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Original Article

Identification and genomic characterization of the first isolate of bluetongue virus serotype 5 detected in Australia

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Abstract

Bluetongue virus (BTV), transmitted by midges (Culicoides sp), is distributed worldwide and causes disease in ruminants. In particular, BT can be a debilitating disease in sheep causing serious trade and socio-economic consequences at both local and global levels. Across Australia, a sentinel cattle herd surveillance program monitors the BTV activity. Prior to 2014, BTV-1, -2, -3, -7, -9, -15, -16, -20, -21 and -23 had been isolated in Australia, but no bluetongue disease has occurred in a commercial Australian flock. We routinely use a combination of serology, virus isolation, RT-PCR and next generation and conventional nucleotide sequencing technologies to detect and phylogenetically characterize incursions of novel BTV strains into Australia. Screening of Northern Territory virus isolates in 2015 revealed BTV-5, a serotype new to Australia. We derived the complete genome of this isolate and determined its phylogenetic relationship with exotic BTV-5 isolates. Gene segments 2, 6, 7 and 10 exhibited a close relationship with the South African prototype isolate RSArrrr/5. This was the first Australian isolation of a Western topotype of segment 10. Serological surveillance data highlighted the antigenic cross-reactivity between BTV-5 and BTV-9. Phylogenetic investigation of segments 2 and 6 of these serotypes confirmed their unconventional relationships within the BTV serogroup. Our results further highlighted a need for a revision of the current serologically based system for BTV strain differentiation and importantly, implied a potential for genome segments of pathogenic Western BTV strains to rapidly enter Southeast Asia. This emphasized a need for continued high-level surveillance of vectors and viruses at strategic locations in the north of Australia The expansion of routine characterization and classification of BTV to a whole genome approach is recommended, to better monitor the presence and level of establishment of novel Western topotype segments within the Australian episystem.

Keywords: bluetongue virus, serology, phenotype, genotype, topotype, whole genome.

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Introduction

Bluetongue virus (BTV) strains (genus: *Orbivirus*, family *Reoviridae*) have a worldwide distribution and are responsible for a potentially debilitating disease in ruminants expressed as an epithelial cell pathology of varying intensity, primarily involving mucous membranes and the lining of blood vessels. Virus transmission is normally via the bite of a haematophagous midge vector (*Culicoides sp*). A range of livestock and wild animal species are susceptible to infection but rarely exhibit signs of disease (MacLachlan *et al.* 2009). However, the capacity for BTV to produce severe disease in sheep can result in serious trade and socio-economic consequences for affected local communities and global trade (Saegerman *et al.* 2008). Cattle rarely show clinical disease but are readily infected and are an epidemiologically important reservoir for BTV (Barrattboyes & MacLachlan 1994; MacLachlan 1994). The BTV

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genome contains ten segments of double-stranded RNA that encode an RNA polymerase, expressed by segment 1(Seg-1) and two enzymatic proteins involved in viral genome assembly, VP4 and VP6 (Seg-4 and -9 respectively), which are encased within an icosahedral virus capsid. This comprises the inner core VP3 protein (Seg-3), a further outer core protein, VP7 (Seg-7) arranged in interconnected trimeric structures and a diffuse outercoat comprising proteins VP2 and VP5 (Seg-2 and Seg-6 respectively) (Hewat *et al.* 1992, 1994). Gene segments 5, 8 and 10 code for three non-structural proteins (NS1, NS2 and NS3 respectively) (Mertens *et al.* 1984; Vandijk & Huismans 1988; Roy 1989, 1992) and a second truncated form of NS3, lacking the first 13 aa (NS3a), is generated from a second initiation site (French *et al.* 1989). A fourth non-structural protein (NS4) and a putative fifth non-structural protein (NS5) are expressed from overlapping ORF's (+ 1 reading frame shift) present on Seg-9 and Seg-10 respectively (Belhouchet *et al.* 2011; Stewart *et al.* 2015).

The outercoat protein VP2 possesses virus neutralization epitopes which elicit the unique immune responses that are serologically assayed to differentiate individual BTV serotypes (Howell & Verwoerd 1971; Huismans & Erasmus 1981). At least 27 serotypes have been documented to date (Erasmus 1990; Maan *et al.* 2016). However, the rapid output from next-generation nucleic acid sequencing technologies has enabled the development of BTV-specific quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays which, together with the application of phenotyping software algorithms, play an increasingly significant role in initial BTV detection and serotype confirmation (Maan *et al.* 2007, 2015; Rao *et al.* 2013, 2016). This work has also revealed that two broad ancestral lineages exist worldwide. Western isolates encompass Africa, Europe and the Americas, and Eastern isolates predominate in Australia, Japan, China, India and Southeast Asia (Mertens *et al.* 2007; Maan *et al.* 2008, 2015; Rao *et al.* 2013). Further genotypic sub-groups have also been documented within each region (Bonneau *et al.* 1999; Van Niekerk *et al.* 2003). In the Australian –Southeast Asian region, at least five such genotypes have been proposed (Pritchard *et al.* 2004). These studies, alongside more recent evidence, have clearly demonstrated that BTV continually enters northern Australia via infected *Culicoides* (of varying species) transported on seasonally occurring, prevailing winds from specific island sites within Southeast Asia (Eagles *et al.* 2012, 2013; Bellis *et al.* 2015). However a clear attribution of geographic origin to individual virus isolates can be problematic due to a very high level of gene segment reassortment resulting from mixed infections in both vector and host (Carpi *et al.* 2010; Boyle *et al.* 2012, 2014; Nomikou *et al.* 2015). Hence a new serotype incursion may rapidly reassort with genes from an endemic and or another recently introduced exotic strain or even vaccine strains (Bréard *et al.* 2003; Batten *et al.* 2008; Maan *et al.* 2010).

In Australia, eight serotypes of BTV were identified between 1975 and 1986 (BTV-1, -3, - 9, -15, -16, -20, -21 and -23) (St George *et al.* 2001). Two decades later, concomitant with increasing reports of BTV transmission throughout the world (Purse *et al.* 2005; Carpenter *et al.* 2009; MacLachlan *et al.* 2013), Australia experienced further incursions of three new BTV serotypes, BTV-7 (2007), BTV-2 (2008) and most recently, BTV-12 (2015) (Eagles *et al.* 2014; Lunt *et al.* 2009; Maan *et al.* 2015; National Arbovirus Monitoring Program 2015). Despite the prolonged presence of BTV in Australia, there have been no reports of established bluetongue disease in commercial sheep populations. This is believed to be primarily due to the major sheep grazing areas existing in the south of the country where there is very limited and/or sporadic overlap with the known range of the most widespread BTV competent vector in Australia, *Culicoides brevitarsis* (St.George 1985; Standfast *et al.* 1992). Additionally, the majority of BTV serotypes appear to be exclusively concentrated in the far northern regions of the Northern Territory and Western Australia, with a more limited group of serotypes periodically entering Northern Queensland (BTV-1, -2, -15, -16, -21) with only BTV-1 and -21 being consistently isolated in greater Queensland and further south in New South Wales, over a prolonged time period (Firth *et al.* 2017).

In this report, we describe the first isolation and genomic characterization of another BTV serotype

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novel to Australia and investigate its genetic relatedness to previously documented Australian, Eastern and Western regional BTV gene pools. We also provide retrospective serological and molecular evidence of incursions of BTV-5 into Western Australia prior to its first formal identification.

Methods

Virus isolation and propagation

Blood was collected routinely from cattle at National Arbovirus Monitoring Program (NAMP) managed sentinel herds held at Beatrice Hill (12° 34'S 131° 6'E), Northern Territory and periodically from herds near Kununurra (15° 46'S 128° E), Western Australia and from Northern Australia Quarantine Strategy (NAQS) managed herds around Kalumburu (14° 30'S 126° 64'E), Western Australia. Virus isolation on the Beatrice Hill collections was conducted at Berrimah Veterinary Laboratories where 50 μL of whole blood was lysed in 450 μL of sterile distilled water and inoculated into 10-11 day old embryonated chicken eggs as previously described (Gard & Kirkland 1993). This was followed by one passage in C6/36 cells at 24°C in MEM medium (Invitrogen, Australia) supplemented with 10% foetal bovine serum (Serana, Australia) and two passages in BHK-21 BSR cells at 37°C in MEM supplemented with 10% foetal bovine serum. Cattle bloods collected at both Beatrice Hill and Kununurra; and the Beatrice Hill virus isolations were sent for confirmatory testing to the Australian Animal Health Laboratory (AAHL), where they were again passaged and further titrated in BHK-21 BSR cells. Viral titres were calculated as previously described (Reed & Muench 1938).

Antigen detection and serotyping

The presence of BTV strains amongst the viruses isolated from blood samples were detected using an immunofluorescent antibody (IFA) test (Blacksell & Lunt 1993) and/or an antigen detection ELISA (Stanislawek *et al.* 1996) at the Berrimah Veterinary Laboratories and further characterized using virus neutralization tests (Gard & Kirkland 1993). The serum neutralization test (SNT) incorporated serotype-specific reference antiserum sourced from the Onderstepoort Veterinary Institute, Republic of South Africa (RSA). Antisera collected from NAMP sentinel herds were tested for the BTV type-specific antibodies using the virus neutralization test (VNT) where virus concentration remained constant (100 $TCID_{50}$). Where cross reaction between individual serotypes occurred, a plaque reduction neutralization test (PRNT) (Della-Porta *et al.* 1981) was employed to resolve serotype identification.

Molecular characterization

Viral dsRNA isolation

Total RNA was extracted from either original blood samples and or virus infected cell cultures using the MagMAX - 96 Viral RNA Kit (Ambion, Life Technologies, USA) and a MagMAX Express – 96 nucleic acid extraction device (ThermoFisher, Australia).

BTV genome confirmation

Initial confirmation of the presence of viral genome was via a BTV serogroup generic TaqMan assay targeting Seg-10, essentially following a standard protocol (Hofmann *et al.* 2008). Briefly, the AgPath-ID™ One-Step RT-PCR system (Applied Biosystems) was used with the following run conditions, 1 cycle [45°C for 10 min, 95°C for 10 min] then 45 cycles [95°C for 15 sec, 60°C for 45 sec] and a final hold at 4°C. Cycle threshold (Ct) values <37.0 were deemed positive.

Capillary sequencing

Conventional PCR was performed on RNA extracts of positive samples using in-house designed serotype-restricted primer sets, each targeting Seg-2 and Seg-6. Following electrophoresis on 2% agarose gels, bands of the expected size were excised and purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The concentration of purified dsRNA was determined using a NanoDrop UV Spectrophotometer (NanoDrop Technologies, Rockland, DE). Viral dsRNA was denatured by heating

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at 95°C for 5 min and immediately placing on ice. Denatured RNA was then transcribed into cDNA and amplified using the Superscript III One Step RT-PCR System incorporating Platinum Taq DNA polymerase (Life Technologies) following the manufacturer's instructions. Resultant amplicons were subjected to Sanger sequencing carried out using the BigDye terminator v3.1 kit on an Applied Biosystems 3130xl Genetic Analyser. Trace files were analysed using ChromasPro 1.34 (Technelysium).

Genotyping

BTV positive samples were further tested to determine their Seg-3 genotype as previously described (Pritchard *et al.* 2004). Briefly, the Seg-3 specific primers, BTV A196Fwd (5' - ACCGCACAG-CAGCTTAATGATGTTAG - 3') and BTV A203Rev (5'- ATACGCTGCCTCCGAGTCCTTACC - 3') were used to amplify a 384 bp region. Amplification utilized a SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). Cycling conditions were 1 cycle [50°C for 30 min, 95°C for 2 min], 35 cycles [94°C for 15 sec, 50°C for 30 sec and 68°C for 1 min], 1 cycle 68°C for 5 min, and a final hold at 4°C. The resultant amplicon was sequenced and analysed.

Whole genome sequencing

Isolation of BTV dsRNA. Two 150-cm^2 cultures of BHK-21 BSR cells at 80% confluence were inoculated at a multiplicity of infection of 0.1 for each virus isolate. The infected cells were harvested 4–5 days post-inoculation when CPE had reached 50–70%. Adherent cells were removed by a cell scraper and pelleted by centrifugation at 1000*g* for 5 min at room temperature. The pellet was dissolved in RLT plus buffer and RNA was extracted using an RNeasy Plus Mini Kit (QIAGEN), following manufacturer's instructions.

Preparation of BTV ds cDNA. The extracted nucleic acid was treated with RQ1 DNase (Promega), heat denatured at 95°C for 5 min then snap cooled on ice. Complementary DNA was reverse transcribed using the Superscript III system (Invitrogen) in the presence of 50 ng of random hexamers and 0.5 ng of primers specific to the 5' and 3' ends common to all BTV genome segments (5' = GTTAAAN = 3' = and 5' = GTAAGTN = 3' = respectively) (Mertens & Sangar 1985) and digested with RNase H (New England Biolabs). Double-stranded cDNA was prepared by treatment of the cDNA with the NEB Klenow fragment, following the manufacturer's instructions. The cDNA was purified using a MiniElute PCR Purification Kit (Qiagen) and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific), prior to library preparation for next generation sequencing.

High-throughput genomic sequencing and sequence assembly. DNA library preparation was performed using 1 ng of cDNA product with the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA), according to the manufacturer's instructions. Paired-end sequencing of 250 bp fragments was performed using the MiSeq reagent kit V2 (500 cycles) in a multiplex format (30×) and MiSeq Sequencing System (Illumina). Bioinformatics analysis of sequence data was conducted using CLC Genomics Workbench (v9.5.3 Qiagen) with default parameters. After quality control and quality trimming, a combination of read mapping and *de novo* assembly methods was performed to construct a consensus sequence for each BTV genome segment. Where necessary a small proportion of gap filling was provided by referring to the capillary sequence data previously obtained for the relevant segments. Final consensus sequences are available in the GenBank database under the accession numbers MG924986 through MG924995.

Sequence and phylogenetic analyses

Bluetongue virus sequences from this study were aligned with reference sequences using the MUSCLE algorithm implemented in MEGA7 (Kumar *et al.* 2016). Maximum Likelihood (ML) trees were constructed for complete ORF sequences of Seg-2 (position 18-2885 nt) and Seg-6 (29-1609 nt) and partial Seg-3 sequence (1123-1506 nt) using best fit substitution models estimated in MEGA7 for each alignment. Reliability of the inferred trees was tested

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by the bootstrap method using 1000 replicates. Pairwise distances were determined at the nucleotide and amino acid level for each gene segment using the p-distance model in MEGA7.

Results

Virus isolation and serological characterization

Virus isolations were made in 2014–2015 primarily at the Berrimah Veterinary Laboratories, Northern Territory, from sentinel cattle held at Beatrice Hill Farm (BHF) near Darwin. A smaller number of isolates came from cattle held at both Berrimah Research Farm and Douglas Daly Research Farm (DDRF), the latter located approximately 200 km further south of Darwin (13° 50′S, 131° 11′E). Two separate batches of virus isolates were received at the Australian Animal Health Laboratory (AAHL) in March (comprising bloods collected in January 2015) and May (bloods collected between September 2014 and April 2015) for serotype identification and genetic characterization. Testing against reference antiserum to all available exotic and Australian serotypes in the SNT revealed 15 isolates were neutralized by sheep antisera raised to the South African reference strain RSArrrr/05 (BTV-5 RSA). Several isolates were also neutralized by RSArrrr/09 (BTV-9 RSA) antisera (Table S1). Where cross reactions were observed, SNT titres generally were ≥ fourfold higher to BTV 5 RSA reference sera than to BTV-9 RSA antisera. All of these isolates were positive for BTV when tested in the generic BTV TaqMan assay (Ct values ranging from 10.9 to 14.2). Five isolates (V9230, V9233 and V9235 from BHF, and V9323 and V9324 from DDRF) were selected for subsequent molecular analyses.

Molecular characterization

Seg-2 sequence analysis

As a result of the SNT findings, Seg-2 specific primers were designed to detect BTV-5 based upon existing GenBank sequence data for South African (JX272570, KP821101, AJ585126), African (AJ585182, AJ585181) and French (Gaudeloupe Island) (HQ241072) isolates. Due to the observation of significant cross reaction of isolates with antisera raised to BTV-9 in the SNT assay, similar primer sets were also designed for detection of BTV-9, accommodating both the Australian isolate DPP837 and a number of selected exotic isolates (Table 1). Both BTV-5 and BTV-9 specific primer sets amplified approximately 98% of Seg-2 of the respective RSA reference strains where these were used as positive controls (results not shown). Amplicons were obtained for three isolates V9230, V9233 and V9235, using BTV-5-specific primers alone and analysed by capillary sequencing. The resultant consensus sequence for each isolate covered at least 97% of the coding region of Seg-2 and were used in a BLAST (NCBI) search. High-nucleotide identity levels were observed (≥98.7%) with four recently published BTV-5 isolates from China (GenBank Accession numbers KT945042 through to KT945045) (Yang *et al.* 2017), the prototype RSA strain RSArrrr/05 (95.3%) and a Cameroon strain of BTV-5 (CAR1982/02) (95.9%) (Table S2). The earliest evidence for BTV-5 presence in Australia based upon SNT data was isolate V9302 derived from blood collected at BHF on 27th November 2014. However isolate V9230 from blood collected in January 2015 at BHS (V9230) was selected as the prototype Australian strain and therefore reassigned the isolate number DPP9230.

Genotyping by Seg-3 sequence analysis

Genotyping analysis using a 384 bp product amplified from Seg-3, revealed the three Australian BTV-5 isolates from the February submission (DPP9230, V9233 and V9235) plus a further two isolates serologically positive for BTV-5 from the May submission (V9323 and V9324) (GenBank accession numbers MG924988, MH4430224, MH4430225, MH4430222 and MH4430223 respectively), all occupied the Malaysia A sub-group within the Eastern BTV topotype. (Pritchard *et al.* 2004) (Figure 1). Highest levels of nucleotide sequence identity were observed with Seg-3 sequences from the Malaysian isolate BTV-1/53 (96.9–97.1%) and the Australian BTV-20 isolate V3598 (96.6–96.9%).

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Table 1. Primer sets used in capillary sequencing reactions for detection of the Seg-2 gene of BTV-5 and BTV-9

Serotype	Primer Sets	Sequence 5' - 3'	Region amplified (Start - Finish)	Product size (bp)
BTV-5	FOR-VP2-8	GCTTCTCAGGATGAGTTCGGT	12–1039	1027
	REV-5-VP2-1060a	TCCGTAGCGCAGATCAGCAAT		
	REV-5-VP2-1060b	TCAGTGGCACAAATCAGCAAT		
	FOR- 5-VP2-1570a	CGATCTTTAGCACGAGGTACA	1590–2107	517
	REV-5-VP2-2108	GTCTGCCATCACCTCTCGAT		
	FOR-5-VP2-894	TGGAAGAAGAACCTAAGGATGA	917–2876	1959
	REV-5-VP2-2900	CTCATGTCACTGAGACTAAACGTTC		
BTV-9	FOR-9-VP2-4	AGTTATCTAGGATGGATGGACGA	9–979	972
	FOR-9-VP2-44	TCGAGCAACGTACCACACGAT	65–979	914
	REV-9-VP2-980	CTCACATTCTGCATGATGTTGGT		
	FOR-9-VP2-1636	CTAAGAGTCCAGATCCAGCAGT	1635–2089	454
	REV-9-VP2 2080	GCTTGATGAGCGAATGATATG		
	FOR-9-VP2-894	ATGCAGAGGAAGAACCCAAGGAC	911–2871	1960
	REV-9-VP2-2900	CTCATGTCACTGGGACTATACATTC		

Whole genome sequencing

The open reading frames of all ten genome segments of DPP9230 (herein designated BTV-5AUS) were sequenced using next-generation sequencing methods (GenBank accession numbers MG924986 through MG924995 respectively). Sequences for Seg-2, -3 and -6 were in agreement with those obtained from capillary sequencing. BLASTn® analysis of each of the 10 genome segments revealed a mixture of potential ancestral lineages. Segments 1,5 and 8 all had high-nucleotide sequence identity (≥ 98%) with the analogous genes of the prototype Australian BTV-7 isolate (DPP6936) and Australian BTV-1 isolates (Table 2). Additionally, Seg-4 had highest levels of nucleotide identity with a Chinese isolate of BTV-1 (Y863) and the Australian prototype strain of BTV-2 (DPP7291). Segment 9 also showed strong alignment with Taiwanese isolates of BTV-2 and BTV-12. For Seg-6, Seg-7 and Seg-10, the closest genetic identities were with viruses of Western origin (RSA). This represents the first documented account of a Western topotype Seg-10 in Australia.

To further investigate the relative genetic relationships of Australian serotypes with those from the Western region, a comparison of Seg-2 sequence data for representative isolates from both regions was undertaken. This revealed that Australian isolates of BTV-5 and BTV-7 possessed much greater nucleotide similarities with their equivalent Western lineage prototypes (94.9% and 92.8% respectively) than the remaining endemic serotypes, BTV-1, -2, -3, -9 and -15 (collectively <75% nt) (Table S3).

Retrospective serological and molecular testing of sentinel herd virus isolates

The observed serological cross reaction of BTV-5 AUS isolates with reference antiserum to BTV-9 RSA (Table S1), led us to re-analyse serum samples taken during 2014 (from July to November) from sentinel herds stationed at Kununurra and Kalumburu in the northeast of Western Australia. These field sera were previously only tested in the VNT using the known complement of Australian BTV serotypes and only demonstrated significant reactivity with BTV-9 AUS. Upon retesting by VNT, all samples previously neutralized by BTV-9 antiserum generally gave equivalent or stronger end-point neutralizing antibody titres to the newly isolated BTV-5 AUS (Table S4). When these samples were further tested in the more sensitive PRNT, antibody titres were highest against BTV-5 AUS. Where reactions with BTV-9 were observed, titres were ≤ fourfold lower than those involving BTV-5. Furthermore, 15 blood clots associated with the samples taken from animals stationed near Kununurra in October 2014 were re-tested in the generic BTV TaqMan

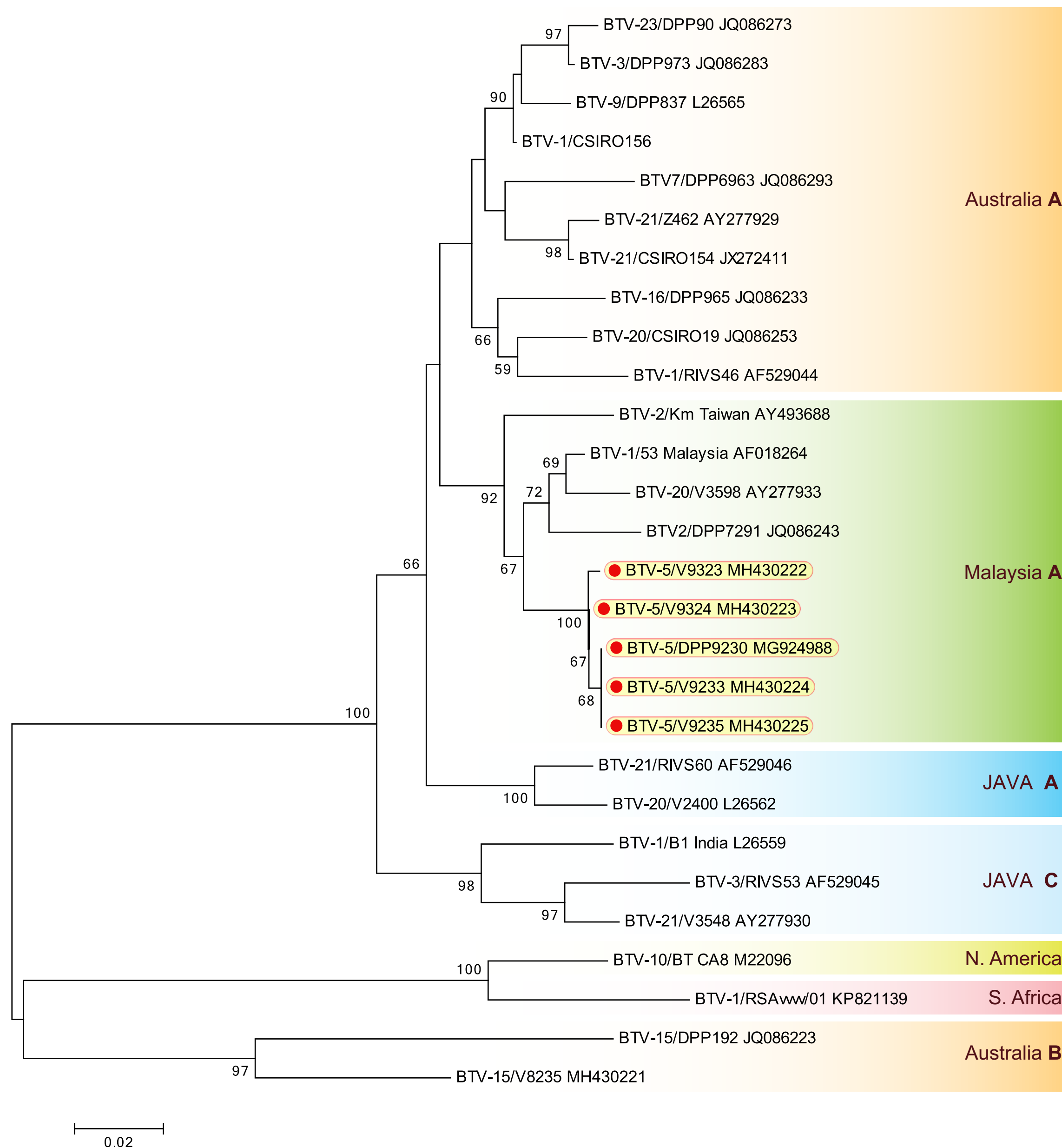

Fig. 1. Maximum likelihood phylogenetic tree showing the relationships of partial RNA Seg-3 sequences (384 nt.) of the Australian BTV-5 prototype isolate DPP9230 and related isolates (highlighted by closed circles) with corresponding reference sequences belonging to discrete genotypes within the Eastern topotype (Pritchard *et al.* 2004). The tree was drawn using Tamara-3 parameter model with invariant sites and rooted using the North American, South African and Australia B lineages. Numbers at the nodes represent bootstrap support as a percentage of 1000 replicates; only values >50% are shown. The scale bar represents 0.02 nucleotide substitutions per site.

assay and RNA extracts from two resultant BTV positive samples (Ct values 34.8 and 32.2) were used as template for BTV-5 Seg-2 specific RT-PCR (Table 1). Nucleotide sequence matches of 100% with the Seg-2 sequence of the prototype isolate DPP9230 over a 517 bp region were found for both samples, however virus isolation proved unsuccessful. These data and the earlier SNT data (Table S1),

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Table 2. Details of whole genome sequencing outputs for each segment of BTV-5 AUS isolate V9320

Seg.	Size [bp]	Viral protein [aa's Start-End]	Closest ORF maximum identity Access. No.	Isolate [origin-isolate no. (year)]	Serotype	Nucleotide Identity†	Topotype‡
1	3916	vp1 RNA polymerase 1-1302	JQ086291	AUSTRALIA-DPP6963 (2007)	7	99	Eastern
			JQ086301	AUSTRALIA-DPP837 (1985)	9	98	
2	2893	vp2 Outercoat -Neut. 1-955	KT945042	CHINA-V084 (2012)	5	99	***Western***
			AJ585181	SOUTH AFRICA-RSArrrr/05 (1953)	5	95	
3	2706	vp3 Inner Core 1-901	DQ186816	MALAYSIA-MAY1987/01	1	96	Eastern
			AF529047	WEST JAVA-RIVS 63 (1990)	6	96	
4	1937	vp4 Capping enzyme 1-644	KC879618	CHINA-Y863 (1979)	1	98	Eastern
			JQ86244	AUSTRALIA-DPP7291 (2008)	2	96	
5	1659	NS1 Intra-cell tubules 1-552	JQ086295	AUSTRALIA-DPP6963 (2007)	7	99	Eastern
			KM099575	AUSTRALIA-DPP1000 (1986)	1	98	
6	1581	vp5 Outercoat-Fusion 1-526	AJ586701	CAMEROON-CAR1982/02	5	95	***Western***
			AJ586700	SOUTH AFRICA-RSArrrr/05 (1953)	5	95	
7	1053	vp7 Outer Core – Group antigen 1-349	JX272575	SOUTH AFRICA-RSArrrr/05 (1953)	5	97	***Western***
			AY485667	INDIAN OCEAN-LA REUNION (2003)	3	96	
8	1065	NS2 Viral inclusion body 1-354	JQ086298	AUSTRALIA-DPP6963 (2007)	7	98	Eastern
			KM099631	AUSTRALIA-DPP4032 (1996)	1	98	
9	993	NS4 Helicase 1-330	GU390662	TAIWAN-BTV12/PT/2003	12	97	Eastern
			AY493691	TAIWAN-KM (2003)	2	97	
10	714	NS3 Membrane egress 1-229	JX272488	SOUTH AFRICA-BT87/59 (1959)	14	97	***Western***
			GQ506481	HOLLAND-NET2008/05	6	97	
			JN255941	SOUTH AFRICA-RSArrrr/05 (1953)	2	97	

†BLASTn® derived percentage nt identity, corrected to the nearest whole number. ‡Genome segments with a Western topotype are highlighted in bold, italicized text.

indicated BTV-5 was present in Australia from at least sometime earlier in 2014 and that its initial incursion into the continent was possibly via the northern tip of Western Australia.

Phylogenetic relationships within Seg-2 and Seg-6 genes of BTV-5 and BTV-9 isolates

Given the close antigenic relationship observed between Australian isolates of BTV-5 and BTV-9, we undertook phylogenetic analyses of each of the two BTV genomic segments of these serotypes that encode the outer coat virus proteins (Seg-2 and Seg-6) which are the main determinants of virus serotype. Reference sequences representing Western and Eastern topotypes were used for these analyses. The Seg-2 sequences analysed clustered into three major clades comprising two Western topotypes and a single Eastern topotype (Figure 2a), while BTV-5 sequences all exhibited a Western topotype, BTV-9 sequences occupied both Western and Eastern topotype groups.

Bluetongue virus serotype 5 sequences split into two Western sub-clades (W-1 and W-2). The BTV-5 AUS Seg-2 sequence was most closely related to W-1 sub-clade reference sequences from Chinese, South African and Cameroon BTV-5 isolates (95.3-98.9% nt identities). The W-2 sub-clade comprised USA, Caribbean and Nigerian reference sequences which showed some mutual divergence (W-2). Similarly, within the BTV-9 Western topotype, two sub-clades were observed: South African, Libyan and Italian reference sequences (W-1), and USA and Caribbean reference sequences (W-2). The Eastern BTV-9 clade (E-1) comprised a group of closely related reference sequences from Europe and India and more divergent Seg-2 sequences from Australia and Japan which also displayed a level of mutual divergence.

Phylogenetic analyses of Seg-6 sequences revealed a similar pattern of phylogenetic relationships to that

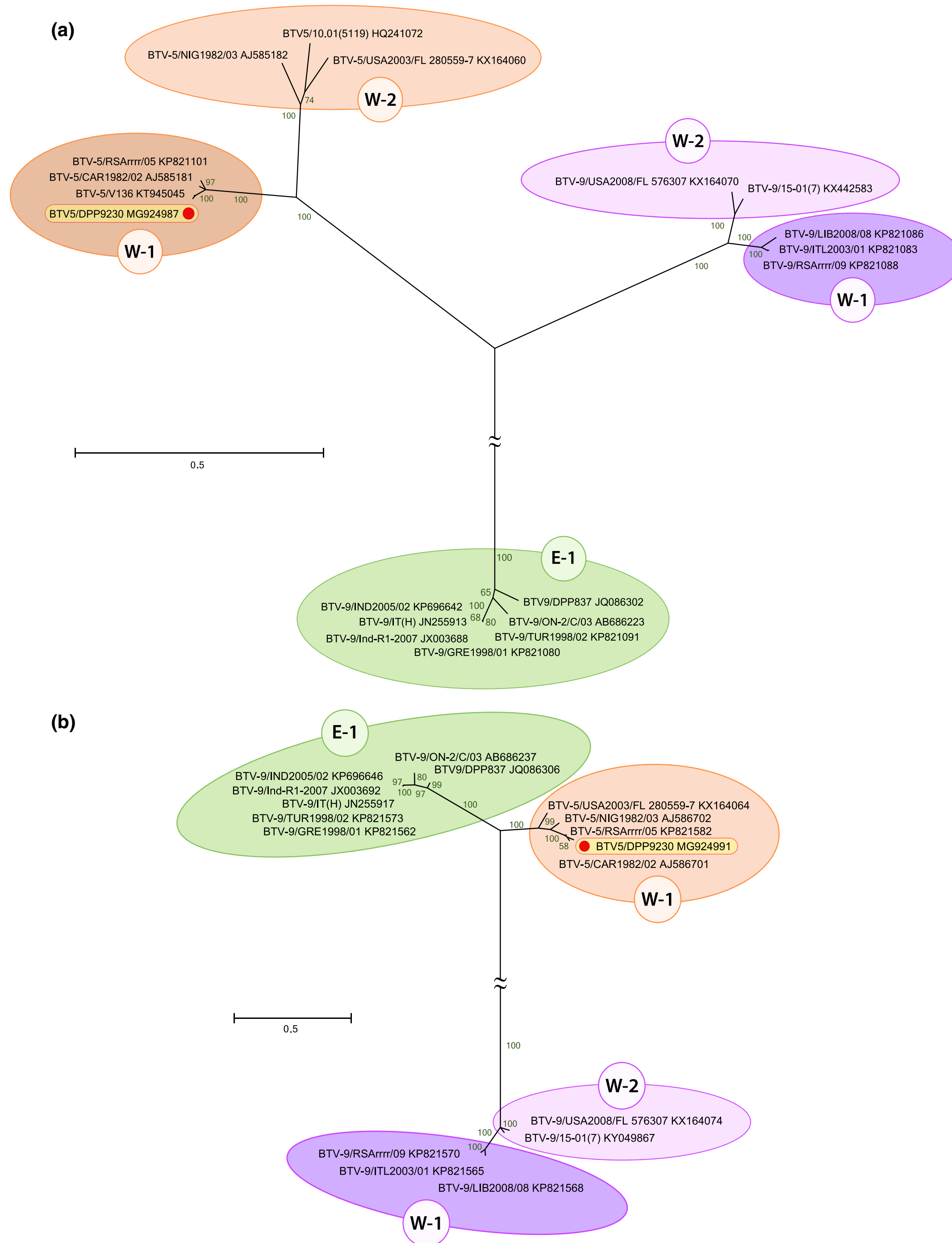

Fig. 2. Maximum likelihood phylogenetic tree showing the relationships of the complete ORF sequences (a) Seg-2 (2868 nt) and (b) Seg-6 (1581 nt) of the Australian prototype BTV-5 isolate DPP9230 (closed circle) and representative BTV-5 and BTV-9 isolates belonging to specific geographic topotypes as indicated by coloured oblongs; Eastern BTV-9 isolates (green), Western BTV-9 isolates (purple shades), Western BTV-5 isolates (tan shades). The trees were drawn using the Tamara-Nei model with a gamma distribution and invariant sites. Numbers at the nodes represent bootstrap support as a percentage of 1000 replicates; only values >50% are shown. In each image, the scale bar represents 0.5 nucleotide substitutions per site and distance truncation symbols represent a 40%(a) and 60%(b) shortening of the true distance respectively.

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found in the Seg-2 analysis, with three major clades described (Figure 2b). However, for BTV-5 sequences only a single Western clade was resolved (W-1). The Seg-6 of isolate BTV-5 AUS was again most closely related to the analogous gene segments of Chinese, South African and Cameroon BTV-5 isolates (95.7% nt identities). Surprisingly though, and in contrast to the Seg-2 data, the BTV-9 Eastern topotype was considerably more closely related to Western BTV-5 topotype sequences than to the Western topotype BTV-9 isolate ($\geq$ 78.5% nt identity to BTV-5 W-1/W-2 compared to $\leq$ 71.3% identity to BTV-9 W-1/W-2). As found with the Seg-2 data analysis, all Eastern BTV-9 isolates occupied a common nucleotype grouping. In contrast, the Seg-6 sequences of Western BTV-9 isolates occupied a distinct and divergent nucleotype group (Maan *et al.* 2015).

Discussion

There are relatively few documented accounts of BTV-5 isolations worldwide (Table S2) and only one account linking this serotype to a limited disease outbreak in Israel (Brenner *et al.* 2010). The very recent first isolation of BTV-5 in China (Yang *et al.* 2017) and India (Hemadri *et al.* 2017) and the close genetic relationship of these isolates with BTV-5 AUS, suggested a possible Eastern Asia origin for this virus. This route of entry into Australia was corroborated by the identification of BTV-5 AUS Seg-3 as a Malaysia A genotype (Pritchard *et al.* 2004). In contrast, there was relatively little genetic variation between the Seg-2, -6 and -7 genes of BTV-5 AUS and the prototype South African BTV-5 Mossop strain RSArrrr/05, isolated in 1953 (Howell 1970) (Table 2 and Table S2). The introduction of such a well preserved complement of ostensibly RSA origin gene segments is unusual within currently documented isolates of Australian BTV serotypes and implies co-segregation of these three genes over a significant geographic range and a relatively short time frame. The Seg-2 gene of all but one (BTV-7) of the remaining Australian BTV serotypes with counterparts originally isolated in a Western episystem (BTV-1,-2,-3,-7,-9 and -15), display <75% nt identity with the equivalent RSA reference isolates (Table S3). Bluetongue virus serotype 7(AUS) possesses nucleotide identities of approximately 92–94% with the RSA prototype strain RSArrrr/07 for Seg-2, -6 and -7 (Boyle *et al.* 2012; Yang *et al.* 2016). These three segments have also consistently co-segregated across time and well separated, geographically distinct, episystems. Bluetongue virus proteins VP5 and VP7, the encoded products of Seg-6 and Seg-7 respectively, are known to be structurally interactive with VP2 on the outer core and surface of the virus particle (Hewat *et al.* 1994; Nason *et al.* 2004) with possible implications for the expression of immunodominant epitopes on VP2 (Mertens *et al.* 1989; Anthony *et al.* 2007). Thus within the context of the total gene constellation currently present in the Northern Australian BTV episystem, co-segregation of Seg-2, -5 and -7 may be necessary for the preservation of essential outer core structural interdependence between their respective expression products, at least for BTV-5 and BTV-7.

Speculation on how rapid BTV gene segment translocations between Western and Eastern episystems might occur has included trade in live animals, semen and embryos (Mintiens *et al.* 2008; Rao *et al.* 2012a; Krishnajyothi *et al.* 2016), live vaccine use and importation of vaccinated animals (Maan *et al.* 2015). Sequential vector species expansion events due to extraordinary climatic conditions and long distance wind dispersals, causing overlap with and allowing novel BTV gene incursions into neighbouring episystems, are also thought to play a major role (Hendrickx 2009; Eagles *et al.* 2012, 2013, 2014; Purse *et al.* 2015). Anthropogenic factors may also contribute to vector abundance and spread (MacLachlan *et al.* 2013). The role of novel *Culicoides* species not tested for BTV competency and those previously thought to be incompetent vectors for BTV, can also not be disregarded in enabling establishment of novel BTV isolates in new regions (Mellor 2004; Carpenter *et al.* 2008, 2015; Bellis *et al.* 2015; Gopurenko *et al.* 2015).

There has been limited serological evidence for a longer term presence of BTV-5 in Southeast Asia (Della-Porta *et al.* 1983; Daniels *et al.* 2004; Zhang *et al.* 2004). The considerable degree of cross neutralization between BTV-9 and BTV-5 found in this

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study (Tables S1 and S4), and previously documented by others (Rao *et al*. 2012b), could explain the perceived existence of BTV-5 antibodies in host animals in Eastern lineage regions. Our phylogenetic analysis of the relationships between Seg-2 and Seg-6 of BTV-5 isolates showed that these belonged to Western topotype lineages (W-1/W-2) whereas for BTV-9 isolates, both Western and Eastern lineage clades exist for this serotype, involving two Western lineages (W-1/W-2) and a single Eastern lineage (E-1) (Figures 2a & 2b).

Of note was the finding in this study and by others previously (Rao *et al.*, 2012c; Rao *et al.* 2012b; Shirafuji *et al.* 2012; Maan *et al.* 2015), that Seg-2 sequences from BTV-9(W) isolates showed greater similarity with BTV-5 isolates than with BTV-9(E) viruses (Figure 2a; Table S5). However the opposite condition existed for Seg-6 (Maan *et al.* 2010, 2015) (Figure 2b; Table S6), further confusing the ancestral relationships between these serotypes. The phylogenetic associations revealed that the Seg-6 of BTV-5 RSA and BTV-5 AUS isolates were significantly more closely related to BTV-9(E) isolates than to those from a BTV-9(W) lineage (>79% compared to <71% nt identities respectively). Furthermore, the Seg-6 sequences from these separate BTV-9 topotypes fell into phylogenetically well-separated lineages normally indicative of separate serotypes (Maan *et al.* 2008). Our phylogenetic analyses thus concur with previous studies that have suggested BTV-9(E) isolates meet the criteria for designation as a separate serotype within the BTV5/BTV-9 Seg-2 nucleotype E cluster (Rao *et al.* 2012c; Maan *et al.* 2015).

The collective BTV-5 and BTV-9 sequence data presented in this study and the earlier work of others (Maan *et al.* 2009, 2016; Rao *et al.* 2012b) have further highlighted the apparent discrepancies between the current internationally recognized serological tests for BTV serotype designation (Huismans & Erasmus 1981; Erasmus 1990) and the range of percentage nucleotide identities that have subsequently been found to exist between the Seg-2 genes of different isolates of the same serotypes. From the mid -1990s more extensive vector and host screening programs were established globally which drove an exponential growth in virus isolations and the publication of whole genome data for a diverse range of bluetongue viruses (Boyle *et al.* 2012, 2014; Maan *et al.* 2015; Nomikou *et al.* 2015). Subsequent thorough interrogation of these data has, in hindsight, suggested analysis by serology alone has potentially interpreted intra-serotype variants isolated between distant geographic regions as 'new' serotypes (Della-Porta *et al.* 1981; Groocock *et al.* 1982) while also potentially failing to detect truly novel BTV strains. In the context of this study, it is noteworthy that the levels of nucleotide identity for Seg-2 between the South African prototype strain of BTV-9 (RSArrrr/09) isolated in 1942 and several Eastern topotype BTV-9 isolates (<70% nt) (Table S5) are the lowest so far observed for intra-serotypic variation within a BTV serotype (Maan *et al.* 2016). In addition, some Indian isolates of BTV-9 failed to be neutralized by reference antiserum raised to BTV-9 RSA (Rao *et al.* 2012b).

Molecular approaches applied to BTV diagnosis are now sufficiently rapid and substantially more informative in providing a more thorough identification of BTV field isolates (Rao *et al.* 2013; Maan *et al.* 2015, 2016; Krishnajyothi *et al.* 2016). This will remove the potential shortcomings that can result from a primary reliance on serological BTV characterization of epitopes present on the gene product of Seg-2 alone. However, there is a growing number of reports of BTV isolates that comprise mixtures of gene segments of both Western and Eastern heritage. This makes definitive topotyping at the isolate level, based upon individual more highly conserved genome segments such as Seg-3 and Seg-10 alone, increasingly problematic. To ultimately provide an improved understanding of the ancestry and inter-regional relationships between individual BTV isolates, the complete genomes of each must be considered. The effort to establish a genotype-based classification system for rotavirus (Matthijnssens *et al.* 2008, 2011) plus similar suggestions by others (Nomikou *et al.* 2015), would therefore be useful frameworks to consider when a revised system for BTV classification is inevitably addressed.

Classic BTV serotyping methodologies still retain an essential role in determining vaccine strategies in

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preparation for and response to disease outbreak situations. Serological screening for both group reactive and neutralizing antibodies also provides vital support for virus isolation and epidemiological studies to provide early warning of BTV activity in specific regions (St George *et al.* 2001; Melville 2004; Geoghegan *et al.* 2014). Indeed, the combined retrospective serological testing and subsequent virus genome characterization data from Kalumburu sentinel herd samples, further implicated the northern tip of Western Australia as a 'hotspot' for BTV emergence on the Australian mainland (Eagles *et al.* 2012, 2013, 2014).

This study has provided a full genomic characterization of a BTV serotype (BTV-5) new to Australia and shown that it possesses a genome composition related to recent virus isolates belonging to the Western lineage episystem. The Seg-10 of BTV-5 is also the first documented report of a Western topotype of this gene entering Australia. Segment 10 has recently been strongly implicated as a significant modulator of BTV pathogenicity (Feenstra *et al.* 2014; Ftaich *et al.* 2015; Pullinger *et al.* 2016). Collectively, these data implied a potential for BTV genome segments that have been associated with highly pathogenic BTV serotypes in the Western episystem (Maan *et al.* 2008, 2010) to be more rapidly transported to Southeast Asian episystems than previously thought plausible. This possibility emphasized the need for maintenance of appropriate levels of surveillance of both vectors and viruses at several locations in the far north of Australia (Klingseisen *et al.* 2013). In particular, the expansion of molecular characterization of BTV to a whole of genome approach is recommended to continually monitor the presence and level of establishment of novel Western topotype segments within the Australian episystem.

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Conflict of interest

The authors have no conflicts of interest to declare.

Contributions

Study design and manuscript preparation: JRW, DTW, DE, JW. Laboratory work: JRW, JW, HC, GH, AR AC, RPW, SSD, LFM, RAL. Data analyses: JRW, DTW, JW. Manuscript review: JRW, DTW, DE, RAL, RPW.

Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate internal ethics review committee approvals has been received.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Reciprocal end-point titres in the SNT for reaction between the Berrimah virus isolates and reference sheep antisera raised to RSA prototype isolates BTV-5 and BTV-9.

Table S2. Percentage nucleotide identities between Seg-2 sequences of Australian and exotic isolates of BTV-5.

Table S3. Comparison of Seg-2 nucleotide and amino acid sequence identities between South African (RSA) prototype BTV serotypes and reference

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isolates of analogous serotypes isolated in Australia (AUS).

Table S4. Screening of Western Australian sentinel cattle herd sera against Australian BTV serotypes, BTV-6 (RSA) and BTV-5 AUS in the VNT and PRNT tests (reciprocal titres shown).

Table S5. Percentage nucleotide identity matrix of the Seg-2 gene between groups of Eastern and Western topotypes of BTV-5 and BTV-9 isolates.

Table S6. Percentage nucleotide identity matrix for the Seg-6 gene between groups of Eastern and Western topotypes of BTV-5 and BTV-9 isolates.

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