Genetic diversity of bluetongue viruses in Australasia

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Summary

The authors have characterised the genetic diversity of the bluetongue virus (BTV) RNA segments 3 and 10 from Indonesia, Malaysia and Australia. Analysis of RNA segment 3, which codes for the core protein VP3, showed conserved sequences in the previously defined Australasian topotype, but which further divided into four distinct clades or genotypes. Certain genotypes appeared to be geographically restricted while others were distributed widely throughout South-East Asia. Ongoing surveillance programmes in Australia have identified the movement of Indonesian genotypes into northern Australia and possible reassortment among them. Similarly, analysis of RNA segment 10, which codes for the non-structural protein NS3/3A, showed they were also conserved and grouped into five clades or genotypes, three Asian and two North American/South African.

Keywords

Australasia – Bluetongue – Core protein – Genetics – Non-structural 3/3A protein – *Orbivirus* – Phylogenetic – Viral protein – Virus.

Introduction

Bluetongue (BT) is an arthropod-transmitted disease of wild and domestic ruminants caused by BT virus (BTV). BTV is a member of the *Orbivirus* genus, one of nine genera in the family *Reoviridae* (22). They have a segmented, double-stranded RNA (dsRNA) genome (32) and 24 serotypes have been described (8). BTV is transmitted between vertebrate hosts by *Culicoides* biting midges (18). The virus particle contains three protein layers, the outer capsid layer comprised of two proteins, VP2 and VP5 (11, 20), where VP2 is the major neutralising protein and determinant of serotype specificity (12). The bi-layered core particle is made up of two proteins, VP7 and VP3, three minor proteins, VP1, VP4, VP6, and ten species of dsRNA (20). There are three non-structural proteins, NS1, NS2 and NS3/3A, which are expressed in virus-infected cells (19). The RNA segment 10 codes for NS3/3A protein which mediates the release of virus particles from infected cells (14).

The BTVs have a wide distribution throughout both tropical and sub-tropical regions. In Australia, BTV was first isolated in 1975 (29) and currently eight serotypes have been reported. Six of these (3, 9, 15, 16, 20, 23) have only been found in the north of the Northern Territory, while two serotypes (1, 21) are widely distributed across the northern and eastern coastal regions of Australia (33).

In Indonesia, an outbreak of BT occurred in 1981 in Suffolk sheep imported from South Australia. Subsequently BTV was shown to be widespread throughout Indonesia with seasonal patterns of infection (21, 26). Serotypes 1, 7, 9, 12, 21 and 23 have been isolated from sentinel cattle or *Culicoides* species (26) with serological evidence in cattle, buffalo, goats and sheep (26). The presence of BTV in Malaysia was first indicated serologically in 1977 and clinical BT was reported in imported Australian sheep (4, 27). Subsequently, serotypes 1, 2, 3, 9, 16 and 23 were isolated from sentinel cattle (27). Eleven of the 24 known serotypes of BTV have now been confirmed in Australia, Indonesia and Malaysia.

Molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) (5, 16, 34, 35) have been used to show that geographic separation has resulted in significant divergence in RNA segment 3 sequences (9, 10, 25). Significantly, the

RNA segment 3 sequences could be used to determine the topotype of the BTV isolates (9). The three topotypes identified were the Australasian topotype, North American/South African topotype and another topotype characterised by BTV serotype 15 isolates in Australia (9). The nucleotide sequence variation between topotypes was defined as greater than 15%. Similarly analysis of the RNA segment 10 of field isolates of BTV from the United States of America (USA) demonstrated that virus strains isolated in a restricted geographic region had evolved independently (24) and could be segregated into three distinct monophyletic groups, including two USA groups and one Asian group (3). More recently, the South African BTV isolates have grouped with the North American clusters (I and II), while the Indian BTV isolates grouped with the Asian cluster (III) (31).

We characterised the pattern of distribution and the genetic relationships among BTV isolates from the Australasian region using the nucleotide sequences of RNA segments 3 and 10.

Methods

Virus isolation and identification

Viruses were isolated from sentinel cattle in embryonated chicken eggs, *Aedes albopictus* C6/36 and baby hamster kidney 21 (BHK-21) cells. The serotypes were determined by plaque reduction neutralisation tests (7). Sera from sentinel cattle were tested for BTV-specific antibodies by competitive enzyme-linked immunosorbent assay (c-ELISA) (15).

Molecular analysis

Nucleic acids were extracted from BTV-infected cells using the RNeasy kit (Qiagen) in accordance with the instructions of the manufacturer. The RT-PCR was performed with the One-Step RT-PCR kit (Qiagen) and BTV-specific primers used were A196 (5′accgcacagcagcttaatgatgttag3′) and A203 (5′atacgctgcctccgagtccttacc3′) for RNA segment 3 and B49 (5′gttaaaaagtgtcgctgccatgct3′)/B50 (5′gtaagtgtatagcgccgcaca3′) for RNA segment 10. PCR products from RNA segments 3 and 10 were purified from agarose gels with Qiaquick PCR kits (Qiagen), and sequenced on an AB377 automated sequencer. BTV sequences were aligned using Clustalw 1.6 (30), and the phylogenetic relationships were determined using programs in the Phylip package (6) and the TreeView program (23).

Results

Sequence analysis of RNA gene segment 3

BTVs from Australia, Indonesia and Malaysia were isolated over a number of years in an ongoing surveillance programme (Table I). The majority of nucleotide changes for RNA segments 3 and 10 were synonymous as was shown previously (9, 17, 25). The history of isolation, genotype and GenBank accession numbers are listed in Table I. Genetic analysis showed that the RNA segment 3 sequences in this study could be grouped into the Australasian group or topotype with further groupings into genotypes (Table II; Fig. 1). The genotypes were given names based on the region of first isolation and within a genotype we observed nucleotide sequence variation of less than 6%, while between genotypes the variation was greater than 6% (Table II). Nucleotide sequence analysis of their RNA segment 3 showed the Malaysian BTV isolates, all from 1991, were almost identical and comprised a single genotype, named Malaysia A, while the Indonesian BTV isolates, from 1988-1992, showed much greater genetic variation, and grouped into several genotypes, Java A, C and Australia A (Fig. 1).

Movement of Indonesian genotypes into Australia

Since BTV was first isolated in Australia, the predominant genotype has been Australia A (Table III). This was from 1975 when BTV serotype 20 was first isolated until 1992, 17 years later, when BTV-20 was again isolated (Table III). This BTV serotype 20 had RNA 3 sequences closely related to a Java A genotype from Indonesia. Then further isolations of BTV serotype 20 were obtained in 1995 with the Malaysia A genotype. This genotype had been isolated in Malaysia and Indonesia (Table I). Neither of these genotypes were isolated again.

Similarly, BTV-21 was not isolated for 10 years, between 1984 and 1994. In 1993/1994, early virus isolations of serotypes 1 and 21 had the Australia A genotype. However, towards the end of the wet season, in June 1994, a novel serotype 21 was isolated with a sequence closely related to another Indonesian genotype Java C (Table III).

During the next wet season, serotype 21 viruses with genotype Java C were the most abundant, until March 1995, when the serotype 20 viruses were again isolated with the Malaysia A genotype (Table III). At the end of this wet season we isolated a BTV serotype 20 with the Java C genotype and RNA gene segments 2, 9 and 10 with altered electrophoretic mobilities in polyacrylamide gels (data not shown).

Table I
Genotype, GenBank accession numbers and origin of bluetongue virus isolates

Isolate	Type	Source	Year	Genotype	Accession No.	
					RNA 3	RNA 10
CS156	1	Australia	1979	Australia A	AY322428	
V3036	1	Australia	1994	Australia A	AY277935	AF529052
DPP973	3	Australia	1986	Australia A	L26566	(Ref. 17)
DPP836	9	Australia	1985	Australia A	L26565	(Ref. 17)
DPP965	16	Australia	1986	Australia A	L26557	(Ref. 17)
CS19	20	Australia	1985	Australia A	L26563	(Ref. 17)
CS154	21	Australia	1979	Australia A	L26564	(Ref. 17)
V3209	21	Australia	1994	Australia A	AY277931	AF529053
Z462	21	Australia	1998	Australia A	AY277929	AF529058
DPP90	23	Australia	1982	Australia A	L26568	(Ref. 17)
RIVS46	1	West Java	1990	Australia A	AF529044	AF529049
1163	1	Irian Jaya	1990	Australia A		
B1 Malaysia	1	Malaysia	ND	Malaysia A	L26560	(Ref. 17)
53	1	Malaysia	1991	Malaysia A	AF018264	
55	1	Malaysia	1991	Malaysia A		
49	2	Malaysia	1991	Malaysia A		
54	3	Malaysia	1991	Malaysia A		
41	9	Malaysia	1991	Malaysia A		
44	16	Malaysia	1991	Malaysia A		
57	23	Malaysia	1992	Malaysia A		
Fulvus	1	West Java	1992	Malaysia A		
RIVS178	16	Irian Jaya	1988	Malaysia A		
RIVS63	21	West Java	1990	Malaysia A	AF529047	
Peregrinus	21	West Java	1990	Malaysia A		
RIVS137	21	West Java	1991	Malaysia A		
RIVS113	21	Irian Jaya	1991	Malaysia A		
RIVS39	23	West Java	1991	Malaysia A	AY277928	AF529051
V3594	20	Australia	1994	Malaysia A		
V3598	20	Australia	1995	Malaysia A	AY277933	AF529055
RIVS177	9	West Java	1988	Java A		
RIVS60	21	West Java	1990	Java A	AF529046	AF529059
RIVS66	21	West Java	1989	Java A		
D151-21	21	West Java	1990	Java A		
D151-23	21	West Java	1990	Java A		
V2450	20	Australia	1992	Java A		
RIVS53	3	West Java	1990	Java C	AF529045	AF529050
D153	3	West Java	1990	Java C		
RIVS74	9	West Java	1991	Java C		
RIVS106	23	Irian Jaya	1989	Java C		
B1 India	1	India	ND	Java C	L26559	(Ref. 17)
V3692	1	Australia	1995	Java C	AY277932	AF529056
V4014	1	Australia	1996	Java C	AY277934	AF529057
V4445	1	Australia	1998	Java C	AF529048	
V3217	21	Australia	1994	Java C		
V3548	21	Australia	1995	Java C	AY277930	AF529054
DPP192	15	Australia	1982	Australia B	AY322427	

ND not done

Table II
Per cent nucleotide sequence variation for RNA segment 3 among South-East Asian and Australian bluetongue virus genotypes
Figures in brackets are the percent nucleotide variation within each genotype

Genotype	Java A	Java C	Malaysia A	Australia A	Australia B
Java A (6)	–	7-11	7-8	6-8	24
Java C (4)		–	7-12	9-12	24
Malaysia A (5)			–	6-8	21
Australia A (5)				–	22
Australia B (0)					–

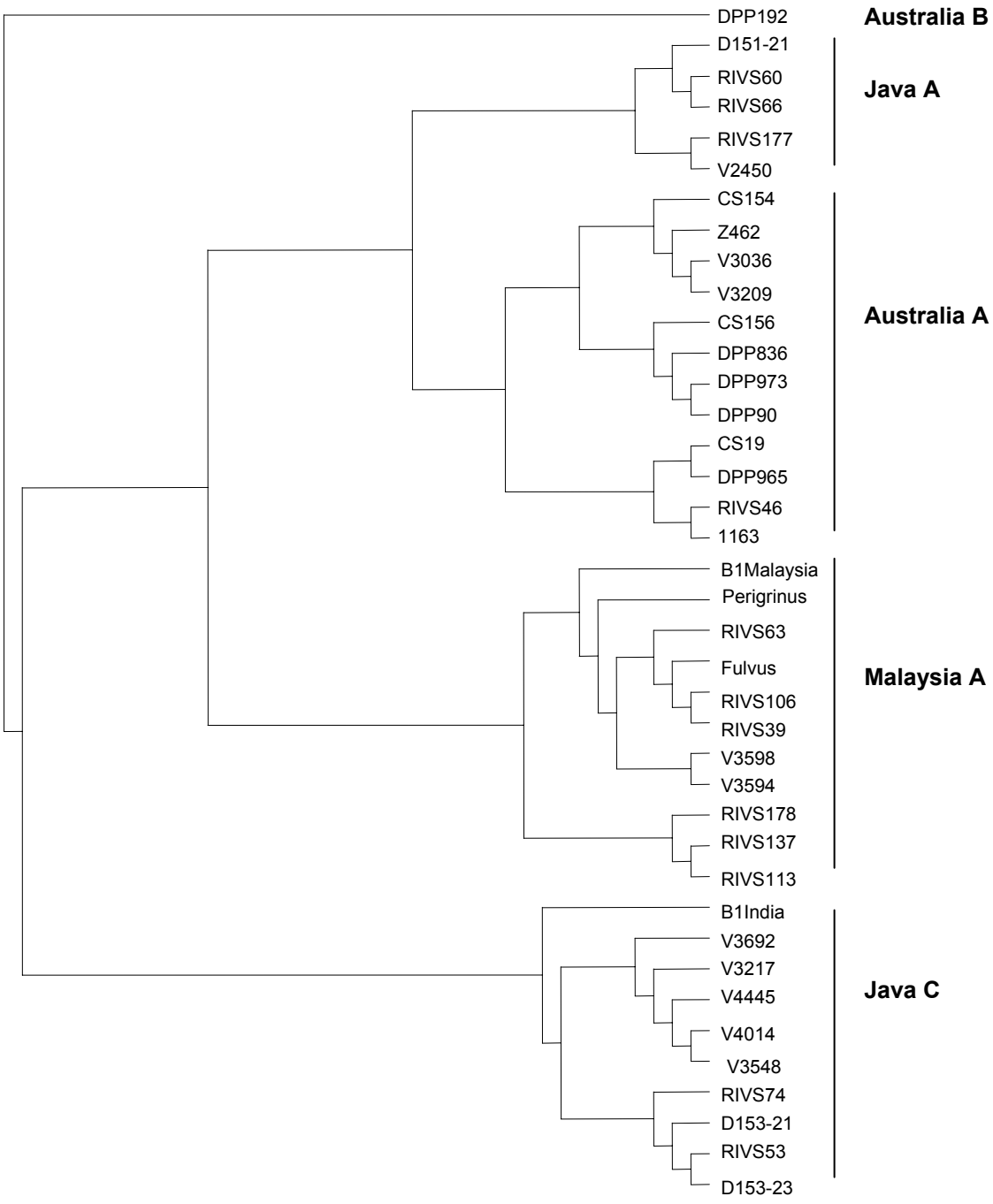

Figure 1
Phylogenetic analysis of bluetongue virus isolates using RNA segment 3
Bootstrap values were not included for clarity
Additional sequences were obtained from McColl and Gould (16)

Table III
Genotypes of bluetongue virus from surveillance in Australia

Year	Northern Territory	New South Wales	Queensland	Western Australia
1975	Australia A			
1979	Australia A			
1981			Australia A	
1982	Australia A Australia B			
1983	Australia A		Australia A	
1984				
1985	Australia A			
1986	Australia A			
1987				
1988	Australia A			
1989	Australia A	Australia A		
1990				
1991	Australia A			
1992	Australia A Java A			
1993	Australia A	Australia A		Australia A
1994	Australia A Java C			
1995	Java C Malaysia A			
1996	Java C			
1997	Java C	Australia A		
1998	Java C	Australia A	Australia A	
1999	Java C		Australia A	
2000	Java C Malaysia A		Australia A	
2001	Java C Malaysia A Australia A		Australia A	
2002	Java C			
2003	Java C			

Sequence data showed that the RNA gene segments 9 and 10 of this virus were also closely related to Indonesian viruses (data not shown). A reassortment appears to have occurred which possibly conferred a selective advantage on this virus in this region.

Subsequently, in 1995/1996, another reassortment occurred between this virus (serotype 20/Java C genotype) and a serotype 1/Australian A genotype, which resulted in a reassortant BTV serotype 1 with a Java C genotype. Again the sequences of gene segments 3, 9 and 10 were closely related to Indonesian genotypes (Table III).

There has been a long history of certain serotypes being restricted in their movement within Australia. It is known that serotypes 1 and 21 have been established across the top and along the east coast of Australia, whereas serotypes 20, 3, 9, 16, 23 and 15 have been confined to the northern area of the Northern Territory.

Sequence analysis of RNA gene segment 10

The Indonesian BTV RNA segment 10 sequences (NS3/3A) also segregated into the Asian and North American/South African groups, which could be further clustered into genotypes. Significantly, the key structural components of the NS3/3A protein were conserved. These include the N-linked glycosylation site (150) and two hydrophobic domains H1 (118-141) and H2 (162-182), which are potential membrane spanning sequences (1, 13, 24). In addition, there are conserved tryptophan (159), cysteine (137 and 181) and proline residues near the amino terminus. There is also a hypervariable region (153-158) between the two hydrophobic domains, H1 and H2. Greater heterogeneity occurred among the Asian isolates than the North American isolates (Fig. 2) and the Chinese, Indian and Indonesian BTV appear to share a common ancestor and have evolved independently from the North American isolates (Fig. 2).

Discussion

The studies reported in this paper were undertaken to improve the understanding of the regional distribution and molecular epidemiology of BTV in South-East Asia and Australia. Between 1988 and 1992, seven serotypes were isolated from Malaysia and Indonesia. While in Malaysia and Irian Jaya (4 000 km to the east of Java), similar serotypes were isolated during the same period indicating that these viruses are endemic throughout the region.

Previously, partial segment 3 sequences were used to group BTV isolates into three distinct groups or topotypes; Australasian, North American/South African and BTV serotype 15 (9, 10). BTV isolates within the Australasian topotype had nucleotide sequences greater than 15% different from isolates from the Caribbean, North America and South Africa, and from Australian BTV serotype 15 (25). While the RNA segment 3 sequences in this study could be grouped into the Australasian topotype, there were further groupings or clades with nucleotide sequence variation greater than 6%, but less than 15% (Table II). These grouping were defined as genotypes, and have been given names based on the region of first isolation (Table II).

Work conducted under the National Arbovirus Monitoring Program (NAMP) has detected possible incursions of Indonesian genotypes into northern Australia on several occasions, in 1992, 1994, 1995 and 2000. Further studies over a longer time frame

are needed to determine if these genotypes circulate continuously or periodically and would assist in the study of the complex interactions which give rise to the evolution of these viruses, their regionalisation and the role of host/vectors. Phylogenetic analysis of RNA segment 3 has shown the evolutionary relationships among the BTV isolates from South-East Asia and Australia. There appears to have been significant movement of genotypes throughout the region. The progenitor of the Australia A genotype shares a common ancestor with the Java A genotype and Indonesian isolates RIVS46 and 1163 (Fig. 1), but the evolutionary history of the Australia B genotype (BTV-15 Australia) is not yet known (25). Similarly, some Japanese encephalitis (JE) virus genotypes in this region have spread across Asia while others are localised and it has been suggested that JE virus originated from its ancestral virus in the Indonesia-Malaysia region (28).

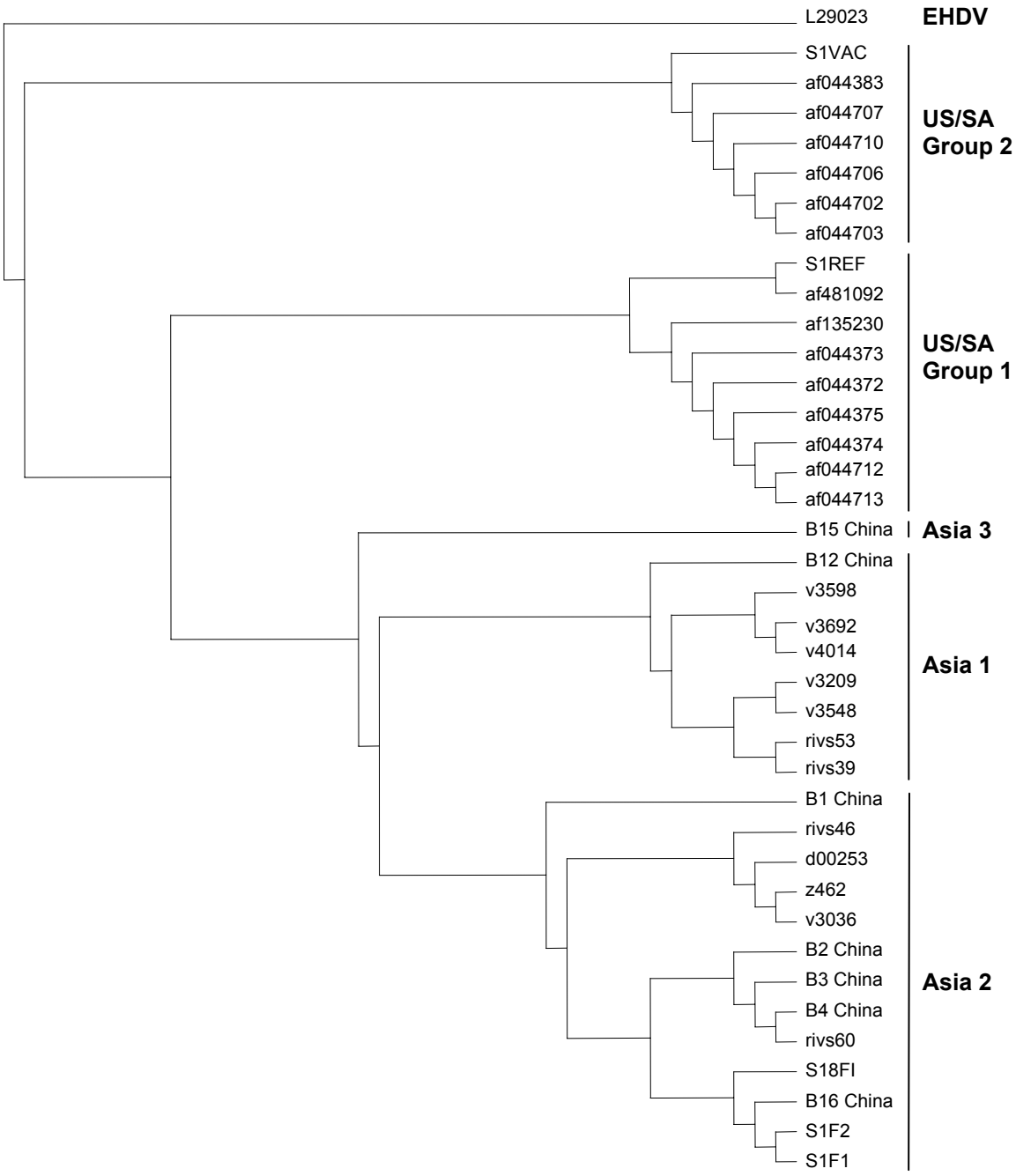

Figure 2
Phylogenetic analysis of bluetongue virus isolates using gene segment 10
Boostrap values were not included for clarity
Additional sequences were obtained from Bonneau *et al.* (3) and Van Niekerk *et al.* (31)

The BTV RNA segment 10 sequences, which code for the non-structural proteins NS3/3A, were also analysed and appeared to cluster according to their geographic origin (3) and may be related to distinct vector species (31). The North American prototype viruses were more closely related to the Asian viruses, while BTV serotype 12 and 15 from China formed independent branches of the Asian group (3). Greater heterogeneity was observed among the Australasian segment 10 sequences than that shown for the North American isolates. Clearly the Chinese, Indian and Australasian viruses share a common ancestor and have evolved independently of the North American viruses. Significantly, the key structural components of the NS3/3A protein were conserved. The NS3/3A protein mediates the release of virus from infected cells and the correct folding at residues 142-161 is thought to be required for transport from the endoplasmic reticulum (ER) to the golgi and then to the cell surface (1, 2). It has been proposed that the RNA segment 10 may have co-evolved with insect vectors (3, 31).

As shown previously, Australasian BTV isolates appear to have evolved from a gene pool distinct from those in other parts of the world. In this region, they appear to have evolved into closely related genotypes primarily based on their geographic isolation. The distribution of BTV within Australasia is also dependent on the movement of infected vectors and/or hosts within naturally constrained ecosystems. Some genotypes were found to be widespread throughout Australasia, while others were confined to discrete niches. Furthermore, the genetic variation of RNA segment 10 sequences may be related to their geographic region and/or vector species responsible for propagation since different vector species predominate in different regions. There appears to be a complex relationship between these viruses and their environment.

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