ0701	10	9	8	11	11	12	10	11	12	11	13	
1.18 (P) XY-3	11	10	9	12	12	13	11	12	13	12	14	

Continued

	EN-6	T4-32	T4-31-2	GA190608	BJ09_26	HY-3	JS12/02	HN1626	HB-03	XH-1	MF-3	
1.23.1 (U) 130/15-5364	16	16	16	17	17	17	16	17	17	17	19	Divergent
1.23.2 (U) m31182	18	18	18	19	19	19	18	19	19	19	21	
1.23.2 (U) 441/09	17	17	17	18	18	18	17	18	18	18	20	
1.23.2 (U) GXBH-EB34	15	15	15	16	16	16	15	16	16	16	18	
1.23.2 (U) JS-00108	16	16	16	17	17	17	16	17	17	17	19	
1.23.2 (U) QHQL-252	16	16	16	17	17	17	16	17	17	17	19	
1.23.2 (U) LN309-5	16	16	16	17	17	17	16	17	17	17	19	
1.23.2 (U) GXCZ-FB22	17	17	17	18	18	18	17	18	18	18	20	
1.25 (P) S153	20	21	21	20	20	20	20	21	20	19	21	Divergent
T6-18 (W)	9	9	10	10	10	11	9	11	10	12	14	Divergent
TR-Erz-Pst8 (V)	12	12	13	13	13	13	12	13	12	14	16	Divergent
TRElz-4-2021 (V)	11	11	12	12	12	13	11	12	12	13	15	
IR-Shiraz-322 (A)	12	12	13	13	13	14	12	13	13	13	15	
TY8723 (V)	11	11	12	12	12	13	11	12	13	13	15	
IRTV1 (A)	11	11	12	12	12	13	11	12	12	13	15	
TR-Elz-Pst16 (V)	13	13	14	14	14	15	13	14	14	15	17	
EN-6 (V)	-											Homogeneous (From 1 to 7 mean 2.89)
T4-32 (V)	3	-										
T4-31-2 (V)	4	1	-									
GA190608 (V)	2	2	3	-								
BJ09_26 (V)	1	2	3	1	-							
HY-3 (O)	2	3	4	2	1	-						
JS12/02 (O)	1	1	2	1	1	2	-					
HN1626 (O)	1	3	4	2	1	2	2	-				
HB-03 (V)	3	4	5	3	2	3	3	3	-			
XH-1 (O)	3	4	5	3	2	3	3	1	4	-		
MF-3 (O)	5	6	7	5	4	5	5	3	6	2	-	

Continued

	T6-18	TR-Erz-Pst8	TRElz-4-2021	IR-Shiraz-322	TY8723	IRTV1	TR-Elz-Pst16	
1a Singer	13	12	13	13	13	13	15	Divergent
1a NADL	13	11	12	13	12	12	14	
1b1 Draper	14	15	16	17	18	18	20	Divergent
1b1 Sanders	13	13	14	18	16	16	18	
1b2 Osloss	11	12	14	15	13	14	16	
1.6 (N) so CP/75	12	11	12	13	12	12	14	Divergent
1.7 (O) JS10116	11	16	16	13	13	16	18	Divergent
1.8 (C) Bega	14	11	10	11	10	10	11	Divergent
1.10 (Q) SD0803	11	14	13	14	13	13	15	Divergent
1.12.2 (F, R) 11207/98	11	13	14	12	11	13	15	Divergent
1.14 (R) TR70	12	12	14	15	15	15	17	Divergent
1.14 (R) TR75	12	11	14	15	15	15	17	
1.15.1 (M) ZM-95	13	17	15	16	15	15	17	Divergent
1.15.2 (M) HB-060111	14	14	13	13	12	13	15	
1.16 (L) TR72	15	9	11	11	12	11	11	Divergent
1.16 (L) TR-2007-Gu	15	9	11	11	12	11	11	
1.16 (L) TR16	15	12	15	14	14	14	14	
1.18 (P) TJ06	12	17	14	15	14	14	16	Divergent
1.18 (P) BJ0701	14	16	14	14	14	14	16	
1.18 (P) XY-3	13	17	14	15	14	14	16	
1.23.1 (U) 130/15-5364	16	17	15	16	15	16	16	Divergent
1.23.2 (U) m31182	18	19	20	18	17	19	19	
1.23.2 (U) 441/09	17	18	19	17	16	18	18	
1.23.2 (U) GXBH-EB34	16	16	19	17	17	18	20	
1.23.2 (U) JS-00108	17	17	20	18	18	19	21	
1.23.2 (U) QHQL-252	17	17	20	18	18	19	21	
1.23.2 (U) LN309-5	17	17	20	18	18	19	21	
1.23.2 (U) GXCZ-FB22	18	17	20	18	18	19	21	

Continued

	T6-18	TR-Erz-Pst8	TRElz-4-2021	IR-Shiraz-322	TY8723	IRTV1	TR-Elz-Pst16	
1.25 (P) S153	23	25	27	24	23	25	26	Divergent
T6-18 (W)	-							
TR-Erz-Pst8 (V)	15	-						Homogeneous (From 1 to 8; mean 4.2)
TRElz-4-2021 (V)	13	8	-					
IR-Shiraz-322 (A)	14	7	4	-				
TY8723 (V)	13	7	3	3	-			Divergent from T6-18
IRTV1 (A)	13	6	2	2	1	-		
TR-Elz-Pst16 (V)	15	7	4	4	3	2	-	

Values exceeding limit value 9 for genotype determination indicated divergence.