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Effects of methodology and analysis strategy on robustness of pestivirus phylogeny

Lihong Liu[a,*], Hongyan Xia[a,b], Claudia Baule[a], Sándor Belák[a,b], Niklas Wahlberg[c]

[a] Joint R&D Division in Virology, The National Veterinary Institute & The Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[b] Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, SE-751 89 Uppsala, Sweden
[c] Laboratory of Genetics, Department of Biology, University of Turku, 20014 Turku, Finland

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ABSTRACT

Phylogenetic analysis of pestiviruses is a useful tool for classifying novel pestiviruses and for revealing their phylogenetic relationships. In this study, robustness of pestivirus phylogenies has been compared by analyses of the 5′UTR, and complete N^{pro} and E2 gene regions separately and combined, performed by four methods: neighbour-joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI). The strategy of analysing the combined sequence dataset by BI, ML, and MP methods resulted in a single, well-supported tree topology, indicating a reliable and robust pestivirus phylogeny. By contrast, the single-gene analysis strategy resulted in 12 trees of different topologies, revealing different relationships among pestiviruses. These results indicate that the strategies and methodologies are two vital aspects affecting the robustness of the pestivirus phylogeny. The strategy and methodologies outlined in this paper may have a broader application in inferring phylogeny of other RNA viruses.

1. Introduction

The genus *Pestivirus* of the family *Flaviviridae* consists of four approved species: *Bovine viral diarrhoea virus* 1 (BVDV-1), *Bovine viral diarrhoea virus* 2 (BVDV-2), *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV); and a fifth tentative species, Pestivirus of giraffe (Thiel et al., 2005). The viral single-stranded, positive-sense RNA genome contains two untranslated regions (UTRs) at the 5′ and 3′ ends, and one open reading frame (ORF) encoding a polyprotein, which is co- and post-translationally processed into 12 polypeptides in the following order: N-terminal autoprotease (N^{pro}), capsid protein (C), envelope proteins (E^{rns}, E1, and E2), p7, and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Lindenbach et al., 2007). Of all genetic regions of the viral RNA, the highly conserved 5′UTR has been used historically for genetic typing of pestiviruses (Hofmann et al., 1994; Pellerin et al., 1994; Ridpath et al., 1994). However, it has been observed that the consensus tree based on 5′UTR contains polytomies and fails to confidently establish relationships among isolates (Xia et al., 2007). By contrast, N^{pro} gene and E2 gene that shows the greatest variability, have been reported well suitable for phylogenetic analysis of pestiviruses (Becher et al., 1997, 1999, 2003). Neighbour-joining (NJ), one of the distance-based methods, has been used in analysis of the complete or nearly complete N^{pro} and/or E2 genes (Arnal et al., 2004; Becher et al., 2003; De Mia et al., 2005). Analysis of other less variable genetic regions has been reported, for example, NS3 (Schirrmeier et al., 2004), NS5B (Lowings et al., 1996), and 3′UTR (Vilcek et al., 1999).

The character-based methods, including maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) have also been used in pestivirus phylogeny (Liu et al., 2009b; Jones et al., 2004; Xia et al., 2007). All of these methods evaluate trees based on an optimality criterion, which is calculated from the character (nucleotide) at each position of a set of sequences, and the most optimal tree(s) is chosen to represent the phylogenetic relationships of the taxa of interest. The three methods differ in the optimality criterion, as well as how the optimality criterion is used. In MP, the most optimal tree(s) is the one that requires the least number of changes, i.e. the most parsimonious explanation of the evolutionary history of a given dataset. In ML, a model of the evolution of the data over time is invoked and the parameters and topology that maximize the likelihood of the model are chosen. In BI, the likelihood of the model is calculated in the same way as in ML, but the method attempts to sample all parameter values and topologies from their true distributions, but as this is currently impossible computationally, the so-called

* Corresponding author at: Department of Virology, Immunobiology and Parasitology (VIP), The National Veterinary Institute (SVA), Travvaegen 22, SE-751 89 Uppsala, Sweden. Tel.: +46 18 674689; fax: +46 18 674669.
E-mail address: Lihong.Liu@sva.se (L. Liu).

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posterior probability is approximated using standard statistical methods.

Two strategies, depending on analysis of the genetic regions individually or combined, have been applied in pestivirus phylogeny (Xia et al., 2007; Liu et al., 2009b). Discrepancies in pestivirus phylogenies have been observed in the analysis under the two strategies (Xia et al., 2007). However, the effects of the methodologies on the robustness of pestivirus phylogenies have never been evaluated. It is evident that such discrepancies would lead to different interpretations of the phylogenetic relationships among pestiviruses. Therefore, the aim of this study was to compare the robustness of the topology of pestivirus phylogenies inferred by four methods: BI, ML, NJ, and MP using two strategies: analyses of the 5′UTR and the complete N^{pro} and E2 gene regions individually or combined. A further Partitioned Bremer Support (PBS) analysis was performed to find possible conflict and the genetic regions in the combined dataset that are supporting the pestivirus phylogeny.

2. Materials and methods

2.1. Nucleotide sequence alignment and model selection

An alignment of 1899 characters including gaps and 52 pestiviruses was used in this study, including all reference strains

Table 1
List of pestiviruses.

Species[a]	Genotype/subgenotype	Virus name	Accession numbers[b]		
			5′UTR	N^{pro}	E2
BVDV-1	1a	BVDV-1/Ref	NC_001461	–	–
		SD1	M96751	–	–
		NADL	AJ133738	–	–
		Oregon C24V	AF041040	–	–
		Singer Arg	DQ088995	–	–
	1b	CP7	U63479	–	–
		Osloss	M96687	–	–
		KE9	EF101530	–	–
		VEDEVAC	AJ585412	–	–
	1c	Trangie Y546	AF049222	–	–
		Deer-NZ1	m	U80903	AF144614
		Bega	AF049221	–	–
	1d	SH9	m	AF144473	AF144616
		721	m	AF144463	AF144609
	1j	KS86-1ncp	AB078950	–	–
		Deer-GB1	m	U80902	AF144615
	1m	ZM-95	AF526381	–	–
		11468	m	AY735458	AY734488
BVDV-2		BVDV-2/Ref	NC_002032	–	–
		New York′93	AF502399	–	–
		890	U18059	–	–
		P11Q	AY149215	–	–
BVDV-3		Th/04_KhonKaen	FJ040215	–	–
		CH-KaHo/cont	m	AY895011	EU385605
		D32/00_HoBi	AY489116	AY735486	AY604725
Pestivirus of giraffe		Giraffe-1/Ref	NC_003678	–	–
		PG-2	m	AY163647	AY163654
Antelope virus		Antelope	AY781152	–	–
BDV	1	BDV/Ref	NC_003679	–	–
		BD31	U70263	–	–
		T1802/1	U65046	AY163649	AY163656
	2	466	m	AY163650	AY163657
		AZ79	m	AY163652	AY163659
		17385/00	m	AY163651	AY163658
		Reindeer-1	NC_003677	–	–
		Bison-1	m	AF144476	AF144619
	3	Gifhorn	m	AY163653	AY163660
	4	Chamois-1	AY738080	AY738083	AY738082
CSFV		94.4/IL/94/TWN	AY646427	–	–
		Brescia	M31768	–	–
		CSFV/Ref	NC_002657	–	–
		Alfort/187	X87939	–	–
		SWH	DQ127910	–	–
		0406/CH/01/TWN	AY568569	–	–
		GXWZ02	AY367767	–	–
		cF114	AF333000	–	–
		Riems	AY259122	–	–
		RUCSFPLUM	AY578688	–	–
		HCLV	AF091507	–	–
		Brescia	AF091661	–	–
		39	AF407339	–	–
		Paderborn	AY072924	–	–

[a] Species are named according to Liu et al. (2009b).
[b] "–" stands for the same accession number as for the 5′UTR; "m" stands for sequence that is not available and treated as missing data.

of the four recognised species and most representative strains within species. The nomenclature of BVDV-1 subgenotypes was used according to Nagai et al. (2008). The proposed species BVDV-3 was used in this study to refer atypical bovine pestiviruses (Liu et al., 2009b). The 5′UTR and the complete N^{pro} and E2 gene sequences were retrieved from GenBank, and concatenated using the software WinClada (Nixon, 2002). The name and accession number for each genetic region are summarized in Table 1. As some sequences of the 5′UTR had not been deposited in the GenBank, they were treated as missing data in the combined analyses, and were excluded in the single 5′UTR analyses. Multiple sequence alignment was done with CLUSTAL W (Thompson et al., 1994). The best-fit evolutionary model was selected by the software MrModelTest V.2.2 (Nylander, 2004), as previously described (Xia et al., 2007).

2.2. Inferring pestivirus phylogeny

The analyses were performed using four commonly used methods: BI, ML, NJ, and MP. The NJ analyses were performed with the software MEGA 4 (Tamura et al., 2007), under the widely used Kimura-2 parameter model (Kimura, 1980) with 1000 bootstrap analyses. The ML and BI analyses were performed under the best-fit evolutionary model. PHYML v2.4.4 (Guindon and Gascuel, 2003) was used for phylogeny inference according to ML criterion. The robustness of the hypothesis was tested through 1000 non-parametric bootstrap analyses. Bayesian inference analysis used the software MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), as previously described (Xia et al., 2007). The Markov chain Monte Carlo (MCMC) search was run with four chains for 3–5 million generations, sampling the Markov chain every 1000 generations. At the end of run, the convergence of the chains was inspected through the average standard deviation of split frequencies. The first 25% trees were discarded as "burn-in". The MP analyses were performed using PAUP ver 4.0b10 (Swofford, 2003). Each analysis was performed at least three times and a representative consensus tree is presented in this paper. A Kishino–Hasegawa test was performed using normal approximation, two-tailed test, which is implemented in PAUP ver 4.0b10 (Swofford, 2003).

The potential conflict in phylogenetic signal among the three genetic regions was investigated in a parsimony framework. The MP analyses were conducted using a heuristic search algorithm in the program TNT (Goloboff et al., 2008) on the combined, equally weighted dataset. The data were subjected to 100 random addition rounds of successive Sectorial, Ratchet, Drift and Tree Fusing searches (Goloboff, 1999; Nixon, 1999; Moilanen, 1999). We evaluated the character support/conflict for the clades in the resulting cladograms using Partitioned Bremer Support, or PBS (Baker and DeSalle, 1997; Gatesy et al., 1999). The scripting feature of TNT was used to calculate these values (Peña et al., 2006) and the data were partitioned according to genetic regions.

3. Results

3.1. Evolutionary model

The General Time Revisible (GTR) with substitution rate heterogeneity and proportion of invariable sites "GTR + I + G" was selected as the best-fit evolutionary model for the single N^{pro} and E2 gene datasets and the combined dataset. For the 5′UTR, the best-fit model was "GTR + G". The gamma shape parameter of the γ-distribution was 0.3692 for the 5′UTR, 0.824 for the N^{pro}, 1.0518 for the E2, and 0.9084 for the combined dataset, indicating different rate variations in each dataset. Regardless of species, a similar proportion of invariable sites was found for the N^{pro} gene (0.2261), the E2 gene (0.189), and the combined sequence dataset (0.2079).

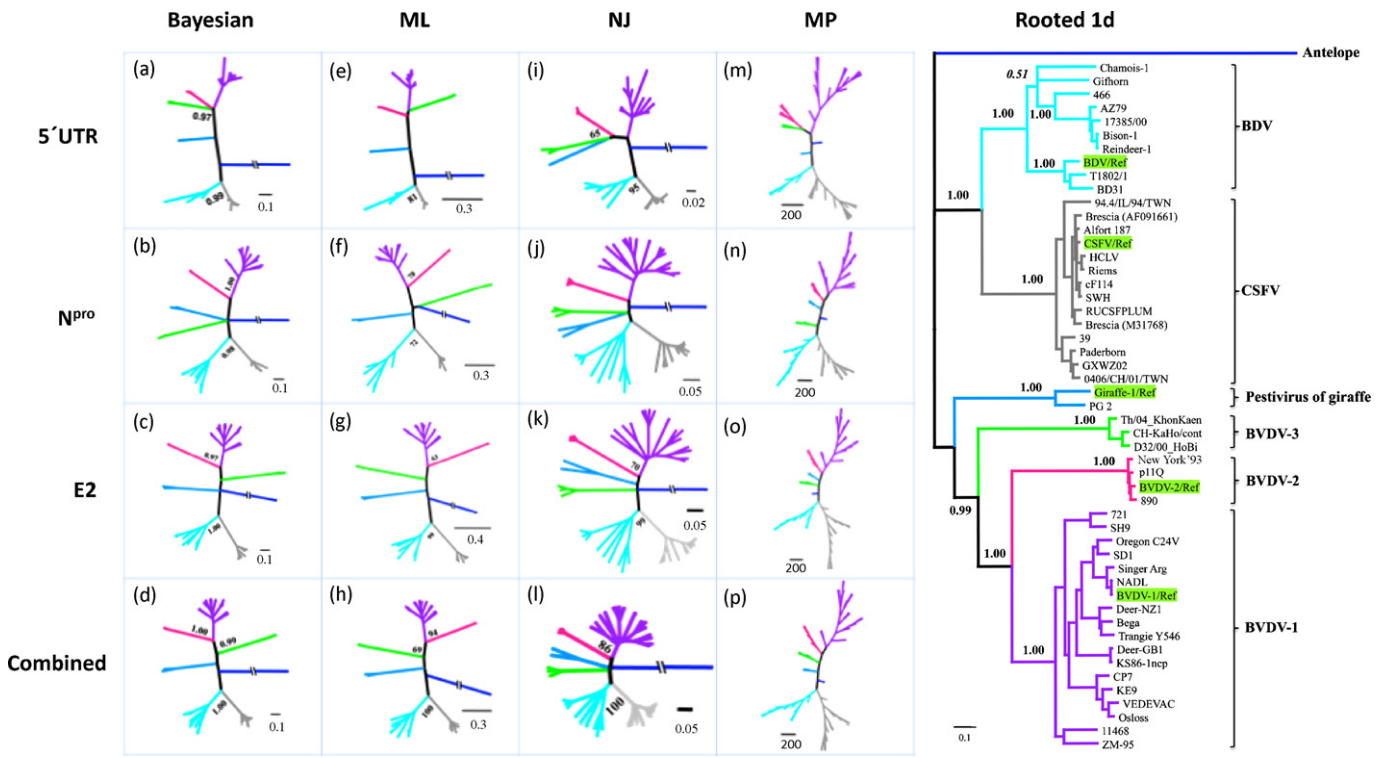

Fig. 1. Pestivirus phylogenies inferred by Bayesian (BI), maximum likelihood (ML), neighbor-joining (NJ), and maximum parsimony (MP) analyses of the 5′UTR (a, e, i and m), complete N^{pro} gene (b, f, j and n) and E2 gene (c, g, k and o) separately and combined (d, h, l and p). The Antelope virus-rooted Bayesian tree (d) is shown to the right in order to display pestiviruses' name and their relationships. The branches are color-coded for each species, and the reference strains are highlighted in green. Numbers are posterior probability or bootstrap (1000 replicates) values for the major clades. All trees are unrooted. Bar indicates changes per site.

3.2. Pestivirus phylogenies

Phylogenetic analyses of pestiviruses resulted in 12 different tree topologies at the species level, which in turn depicted 12 different relationships among pestiviruses (Fig. 1). A closer relationship between BDV and CSFV was consistently resolved in all analyses, and supported by high posterior probability and bootstrap values (except NJ analysis of N^{pro} gene region). The relationship between BVDV-1 and BVDV-2 was also determined in the analyses of the complete N^{pro} and E2 gene regions and the combined dataset. The relationship among BVDV-3, Pestivirus of giraffe, and Antelope was not consistently established in the single-gene analyses. By contrast, BI, ML and MP analyses of the combined sequence dataset unequivocally demonstrated the relationships among all pestiviruses.

In the 5′UTR phylogeny, all pestiviruses were correctly typed to each species as identified by the reference strains within clades, and strongly supported (Fig. 1a, e, and i). However, the 5′UTR phylogeny of the given nucleotide sequence dataset was unable to resolve relationships among the pestivirus species (except that between BDV and CSFV), even for the relationship between BVDV-1 and BVDV-2.

Analyses of the N^{pro} gene region by BI, ML and MP established the relationship between BVDV-1 and BVDV-2 (Fig. 1b, f and n), which was not supported by NJ analysis (Fig. 1j). However, the close relationship between BVDV-1 and BVDV-2 could be established and strongly supported by a bootstrap value of 86% when the Antelope was removed from the NJ analysis. A similar supporting value was also observed when neither Antelope nor BVDV-3 was included in the NJ analysis. This indicated the dependence of the tree topology and supporting values on the composition of the sequences in a NJ analysis.

Bayesian and ML analyses of the E2 gene region further resolved the relationship between BVDV-3 and the established BVDV-1 and BVDV-2 (Fig. 1c and g). However it was not strongly supported by posterior probability and bootstrap values. ML analysis even established all relationships among pestiviruses with strong supports only for the recognised species. NJ analysis revealed that it is the Pestivirus of giraffe, rather than BVDV-3 that is closer to BVDV-1 and BVDV-2 (Fig. 1k). The similar pattern was also observed in MP analysis, where Pestivirus of giraffe was incorrectly positioned in the tree (Fig. 1o).

BI, ML, and MP analyses of the dataset combining all three genetic regions yielded an identical tree topology (Fig. 1d, h, and p). The close relationship between BVDV-3 and the recognised BVDV-1 and BVDV-2 was strongly supported by a posterior probability value of 0.99 and a bootstrap value of 69%. Relative to the divergent Antelope, the clade of the Pestivirus of giraffe was placed in the same side of the tree as BVDV, indicating a closer relationship among these pestiviruses of mainly bovine-origin. NJ analysis of the combined dataset was still unable to reveal such a relationship (Fig. 1l).

3.3. Kishino–Hasegawa test

The robustness of tree topologies was corroborated by a Kishino–Hasegawa test using ML approach. The ML tree of the combined dataset was the best (−Ln = 35,077), but support for this tree was not significantly different from that (−Ln = 35,082) for the E2 tree as both trees had same topology. However, it differed significantly from support (−Ln = 35.184) for the N^{pro} tree, in which the Antelope branch was incorrectly positioned (Fig. 1f).

3.4. Partitioned Bremer Support analysis

PBS analysis showed no conflict for any species as all three genetic regions agree that each species is monophyletic (Table 2 and Fig. 2). There was also no conflict for the sister species relationship between BDV and CSFV, and between BVDV-1 and BVDV-2. Slight conflict was found from the E2 gene region in the clade consisting of all three BVDV species: BVDV-1, BVDV-2 and BVDV-3, but the Bremer support value was still positive for this clade. The position of giraffe as sister to the BVDV clade was supported only by the E2 gene, but the conflict from the others was weak.

4. Discussion

In this study, 12 tree topologies were inferred from BI, ML, NJ, and MP analyses of three genetic regions separately and combined. It must be mentioned that additional topologies have also been

Table 2
Partitioned Bremer Support (PBS) and total Bremer support (BS) for the selected major nodes in the tree of the combined dataset.

Node	Clade	$PBS_{5'UTR}$	$PBS_{N^{pro}}$	PBS_{E2}	BS_{total}
1	1a	0	9	23	32
2	1c	0	23	31	54
3	**1a + 1c**	**1**	**7.5**	**11.5**	**20**
4	1j	14.5	27.5	68	110
5	**1a + 1c + 1j**	**3**	**3**	**11**	**17**
6	1b	5	−1	59	63
7	**1a + 1c + 1j + 1b**	**4**	**−1**	**10**	**13**
8	1m	0	−1	10	9
9	**1a + 1c + 1j + 1b + 1m**	**0**	**0.5**	**3.5**	**4**
10	1d	0	13	29	42
11	**BVDV-1** (**1a + 1c + 1j + 1b + 1m + 1d**)	**5**	**13**	**36**	**54**
12	BVDV-2	26	34	123	183
13	**BVDV-1 + BVDV-2**	**1**	**3**	**8**	**12**
14	BVDV-3	20	37	75	132
15	**BVDV-1 + BVDV-2 + BVDV-3**	**9**	**4**	**−7**	**6**
16	Pestivirus of giraffe	3	32	43	78
17	**BVDV-1 + BVDV-2 + BVDV3 + Pestivirus of giraffe**	**−1**	**−2**	**6**	**3**
18	CSFV	10.5	20.5	61	92
19	BDV-2	3	4	7	14
20	**BDV-2 + BDV-3**	**3**	**0.5**	**1.5**	**5**
21	BDV-1	7	13	35	55
22	**BDV-2 + BDV-3 + BDV-1**	**3**	**5**	**−5**	**3**
23	**BDV** (**BDV-2 + BDV-3 + BDV-1 + BDV-4**)	**6**	**9**	**16**	**31**
24	**BDV + CSFV**	**6.5**	**1.5**	**18**	**26**

Bold values are not judged statistically, they are only indicative of support (positive value) or conflict (negative value).

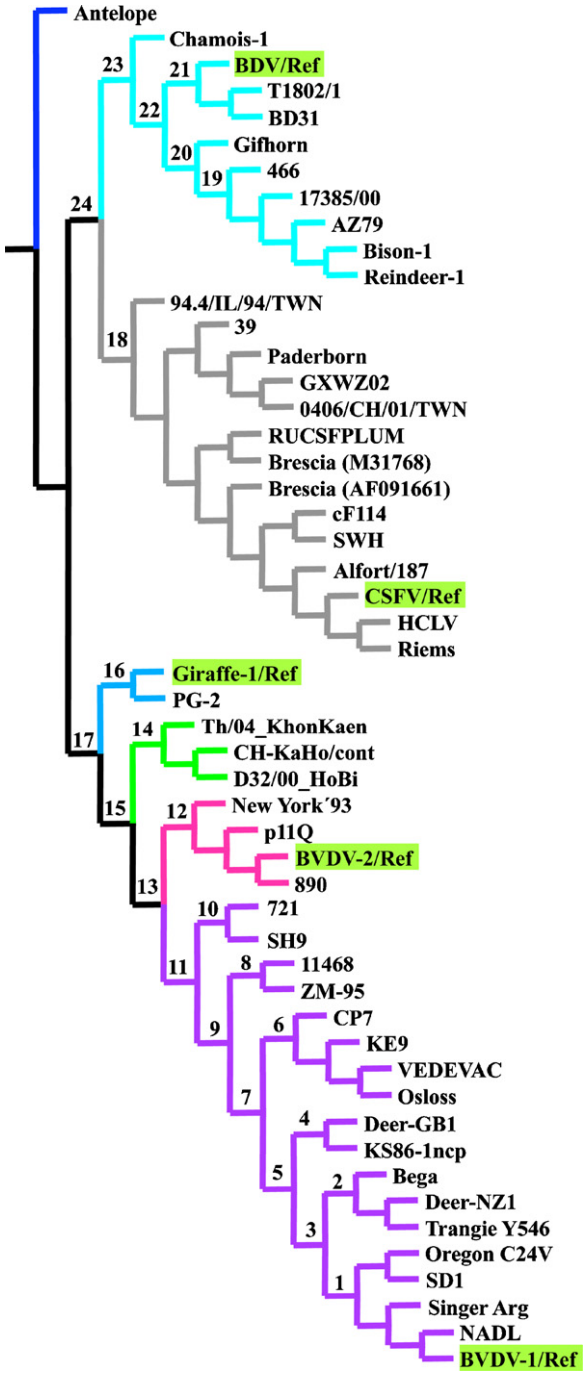

Fig. 2. Maximum parsimony tree of the dataset combining the 5′UTR, and the complete N^{pro} and E2 gene regions. The branches are color-coded for each species with the clades of interest numbered. Partitioned Bremer Support values are summarized in Table 2.

reported, for example, the NJ tree of N^{pro} gene region (Rümenapf and Thiel, 2008). All these topologies present different evolutionary relationships among pestiviruses. For example, the NJ analysis of the N^{pro} gene (Fig. 1j) showed that BDV, CSFV, Pestivirus of giraffe and BVDV-3 share the most recent common ancestor given that the possible root of the tree lies on the Antelope branch (Liu et al., 2009b). However, Bayesian, ML and MP analyses of the combined dataset (Fig. 1d, h and p) showed that BDV and CSFV have the most recent common ancestor, whereas BVDV-1, BVDV-2, BVDV-3, and Pestivirus of giraffe share their own most recent common ancestor. In order to reveal a reliable relationship among pestiviruses, it is crucial to find the most probable tree as the discrepancies in the phylogenies will lead to different, even contradictory relationships.

A comparison of the tree topologies indicated that the strategy and methodology of the analyses are two important factors in determining the phylogenies for the given sequence dataset. The strategy of analysing the combined sequence dataset by BI, ML, and MP methods outperformed the single-gene analysis strategy regardless of the methods used. For instance, the strongly supported unique position of the BVDV-3 clade in the Bayesian and ML analyses of the combined dataset is in agreement to that of the Th/04_KhonKaen in the whole-genome phylogeny (Liu et al., 2009a). This is in sharp contrast to weakly supported, variable positions of the BVDV-3 clade in the analyses of the 5′UTR, N^{pro} and E2 gene regions. Combing all sequence data would increase the amount of phylogenetic signal for a better resolution of phylogenetic relationships in the Bayesian and ML analyses. Furthermore, it has been reported that analysis of a combined dataset can bring out the hidden phylogenetic signal in them (Gatesy et al., 1999; Wahlberg et al., 2005).

For the given dataset, the NJ analyses of the three genetic regions separately and combined with Kimura-2 parameter model were unable to reveal the "true" relationship among pestiviruses at species level. Similar phylogenetic trees were observed with Tamura-3 parameter model (Tamura, 1992). This is supported by further evaluation of the tree topologies by the sum of branch length (SBL), which was 4.83099 for the tree of the combined dataset under Kimura-2 parameter model, and 4.83631 for that under Tamura-3 parameter model. Distance methods, such as NJ, have disadvantages such as reliable estimates of pairwise distances can be hard to obtain for divergent sequences, and that information is lost in compressing sequences into distances, therefore, the observed differences between sequences are not accurate reflections of the evolutionary distances between them (Holder and Lewis, 2003). As a result, the relationships among the pestiviruses were not solved even at the species level.

Single-gene analyses yielded different tree topologies, yet the PBS analysis suggested only weak conflict within the BVDV-giraffe clade (node 17) and within the BVDV clade (node 15). This suggests that the studied gene regions share cryptic phylogenetic signal that is only brought out in a combined analysis (Gatesy et al., 1999). A further finding was the unexpected, positive contribution of the 5′UTR in the combined dataset to all except one clade in the pestivirus phylogeny. This suggests that in order to obtain a confident phylogeny of pestiviruses, it is invaluable to include the 5′UTR in addition to the N^{pro} and E2 gene regions. This is in contrast to two trends in pestivirus phylogeny: one is focusing on N^{pro} and E2 gene regions only arguing that both are giving higher confidence level than 5′UTR; and another is analysing 5′UTR and/or N^{pro} separately arguing that both regions can be easily sequenced. However, as demonstrated in this study, a robust phylogeny was only obtained from analyses of the dataset combining all three genetic regions by BI, ML, and MP methods, and single-gene analyses have a limited capacity to reflect the phylogenetic relationships.

The discrepancies observed in this study were also corroborated by Kishino–Hasegawa test using ML approach. As BI, ML and MP analyses of the combined dataset resulted in a single tree topology, these trees represented the most robust relationships among pestiviruses. The reasons for the discrepancies are still unknown, and RNA recombination has been suggested. However, the discrepancies could not be explained by putative recombination events. When the combined dataset was analysed by Recombination Detection Program v3.22 (Heath et al., 2006), only two possible recombination events were found within species CSFV. However,

the relationship between CSFV and BDV was consistent in all analyses regardless of methods and strategies. It is unlikely that the possible recombination events are causing discrepancies observed in other species whose relationships varied depending on the methods and strategies.

In summary, discrepancies in pestivirus phylogenies at the species level have been observed in the BI, ML, NJ, and MP analyses of three genetic regions separately and combined. The strategy of analysing the combined sequence dataset by BI, ML, and MP methods outperformed the single-gene analysis strategy. This study highlights the importance of the strategy and methodology in pestivirus phylogeny, and may shed light on the improved phylogenetic studies of other RNA viruses.

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