ORIGINAL ARTICLE

Novel marker for recombination in the 3′-untranslated region of members of the species *Human enterovirus A*

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Abstract *Human enterovirus A* (HEV-A) is a species in the genus *Enterovirus*. Viruses belonging to this species are often responsible for hand, foot and mouth disease and associated acute neurological disease. Studies of the 3′ untranslated region (UTR) of human enterovirus 71 (HEV71) revealed a possible role in virus replication. We compared the 3′-UTRs of all members of HEV-A and confirmed the presence of a secondary structure comprising three stem-loop domains (SLDs). SLD-Z is situated closest to the stop codon and has been shown previously to affect plaque morphology. The prototype strains of coxsackieviruses A4 (CVA4), CVA14, and CVA16 carried the longer group I SLD-Z, whilst other CVAs and HEV71 carried the shorter group II SLD-Z. We demonstrate the importance of SLD-Z as a marker for the emergence of newer strains of HEV71 and CVA16 through inter-typic recombination and propose that SLD-Z is a novel evolutionary marker for recombination in HEV-A.

Introduction

Human enterovirus A (HEV-A) is a species in the genus *Enterovirus*, family *Picornaviridae*. Other species in this

genus include HEV-B, -C and -D, *Human rhinovirus* (HRV)-*A*, -*B* and -*C*, *Bovine enterovirus*, *Porcine enterovirus B* and *Simian enterovirus A* [1]. Most enterovirus infections are asymptomatic, but the viruses in HEV-A, particularly coxsackievirus A16 (CVA16) and human enterovirus 71 (HEV71), are often associated with hand, foot and mouth disease in children [2, 3]. HEV71 has also been associated with encephalitis, meningitis and poliomyelitis-like paralysis [4–6]. These viruses contain a positive-stranded RNA genome of approximately 7,500 nucleotides (nt), which consists of an open reading frame (ORF) encoding a single polyprotein. The ORF is divided into three regions: P1 encodes structural proteins, and P2 and P3 encode non-structural proteins. It is flanked by 5′- and 3′- untranslated regions (UTR) with a poly-A tail at the 3′-terminus of the 3′-UTR (reviewed in ref. 7).

The enteroviral 3′-UTR was initially thought to be a highly conserved heteropolymeric sequence consisting of two common stem-loop domains (SLDs) X and Y, as found in poliovirus Mahoney type 1 (PV1M) [8]. However variation in the number of SLDs has been shown to occur in members of other species. The 3′-UTR of coxsackievirus B3 (CVB3) and other members of the HEV-B species have been shown to possess an additional stem-loop structure, SLD-Z [9], whilst the 3′-UTR of the HRVs carried only the SLD-Z [10, 11]. The inter-species heterogeneity of the 3′-UTR secondary structure prompted us to examine whether viruses of other species have unique 3′-UTR structures that can be used as important evolutionary markers. Sequence analyses of the 3′-UTR of some enteroviruses have been reported [8, 12]; however, to our knowledge, there have been no comparative studies on secondary structures.

The functional role of the enterovirus 3′-UTR is still unclear. Together with the poly-A tract, the 3′-UTR has been implicated to be involved in both translation and

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replication [9, 11, 13]. Destabilising point mutations affecting the 3′-UTR tertiary structure have resulted in a lethal phenotype [14, 15]. Structural and biological studies of the CVB3 SLD-Z have demonstrated a relationship to virulence *in vivo* [9]. Nevertheless, translation- and replication-competent picornaviruses have been generated in which the entire 3′-UTR has been deleted [13, 16].

A potentially functional SLD-Z was recently identified in the 3′-UTR of HEV71 [17]. In this study, we performed a detailed analysis of the sequence within the 3′-UTR of all serotypes belonging to HEV-A. Sequence alignment and RNA folding predictions confirmed the presence of a secondary structure comprising three SLDs. Phylogenetic and SimPlot analysis revealed the significance of this region in determining genetic distance and as a marker of recombination.

Materials and methods

Sequence and secondary structure analysis

Sequences representative of HEV-A used in this study are listed in Table 1. Alignment of the sequences was performed using the Clustal W programme [18]. The secondary structure of the 3′-UTR was predicted using the Mfold version 3.2 programme [19]. When more than one structure was predicted, the structure with lower free energy was selected.

Phylogenetic and SimPlot analysis

Sequences were aligned using CLC Main Workbench 6.1.1 (www.clcbio.com) with the following parameters: gap open cost = 10, gap extension cost = 1, end gap cost = as any other. Trees were generated using the neighbour-joining (NJ) algorithm with 100 bootstrap replicates. CVB3 was selected as the out-group in each analysis. To assess potential recombinatorial relationships, nucleotide sequences were analysed by using the bootscanning method implemented in SimPlot v3.5.1 [20]. A window size of 350 base pairs with steps of 10 base pairs was used for each analysis with strict consensus matching.

Results

3′-UTR sequence and secondary structure analysis of HEV-A

The formation of three stem-loop domains within the 3′-UTR of HEV71 was predicted using Mfold version 3.2 [17]. In the current study, we extended this analysis to other HEV-A serotypes. The 3′-UTR sequences from 19 HEV-A prototype strains (representing all serotypes) were obtained from GenBank (Table 1). Alignment of these sequences confirmed the finding that the distal region of the 3′-UTR is the most conserved, and the proximal region the least conserved (Fig. 1a). The newly identified enteroviruses HEV76, HEV89-92, SV43 and SV46 showed a primary sequence and secondary structure that are distinct from those of the other HEV-As (less than 40 % similarity). The 3′-UTRs of HEV76 and HEV89-92 showed greater similarity to each other, particularly in the SLD-X and -Y regions, than to the CVAs and HEV71. The same can be said for SV43 and SV46. However, in the SLD-Z region, these recently identified enteroviruses varied in sequence and predicted secondary structure (data not shown). Interestingly despite a lack of sequence conservation, the 3′-UTR carried three stem-loop structures in all HEV-A serotypes examined.

The SLD-Z can be divided into two main groups based on consensus sequence and secondary structure (Fig. 1a, b). Group I encompassed a longer sequence commencing immediately after the ORF stop codon at the 3′ terminus of the 3D polymerase gene. Group II consisted of a shorter sequence from six nucleotides downstream of the stop codon. Alignment of prototype strains from HEV-A showed that CVA4, CVA14 and CVA16 carried the group I SLD-Z (Fig. 1a). Other prototypes including CVA2, CVA3, CVA5-8, CVA10 and CVA12 and HEV71 carried the group II SLD-Z. The new enteroviruses and simian viruses carried SLD-Z elements of variable size.

Within HEV71, all but two subgenogroups (B3 and C4) carried the group II SLD-Z, similar to HEV71 prototype strain BrCr [17]. Subgenogroups B3 and C4 carried the group I SLD-Z, reminiscent of those of CVA4, CVA14 and CVA16.

Phylogenetic trees were constructed using the SLD-Z sequence. Each of the trees included the corresponding region of CVB3 as the out-group. Analysis of the prototype HEV-A strains revealed a separation into three distinct groups with strong bootstrap support (Fig. 2a). In one group, the majority CVAs and HEV71 showed a close relationship. These genotypes carried the group II SLD-Z. On the other hand, CVA4, CVA14 and CVA16, all of which carried the group I SLD-Z, formed a separate cluster. The newly discovered enteroviruses formed another group, with HEV76 and HEV89-91 in a tight cluster, whilst HEV92 demonstrated divergence. Interestingly, SV43 clustered with the group I SLD-Z, whilst SV46 clustered with the group II SLD-Z.

SLD-Z as a marker for recombination

We were particularly interested in comparing the SLD-Z elements of HEV71 and CVA16, two genotypes of HEV-A

Table 1 List of viruses used in this study

	Strain	Place of isolation	Year of isolation/submission	GenBank accession number
HEV-A genotype				
CVA2	Fleetwood	USA	1947	AY421760
CVA3	Olson	USA	1948	AY421761
CVA4	High Point	USA	1948	AY421762
CVA5	Swartz	USA	1950	AY421763
CVA6	Gdula	USA	1949	AY421764
CVA7	Parker	USA	1949	AY421765
CVA8	Donovan	USA	1949	AY421766
CVA10	Kowalik	USA	1950	AY421767
CVA12	Texas-12	USA	1948	AY421768
CVA14	G14	South Africa	1950	AY421769
CVA16	G10	South Africa	1951	U05876
CVA16	5079-98	Taiwan	1998	AF177911
CVA16	SHZH00-1	China	2000	AY790926
CVA16	SHZH05-1	China	2007*	EU262658
CVA16	KMM/08	China	2008	HQ423141
CVA16	FY18	China	2008	EU812514
CVA16	GZ08	China	2008	FJ198212
CVA16	SZ/HK08-7	China	2008	GQ279371
HEV76	FRA91-10369	France	1991	AY697458
HEV89	BAN00-10359	Bangladesh	2000	AY697459
HEV90	BAN99-10399	Bangladesh	1999	AY697460
HEV91	BAN00-10406	Bangladesh	2000	AY697461
HEV92	RJG7	USA	2007*	EF667344
SV43	OM112t (P12)	USA	2007*	AF326761
SV46	OM22 (P15)	USA	2007*	AF326764
HEV71 subgenogroup				
A	BrCr	USA	1970	U22521
Bl	HUN/1978	Hungary	1978	HQ189392
Bl	Nagoya	Japan	2009*	AB482183
B2	MS/7423/87	USA	1987	U22522
B3	26M/AUS/2/99	Australia	1999	AF376101
B3	SK-EV006	Malaysia	1997	AB550334
B3	EV71/SAR/SHA63	Malaysia	1997	AM396588
B3	3799-SIN-98	Singapore	1998	AF376117
B4	C7/Osaka-Org	Japan	1997	AB550336
B4	EV71/9/97/SHA89	Malaysia	1997	AJ586873
B4	5666/sm/002209	Singapore	2000	AF352027
B5	SB12736-SAR-03	Malaysia	2005*	DQ341362
B5	5511-SIN-00	Singapore	2000	AF376121
B5	N2838-TW03	Taiwan	2003	FJ357378
B5	M0380-TW08	Taiwan	2008	FJ357385
CI	1 M/AUS/12/00	Australia	2000	AY126014
CI	KED005-Org	Malaysia	1997	AB550340
CI	804/NO/03	Norway	2003	DQ452074
C2	6F/AUS/6/99	Australia	1999	DQ381846
C2	ENT/PM/SHA71	Malaysia	1997	AM396585
C2	NCKU9822	Taiwan	1998	AF136379

Table 1 continued

	Strain	Place of isolation	Year of isolation/submission	GenBank accession number
C2	1095-Org	Japan	1997	AB550332
C3	03-KOR-00	Korea	2003	AY125968
C3	06-KOR-00	Korea	2003	AY125970
C4	75-Yamagata-Org	Japan	2003	AB550338
C4	SHZH03	China	2003*	AY465356
C4	1235	Taiwan	2004	DQ133459
C5	E2005125-TW	Taiwan	2006	EF063152
C5	2007-07364	Taiwan	2007	EU527983

* indicates year of submission
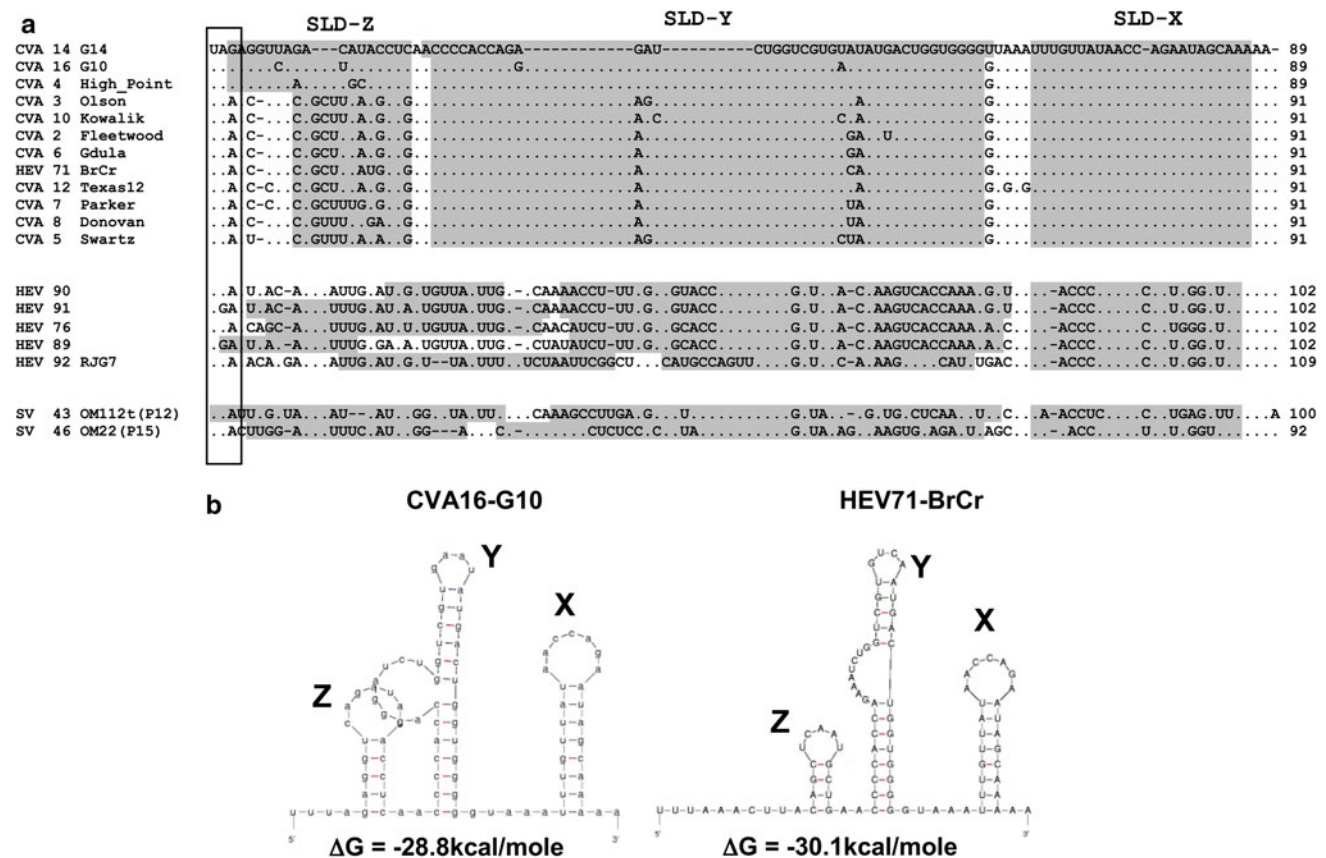
Fig. 1 3′-UTR sequence and secondary structure of HEV-A. (**a**) Alignment of 3′-UTR sequences. Conserved nucleotides are shown as (.); SLD borders determined by Mfold are highlighted in grey; the stop codon is shown in a white box. (**b**) Mfold predictions of the 3′-UTR secondary structure of CVA16-G10 and HEV71-BrCr

that are often co-isolated in HFMD [21–23]. A detailed phylogenetic analysis was carried out using the SLD-Z sequences of HEV71 and CVA16 strains (Fig. 2b). Of the eight strains of CVA16 tested, only two strains, the prototype strain CVA16-G10 and CVA16-FY18, carried the group I SLD-Z. Six other strains (from different outbreaks) carried the group II SLD-Z.

We then utilised SimPlot analysis to verify our findings. Analysis of the 3′ end of HEV71-75-Yamagata-Org (subgenogroup C4) against representative strains of all HEV71 subgenogroups and CVA16-G10 showed highest similarity to CVA16-G10 (Fig. 3a). Bootscan analysis showed up to 95 % sequence similarity at nucleotides 6,300-6,700 and >7,100 (Fig. 3b). SimPlot analysis of the full-length genome confirmed our findings and further revealed a recombination crossing point at approximately nucleotide 3,500 (Fig. 4a, b). Nucleotides 1-3,500 HEV71-75-Yamagata-Org showed a high sequence similarity with subgenogroup C2.
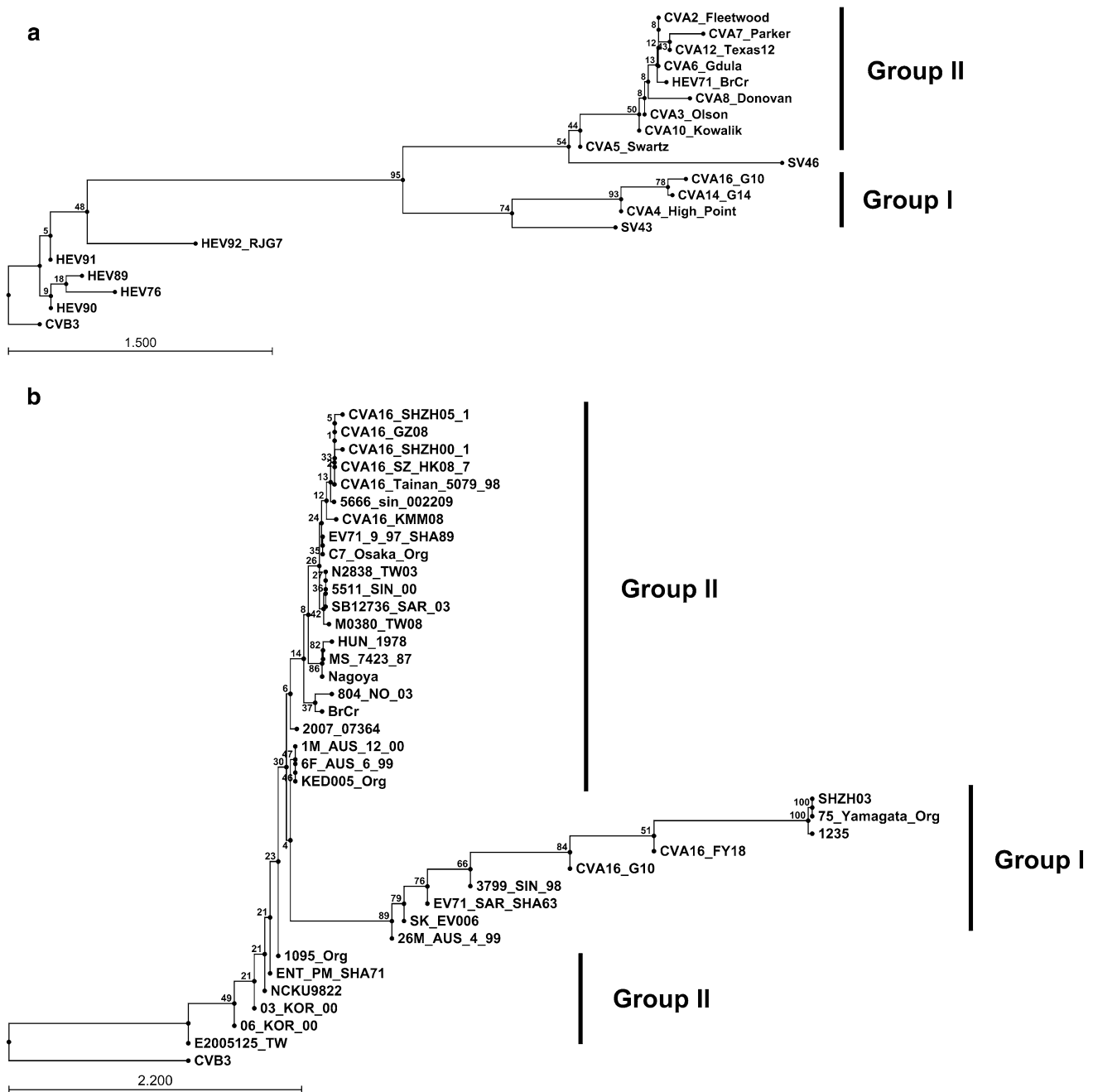
Fig. 2 Phylogenetic analysis based on the SLD-Z nucleotide sequence of (**a**) HEV-A prototypes and (**b**) HEV71 and CVA16 strains. Values at each node indicate bootstrap values, with each bar representing the distance scale for each tree

When HEV71-26M-AUS-4-99 (B3) was used as the query sequence, we again found high sequence similarity to CVA16-G10 (Figure 3c). The region with highest similarity (up to 95 % bootscan value) was after 6,700 (Fig. 3d). When the analysis was extended to the full-length genome, a recombination crossing point was again seen at approximately nucleotide 3,500 (Fig. 4c, d). Upstream of this region, the highest sequence similarity was observed with members of subgenogroup B4/B5.

Next, we compared the CVA16-KMM-08 strain to the various HEV71 subgenogroups and CVA16-G10. This strain clustered with others containing a group II SLD-Z (Fig. 2b). Bootscan analysis showed up to 95 % similarity with HEV71-BrCr (subgenogroup A) 5′ of nucleotide 6,500 and 3′ of nucleotide 6,600 (Fig. 3f). A full-length genome comparison revealed a recombination between CVA16-G10 and HEV71-BrCr with a crossing point at approximately nucleotide 3,500 (Fig. 4e, f).
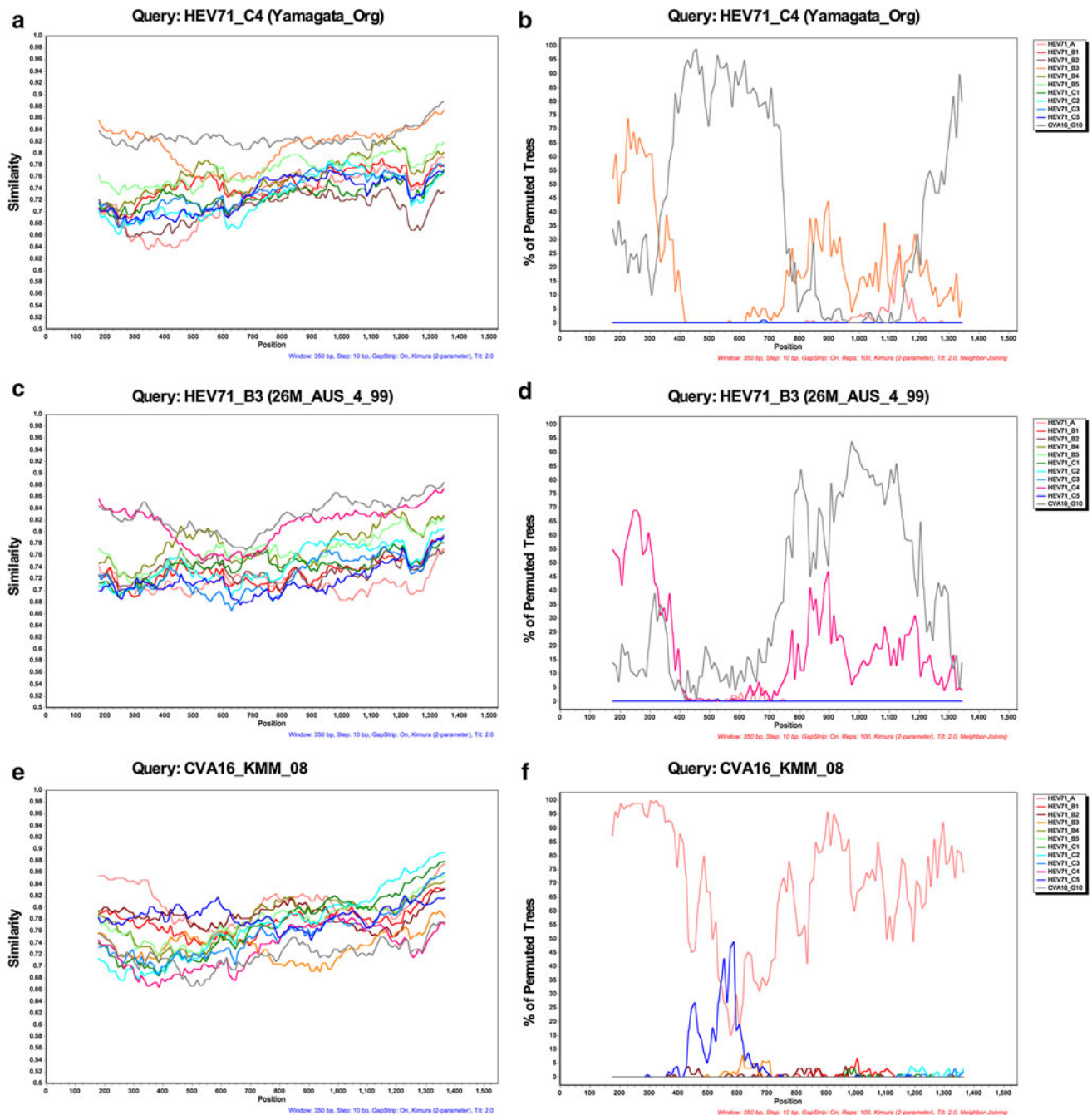
Fig. 3 Simplot analysis based on the 3D and 3′UTR sequence. Similarity plots (left panels) and bootscan analysis (right panels) were prepared using the (**a**, **b**) HEV71-75-Yamagata-Org (**c**, **d**) HEV71-26M and (**e**, **f**) CVA16-KMM-08 as query sequences

Discussion

Unlike the IRES and cloverleaf regions of the 5′-UTR, the 3′-UTR of enteroviruses is a poorly understood region. In early reports, the existence of tRNA-like terminal structures that may play an important role in RNA synthesis was proposed [8, 24]. Other studies have shown discrepancies in elucidating the importance of the 3′-UTR in tissue culture and in animal models [9, 13, 16]. In the current study,

we performed a comparative analysis of published sequences to elucidate the evolutionary significance of the HEV-A 3′-UTR.

Computer-based alignment and secondary structure predictions have proven to be useful in studying the 3′-UTRs of various picornaviruses [8–10, 24]. The Clustal W sequence alignment programme and the Mfold RNA-folding programme were used to derive a model for the secondary structure of the HEV71 3′-UTR, which consists
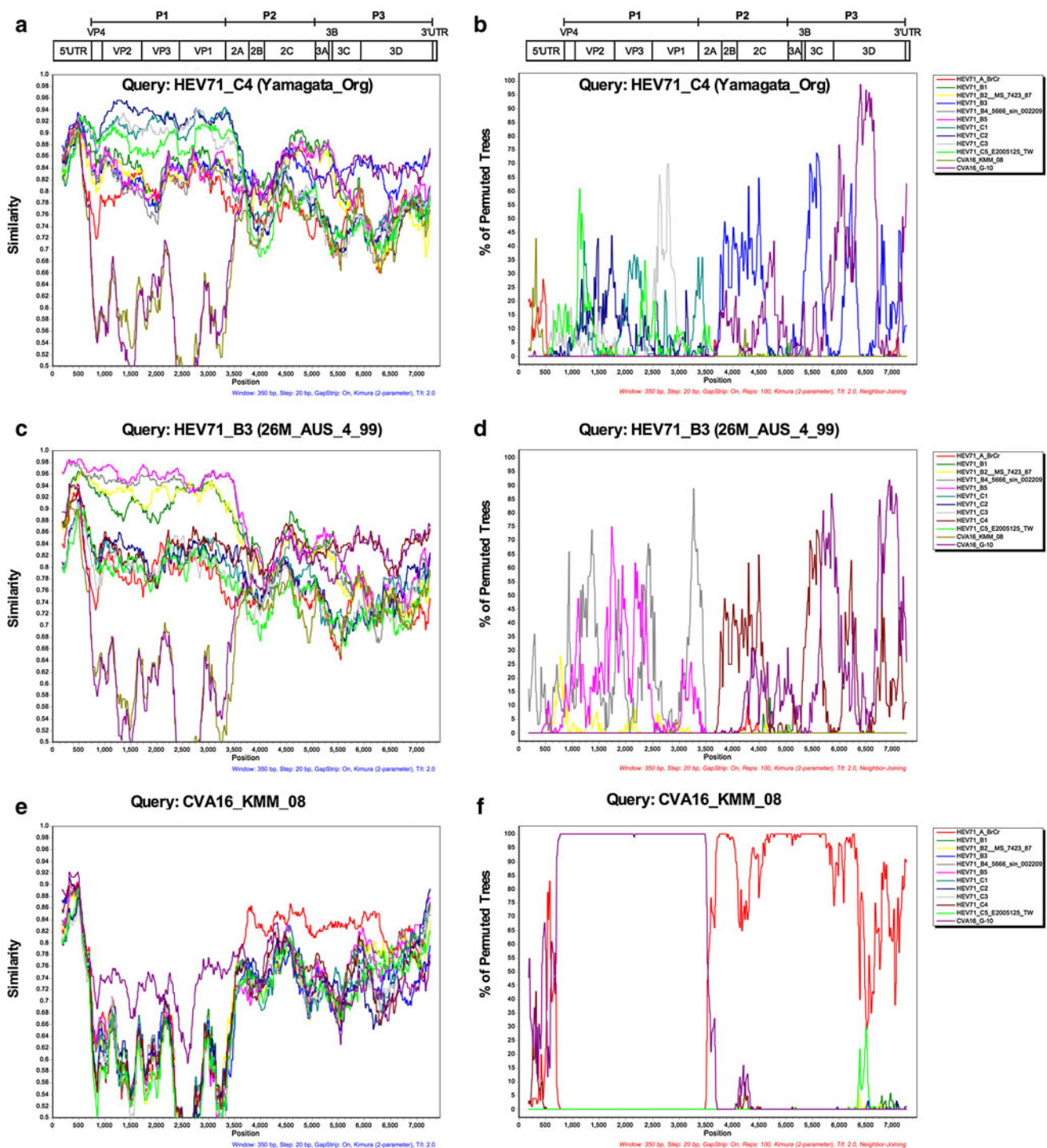
Fig. 4 Simplot analysis based on the full-length sequence. Similarity plots (left panels) and bootscan analysis (right panels) were prepared using the (**a**, **b**) HEV71-75-Yamagata-Org (**c**, **d**) HEV71-26M and (**e**, **f**) CVA16-KMM-08 as query sequences

of three predicted stem-loop structures [17]. In this study, we further showed that the 3′-UTR-associated stem-loop structures exist in all HEV-A serotypes.

The 5′-UTR sequence of HEV-A is closely related to that of HEV-B [25]. Despite a lack of 3′-UTR sequence similarity, our data demonstrated relatedness between

members of these two species in the higher-order RNA structure. The HEV-A SLD-Z is reminiscent of the HEV-B SLD-Z [9], although it is shorter.

Detailed analysis of the HEV-A 3′-UTR revealed two groups of SLD-Z elements based on sequence and secondary structure. CVA4, CVA14 and CVA16 carried the

longer group I SLD-Z, whilst all other serotypes carried the shorter group II stem-loop domain. Within HEV71, B3 and C4 are the only two subgenogroups carrying the group I SLD-Z. All other subgenogroups tested carried the group II SLD-Z. A distinct clustering of the two groups was shown by phylogenetic analysis.

The newly identified enteroviruses HEV76, and HEV89-92, clustered together on a separate branch as expected, due to their low primary sequence similarity to other HEV-As. However all 3′-UTR sequences tested were predicted to fold into three stem-loop domains, which further points to the importance of secondary structure conservation. Interestingly, SV43 clustered with the group I SLD-Z (longer), whilst SV46 clustered with the group II SLD-Z (shorter). The SV43 and SV46 viruses were found to carry a long and short SLD-Z, respectively (data not shown). Taken together, these data suggest that the size and secondary folding of the SLD-Z play an important part in the classification of these viruses.

Phylogenetic analysis of the 3D gene, situated upstream of the 3′-UTR of HEV-A, has been performed previously [26]. A distinct clustering of HEV71-26M (subgenogroup B3) with two other HEV71 strains, HEV71-SHZH03 and HEV71-SHZH98 (both subgenogroup C4), as well as CVA4, CVA14 and CVA16-G10, was evident. Our data extended these findings to the 3′-UTR region and suggest that both the 3D and 3′-UTR regions of HEV71 subgenogroups B3 and C4 were acquired through recombination.

Inter-typic recombination between co-circulating enteroviruses such as between HEV71 and CVA16 has been reported [27], and HEV71-SHZH03 (subgenogroup C4) has been proposed to be a product of recombination between HEV71 subgenogroup C2 and CVA16-G10-like viruses. This is consistent with our data. Based on SimPlot analysis results for the 3D and 3′-UTR regions (Fig. 3c, d) and the complete genome (Fig. 4c, d), we further propose that HEV71-26M-AUS-4-99 (subgenogroup B3) is a product of recombination between HEV71 genogroup B and CVA16-G10-like viruses. Since HEV71 subgenogroups B3 and C4 were isolated more recently compared to other viruses in the same cluster, these are more likely to be the 'recipient' of the group I SLD-Z.

All other HEV71 subgenogroups (including the prototype strain HEV71-BrCr) and HEV-A serotypes tested carried the group II SLD-Z. Six CVA16 strains were also found to carry the group II SLD-Z. Chan and AbuBakar [27] suggested that one of these strains, CVA16 strain Tainan-5079-98 (CVA16-5079-98 in this study), was a product of recombination between CVA16-G10 and HEV71-BrCr. Phuektes et al. [26] further demonstrated the clustering of CVA16-SHZH00 (CVA16-SHZH00-1 in this study) and CVA16-5079 (CVA16-5079-98 in this study) in a branch close to EV71-BrCr, whilst CVA16-Gunnell was

found in another group comprising EV71-MS (subgenogroup B2), EV71-5865 (subgenogroup B4) and CVA5. In the current study, we showed that CVA16-KMM/08 is a recombinant of CVA16-G10 and HEV71-BrCr, confirming previously published results.

Taken together, these data suggest that ancestral strains of HEV71 carried the shorter group II SLD-Z, whilst their counterpart in the CVA16 serotype carried the longer group I SLD-Z. The emergence of newer strains of HEV71 and CVA16 carrying the reciprocal SLD-Z could have occurred through inter-typic recombination. This recombination event was not apparent by conventional screening using VP1 sequencing. Thus, SLD-Z is a useful marker to screen for recombination in HEV-A strains.

In the current study, we have confirmed the presence of three stem-loop domains in members of the species *Human enterovirus A*. We have further revealed two groups of SLD-Z in HEV-A based on the size of the stem-loop domain. Finally, we propose that the SLD-Z is a novel evolutionary marker for recombination in HEV-A.

References

1. ICTV (2011) International Committee on Taxonomy of Viruses. http://ictvonline.org/virusTaxonomy.asp?version=2011. Accessed 22 Aug 2012

2. Chang LY, Lin TY, Huang YC, Tsao KC, Shih SR, Kuo ML, Ning HC, Chung PW, Kang CM (1999) Comparison of enterovirus 71 and coxsackievirus A16 clinical illness during the Taiwan enterovirus epidemic, 1998. Pediatr Infect Dis J 18:1092–1096

3. Hosoya M, Kawasaki Y, Sato M, Honzumi K, Hayashi A, Hiroshima T, Ishiko H, Kato K, Suzuki H (2007) Genetic diversity of coxsackievirus A16 associated with hand, foot, and mouth disease epidemics in Japan from 1983 to 2003. J Clin Microbiol 45:112–120

4. Melnick JL (1984) Enterovirus type 71 infections: a varied clinical pattern sometimes mimicking paralytic poliomyelitis. Rev Infect Dis 6(Suppl 2):S387–S390

5. Gilbert GL, Dickson KE, Waters MJ, Kennett ML, Land SA, Sneddon M (1988) Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr Infect Dis J 7:484–488

6. Alexander JP Jr, Baden L, Pallansch MA, Anderson LJ (1994) Enterovirus 71 infections and neurologic disease – United States, 1977–1991. J Infect Dis 169:905–908

7. McMinn PC (2002) An overview of the evolution of EV71 and its clinical and public health significance. FEMS Microbiol Rev 26:91–107

8. Pilipenko EV, Maslova SV, Sinyakov AN, Agol VI (1992) Towards identification of *cis*-acting elements involved in the replication of enterovirus and rhinovirus RNAs: a proposal for the existence of tRNA-like terminal structures. Nucleic Acids Res 20:1739–1745

9. Merkle I, van Ooij MJM, van Kuppeveld FJM, Glaudemans DHRF, Galama JMD, Henke A, Zell R, Melchers WJG (2002)

Biological significance of a human enterovirus B-specific RNA element in the 3′ nontranslated region. J Virol 76:9900–9909

10. Todd S, Semler BL (1996) Structure-infectivity analysis of the human rhinovirus genomic RNA 3′ non-coding region. Nucleic Acids Res 24:2133–2142

11. Dobrikova EY, Florez P, Bradrick S, Gromeier M (2003) Activity of a type 1 picornavirus internal ribosomal entry site is determined by sequences within the 3′ nontranslated region. PNAS 100:15125–15130

12. Zell R, Stelzner A (1997) Application of genome sequence information to the classification of bovine enteroviruses: the importance of 5′- and 3′-nontranslated regions. Virus Res 51:213–229

13. Dobrikova EY, Grisham RN, Kaizer C, Lin J, Gromeier M (2006) Competitive translation efficiency and the picornavirus type I internal ribosome entry site facilitated by viral cis and trans factors. J Virol 80:3310–3321

14. Melchers WJG, Hoendrop JGJ, Bruins Slot HJ, Pleij CWA, Pilipenko EV, Agol VI, Galama JMD (1997) Kissing of two predominant hairpin loops in the coxsackievirus B virus 3′ untranslated region is the essential structural feature of the origin of replication required for negative-strand RNA synthesis. J Virol 71:686–696

15. Wang J, Bakkers JMJE, Galama JMD, Bruins Slot HJ, Pilipenko EV, Agol VI, Melchers WJ (1999) Structural requirements of the higher order RNA kissing element in the enteroviral 3′UTR. Nucleic Acids Res 27:485–490

16. Todd S, Towner JS, Brown DM, Semler BL (1997) Replication-competent picornaviruses with complete genomic RNA 3′ non-coding region deletions. J Virol 71:8868–8874

17. Kok CC, Phuektes P, Bek E, McMinn PC (2012) Modification of the untranslated regions of human enterovirus 71 impairs growth in a cell-specific manner. J Virol 86:542–552

18. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

19. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415

20. Lole KS, Bollinger RC, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160

21. Lin TY, Twu SJ, Ho MS, Chang LY, Lee CY (2003) Enterovirus 71 outbreaks, Taiwan: occurrence and recognition. Emerg Infect Dis 9:291–293

22. Li L, He Y, Yang H, Zhu J, Xu X, Dong J, Zhu Y, Jin Q (2005) Genetic characteristics of human enterovirus 71 and coxsackievirus A16 circulating from 1999 to 2004 in Shenzhen, People's Republic of China. J Clin Microbiol 43:3835–3839

23. Podin Y, Gias ELM, Ong F, Leong Y-W, Yee S-F, Yusof MA, Perera D, Teo B, Wee T-Y, Yao S-C, Yao S-K, Kiyu A, Arif MT, Cardosa MJ (2006) Sentinel surveillance for human enterovirus 71 in Sarawak, Malaysia: lessons from the first 7 years. BMC Public Health 6:180

24. Rohll JB, Moon DH, Evans DJ, Almond JW (1995) The 3′ untranslated region of picornavirus RNA: features required for efficient genome replication. J Virol 69:7835–7844

25. Oberste MS, Penaranda S, Maher K, Pallansch MA (2004) Complete genome sequences of all members of the species human enterovirus A. J Gen Virol 85:1597–1607

26. Phuektes P, Chua BH, Sanders S, Bek EJ, Kok CC, McMinn PC (2011) Mapping genetic determinants of cell-culture growth phenotype of enterovirus 71. J Gen Virol 92:1380–1390

27. Chan YF, AbuBakar S (2006) Phylogenetic evidence for intertypic recombination in the emergence of human enterovirus 71 subgenotypes. BMC Microbiol 6:1–11