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# Evolutionary history of Simbu serogroup orthobunyaviruses in the Australian episystem

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ABSTRACT

Orthobunyaviruses of the Simbu serogroup are transmitted by insects (primarily biting midges) and infect mammals and/or birds. Many have been associated with disease in livestock or humans. The orthobunyavirus genome comprises three negative-sense RNA segments (L, M and S). We report the complete coding sequences of 57 isolates of Simbu serogroup viruses collected in Australia during 1968–1984. Phylogenetic analysis identified novel genogroups of Akabane virus (AKAV), Aino virus (AINOV) and Peaton virus, and provided evidence of constrained movement of AKAV between epidemiological systems in the northern and eastern regions of the continent. Differential clustering of AKAV isolates in trees inferred from L, M and S segments was indicative of intratypic segment reassortment. Similarly, intertypic segment reassortment was detected between AKAV and Tinaroo virus, and between AINOV and Douglas virus. L segments representing novel genogroups were detected in AINOV reassortants, suggesting the presence of unidentified Simbu group viruses in the episystem.

## 1. Introduction

The Simbu serogroup is one of 18 serogroups of arthropod-borne viruses assigned to the genus *Orthobunyavirus* of the family *Peribunyaviridae,* order *Bunyavirales* (Kinney and Calisher, 1981; Maes et al., 2018). Like all members of the genus *Orthobunyavirus,* Simbu serogroup viruses have a tripartite, negative sense (−) single-stranded (ss) RNA genome composed of L (large), M (medium) and S (small) segments. The L RNA segment encodes a large multifunctional enzyme that includes the RNA-dependent RNA polymerase (Elbayoumy et al., 2013). The M RNA segment encodes a precursor protein that is cleaved to yield two surface glycoproteins (Gn and Gc) and a non-structural protein (NSm) (Fazakerley et al., 1988). The S RNA segment encodes the nucleoprotein (N) and a non-structural protein (NSs) in overlapping reading frames (Fuller et al., 1983). The Gn and Gc proteins embed in the lipid envelope and form projections displayed on the surface of virion. The surface glycoproteins, which associate to form heterodimers, are recognised by virus-specific neutralising antibodies. Of the three RNA segments, the L and S segments are most conserved and the M segment is the most variable (Kobayashi et al., 2007; Saeed et al., 2001a), likely driven by natural selection imposed by the humoral immune response of vertebrate hosts (Fischer et al., 2013; Kobayashi et al., 2007). Based on complete coding sequences of the M segments, over 30 Simbu serogroup viruses fall phylogenetically into two subclades comprising seven virus complexes: Oropouche and Manzanilla (subclade A); and Akabane, Sathuperi, Shamonda, Simbu and Shuni (subclade B) (Briese et al., 2013). Each virus complex (which may comprise several antigenically distinct viruses) has been assigned by the International Committee on Taxonomy of Viruses (ICTV) to represent one or more virus species with 18 species in total currently recognised (Maes et al., 2018).

Simbu serogroup viruses have been isolated from biting midges

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(*Culicoides* spp.) and/or mosquitoes, although biting midges appear to serve as the principal vectors, at least for those viruses for which useful epidemiological data is available (De Regge, 2017; Mellor et al., 2000; Sakkas et al., 2018). Vertebrate hosts vary amongst the viruses but include birds and mammals, and many of the viruses have been associated with diseases of medical or veterinary importance. Oropouche virus (OROV; subclade A) causes an acute febrile illness with associated headache, myalgia and arthralgia in humans in tropical regions of South and Central America (Travassos da Rosa et al., 2017). OROV RNA has also been detected in the cerebrospinal fluid of patients with meningoencephalitis (de Souza Bastos et al., 2012). Although no fatalities have been recorded, approximately 500,000 cases of Oropouche fever have been reported in the Americas since the virus was first isolated from febrile forest workers in Trinidad in 1955 (Azevedo et al., 2007). Iquitos virus (IQTV) and Madre del Dios virus (MDDV) are also assigned to subclade A and have been isolated on multiple occasions from patients with Oropouche fever (Aguilar et al., 2011; Ladner et al., 2014).

In contrast, viruses assigned to subclade B are not known to infect humans or non-human primates but several of the viruses cause important diseases of livestock. Akabane virus (AKAV) infects a wide range of wild ruminants and livestock including cattle, sheep, goats, horses and pigs (Huang et al., 2003; Kirkland, 2002), causing abortion, stillbirth and congenital abnormalities. AKAV is widely distributed across Africa, the Middle-East, East Asia and Australia. During 1972 to 1975, a serious outbreak of Akabane disease in Japan caused more than 31,000 cases of abortion, stillbirth and congenital arthrogyropsis and hydranencephaly (A-H syndrome) (Kurogi et al., 1975). There was also an outbreak of A-H syndrome due to AKAV in Australia in 1974 with more than 8000 cases recorded (Coverdale et al., 1978). Aino virus (AINOV) is also responsible for congenital malformation, abortion and stillbirth in cattle when pregnant ruminants are infected (Coverdale et al., 1978; Yoshida et al., 2000). In late 2011, a new member of the Simbu serogroup, Schmallenberg virus (SBV), emerged in Europe (Hoffmann et al., 2012). SBV has since spread across many countries in Europe, causing abortions, stillbirths and congenital abnormalities in cattle, sheep and goats (Beer et al., 2013). Shuni virus (SHUV) has been associated with neurological disease in horses in South Africa (van Eeden et al., 2012) and with congenital malformations in sheep, goats and cattle in Israel (Golender et al., 2015). More recently, Peaton virus (PEAV) and Shamonda virus (SHAV) have also been associated with congenital abnormalities in ruminants in Japan and Israel (Behar et al., 2019; Hirashima et al., 2017; Matsumori et al., 2018).

Natural genome segment reassortment has been shown to contribute significantly to the evolution of orthobunyaviruses, occurring both between different viruses and between variants of the same virus type (Bowen et al., 2001; Collao et al., 2010; Ding et al., 2013). Intertypic reassortment generally favours co-segregation of the L and S segments with the M segment (determining neutralisation phenotype) segregating more independently (Briese et al., 2013). Within subclade A of the Simbu serogroup, it has been shown that isolates of IQTV and MDDV, as well as Jatobal virus (JATV) isolated from a sloth (*Nasua nasua*) in Brazil in 1985, and Perdoes virus (PERDV) isolated from monkeys in Brazil in 2012, are reassortants (Aguilar et al., 2011; Ladner et al., 2014; Saeed et al., 2001b; Tilston-Lunel et al., 2015). The L and S segments are derived from OROV but the M segments are far more distantly related and appear to be derived by reassortment from other yet unidentified orthobunyaviruses. In subclade B, it has been reported that Australian isolates of Tinaroo virus (TINV) and AINOV are reassortants, deriving their L and S segments from AKAV and PEAV, respectively (Akashi et al., 1997; Kobayashi et al., 2007; Yanase et al., 2010). Similarly, SBV appears to be a reassortant, deriving the M RNA segment from Sathuperi virus (SATV) and the S and L RNA segments from SHAV (Yanase et al., 2012).

Several Simbu serogroup viruses, including AKAV, AINOV, PEAV, TINV, Douglas virus (DOUV), Thimiri virus (THIV) and Facey's paddock virus (FPV), have been isolated in Australia from cattle and/or biting midges (Cybinski, 1984; Doherty et al., 1972, 1979; St George et al., 1978, 1979, 1980; Standfast et al., 1984). Neutralising antibodies to AKAV, AINOV, PEAV, TINV and DOUV have been detected in cattle over a very wide geographic distribution that extends across most of northern tropical Australia and down the east coast to central New South Wales (Cybinski, 1984; Cybinski and St George, 1978; Cybinski et al., 1978; St George et al., 1980). The distribution of these viruses reflects approximately the known distribution of the midge vector, *C. brevitarsis* (Cybinski, 1984; Murray and Nix, 1987). AKAV and AINOV have each been associated with A-H syndrome in Australia (Coverdale et al., 1978) but the role of other Simbu serogroup viruses has not been clearly defined.

In this study we obtained the complete genome sequences of a set of historical isolates of Simbu serogroup viruses from the Australian epidemiological system (episystem), as well as three isolates of Leanyer virus (LEAV) which, although antigenically distinct from the Simbu serogroup, is closely related genetically (Savji et al., 2011). Phylogenetic analysis has revealed the presence of novel genogroups of AKAV, AINOV and PEAV, and has provided evidence that both intertypic and intratypic genome segment reassortment has contributed to the evolution of the viruses in the Australian episystem.

## 2. Materials and methods

### 2.1. Viruses and cells

A total of 57 virus isolates were selected for full genome sequencing in this study (Suppl Table S1). Virus stocks were recovered from storage at −80 °C at the CSIRO Australian Animal Health Laboratory, Geelong, Victoria, and passaged once in Vero cells at 37 °C in minimum essential medium (MEM) supplemented with 10% foetal calf serum, 10 nM HEPES, 500 μg/ml fungizone and 6.7 nM $NaHCO_3$. Passage histories of the viruses prior to recovery from storage are shown in Suppl. Table S1.

### 2.2. Preparation of viral RNA and cDNA

Two 75-$cm^2$ flasks of Vero cells at 70–80% cell confluence were used for each viral infection. Infected cultures were harvested after 4–5 days at 50–70% cytopathic effect, passed through a 0.45 μm filter to remove cellular debris and 1/3 volume of aqueous solution of 30% polyethylene glycol and 23% NaCl was added to the filtrate. After standing overnight at 4 °C, the precipitate was recovered by centrifugation and RNA was extracted using the RNeasy plus mini kit (Qiagen). Viral RNA was reverse transcribed using SuperScript III (Invitrogen) and 50 ng random hexamer primers and 0.5 ng primers specific to the complementary ends of AKAV genome segments ( 5′-AGTAGTGN-3′). Following digestion of the cDNA with RNase H (Invitrogen), dsDNA was synthesized using Klenow fragment (NEB) and then purified using the QIAquick PCR purification kit (Qiagen). The concentration of dsDNA was determined using a Qubit fluorometer (Invitrogen).

### 2.3. High-throughput genome sequencing and analysis

Viral dsDNA samples were prepared for sequencing using the Nextera XT DNA library preparation protocols and adaptors (Illumina) following the manufacturer's instructions. Paired-end, 2x 250- or 300-base read protocols were used for sequencing on an Illumina MiniSeq instrument. Viral genomes were assembled by a combination of *de novo* assembly and mapping to Simbu serogroup reference genomes using CLC Genomics Workbench version 9. Assemblies were then curated and edited manually using Geneious version 10.2, and consensus sequences were generated for each segment.

### 2.4. Phylogenetic analyses

The sequences were aligned in MEGA 7.0 using ClustalW (codons) with default parameters (Kumar et al., 2016). The aligned sequences were then used to infer maximum likelihood phylogenetic trees in MEGA 7.0 using the GTR (general time reversible) nucleotide substitution model and a discrete gamma distribution to model evolutionary rate differences among sites rates of substitution among sites. All positions containing gaps were eliminated. Initial trees for the heuristic search were obtained automatically by applying the Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimates using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log likelihood value. The phylogenetic robustness of each node was determined using 100 bootstrap replicates and nearest-neighbour branch-swapping. Trees were annotated in MEGA 7.0 by anchoring on the selected outgroup and collapsing subtrees as required for best illustration. Final mark-up of trees was conducted in Adobe Illustrator CC.

### 2.5. Nucleotide sequence accession numbers

The nucleotide sequences determined in this study have been deposited in GenBank (Accession Numbers MH734940-MH735110) and are shown in Suppl. Table S1. The accession numbers of all other virus isolates downloaded from Genbank are shown in Figs. 2 and 3 and Suppl. Figures 1-3.

## 3. Results

### 3.1. Whole genome analysis

A set of 57 viruses collected between 1968 and 1991 from the Australian episystem was sequenced. This included 54 isolates of Simbu serogroup viruses (AKAV, TINV, AINOV, PEAV, DOUV, THIV), as well as three isolates of closely related Leanyer virus (LEAV) (Table S1; Fig. 1). Complete coding sequences were determined for the S, M and L RNA segments by a combination of *de novo* assembly and mapping to Simbu serogroup reference genomes. The total number of reads ranged from 94,356 to 6,675,110 and the proportion of total reads representing bunyavirus genome sequences ranged from 13% to 80% among the 57 isolates. The consensus sequences generated in this study displayed moderate to high degrees of similarity with published references sequences for S (> 78.5%), M (> 68.0%) and L (> 78.5%).

Each S RNA segment contained a complete coding region of 703 nt, except for LEAV (708 nt), encoding the nucleoprotein (N) and a non-structural protein (NSs). The L RNA segments contained complete coding regions ranging in size from 6756 nt (AKAV and TINV) to 6782 nt (LEAV), encoding the RNA-dependent RNA polymerase (L). The M RNA segments contained complete coding regions ranging in size from 1400 nt (PEAV) to 1419 nt (LEAV), encoding the envelope glycoproteins (Gn and Gc) and a second non-structural protein (NSm). For isolate CS296, coding sequences were detected for two viruses, with S, M and L segments displaying a high level of identity to AKAV reference sequence and S and M segments displaying a high level of identity to the AINOV reference sequence, indicating co-infection with the two Simbu serogroup viruses.

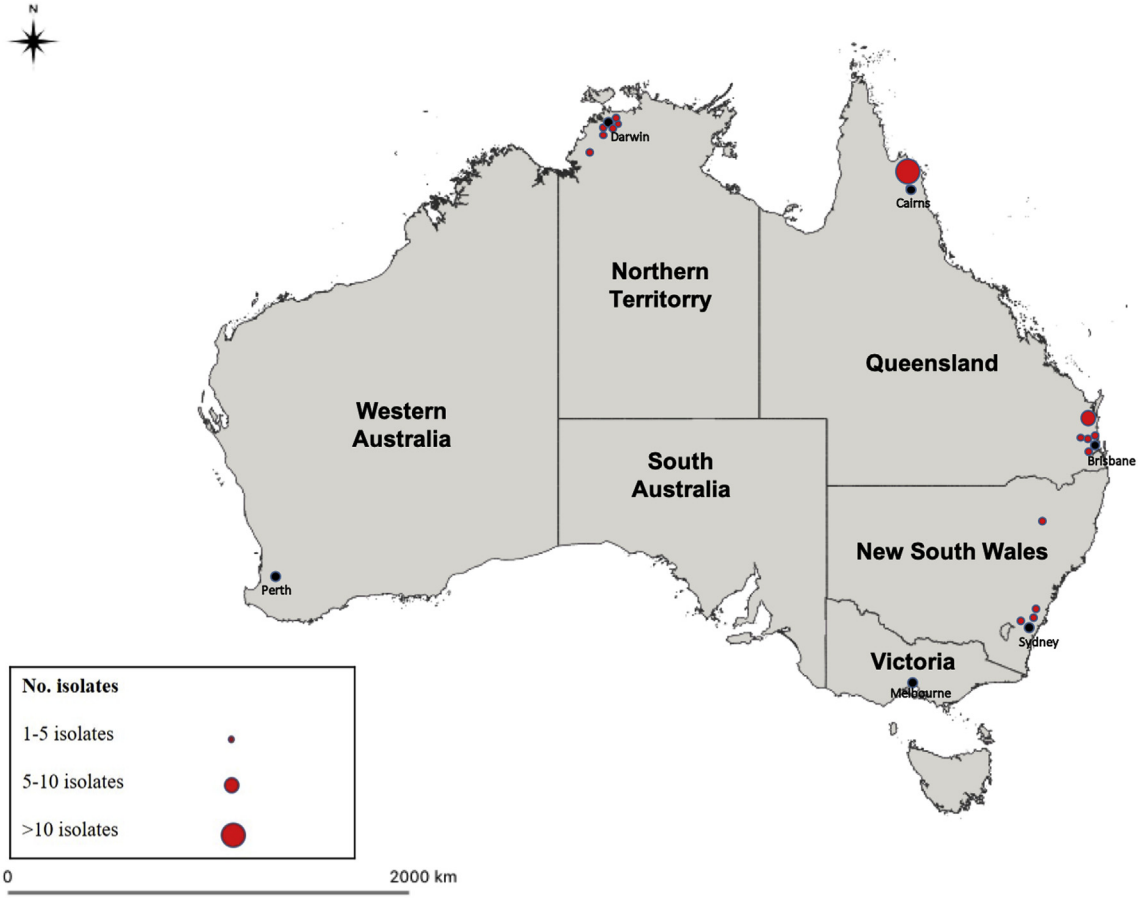


**Fig. 1.** Map of Australia showing the sites of collection of virus isolates sequenced in this study and the approximate numbers of isolates collected at each site. Collection sites in the Northern Territory and the central coast of New South Wales are separated by approximately 4000 km.

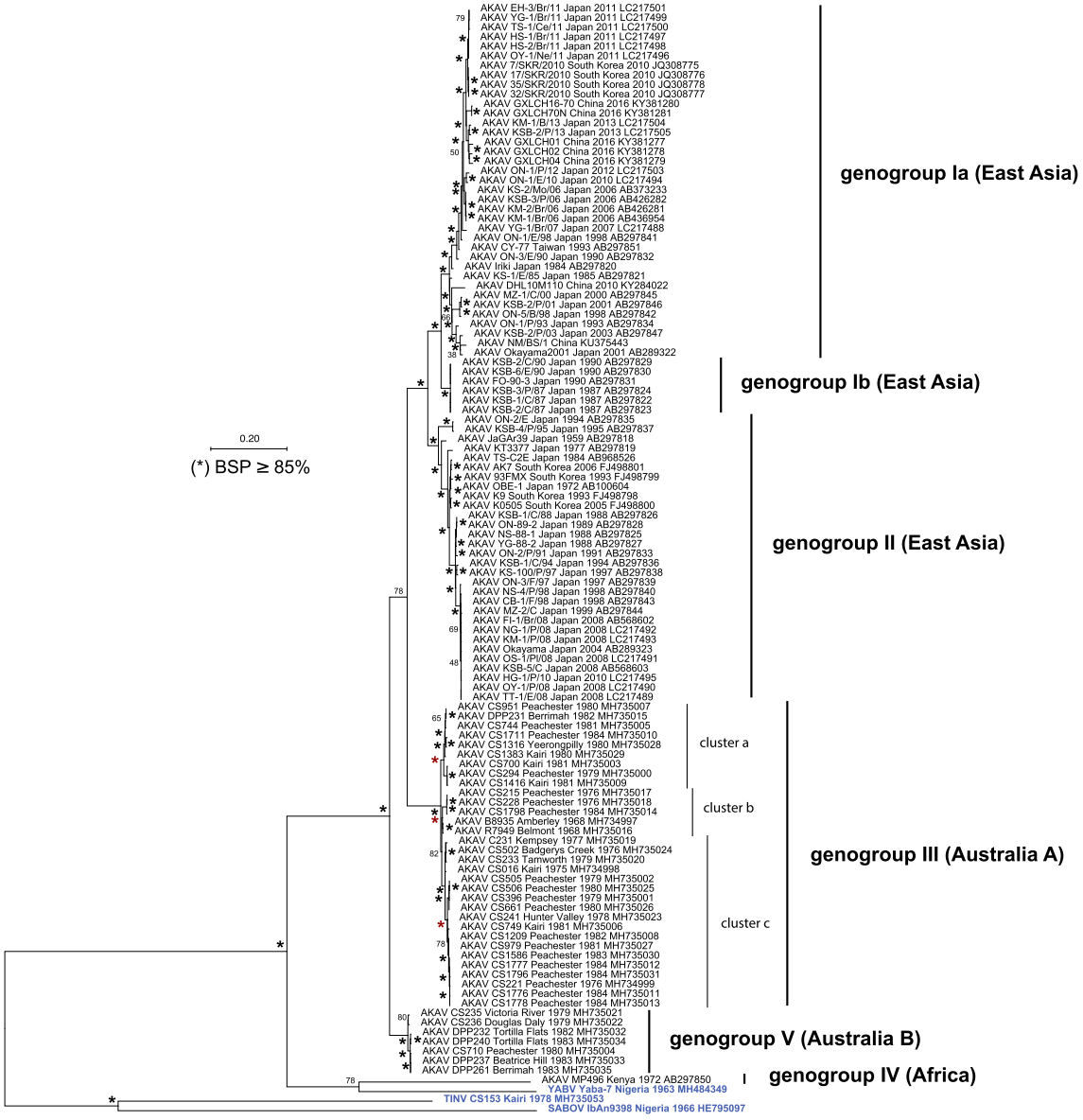


**Fig. 2.** Maximum likelihood phylogenetic trees of all available complete coding sequences of viruses assigned to the species *Akabane orthobunyavirus* (AKAV, TINV, YABV) and Sabo *orthobunyavirus* (SABOV) included as an outgroup. A) M segments (116 sequences). B) S segments (154 sequences). C) L segments (52 sequences). Branch lengths are proportional to the number of nucleotide substitutions per site. Values for bootstrap support proportion (BSP) were determined from 100 bootstrap iterations. The positions in the trees of TINV, SABOV and YABV are shown in blue. Genbank accession numbers of all sequences are as shown.

### 3.2. Phylogenetic analysis of AKAV isolates

Maximum likelihood phylogenetic trees were inferred from the alignments of the sequences of the complete coding regions of the S, M and L segments of 39 Australian isolates of AKAV, the one available Australian isolate of TINV and the corresponding sequences of all AKAV, YABV and SABOV isolates currently deposited in Genbank (Fig. 2). All viruses included in this analysis form the Akabane phylogroup and are assigned to the species *Akabane orthobunyavirus*, as well as closely related SABOV (*Sabo orthobunyavirus*) which was used as an outgroup. Genotype assignments of AKAV isolates from Japan, South Korea, China, Africa and Australia have been assigned in previous phylogenetic analyses (Kobayashi et al., 2007; Oem et al., 2012; Tang et al., 2017; Yamakawa et al., 2006) and these are described below.

Analysis of the M segments indicated that AKAV isolates from East Asia clustered together in three clades corresponding to previously described genogroups Ia, Ib and genogroup II (Fig. 2A). Genogroup Ia included AKAV isolates from Japan, Taiwan, mainland China and South Korea from 1984 to 2016, while genogroup Ib comprised only six viruses isolated in Japan in 1987 and 1990. Genogroup II included isolates from Japan and South Korea collected from 1959 to 2008. The M segments of Australian AKAV isolates formed two clades. Australian clade A (designated genogroup III) was most closely related to both the East Asian clades and includes viruses isolated from Queensland and New South Wales from 1968 to 1984 and a single isolate from the Northern Territory in 1982 (DPP231/Berrimah/1982). There was no evidence of geographic or temporal clustering within eastern Australia. In contrast, Australian clade B (designated genogroup V) fell in a basal position with respect to Australian clade A (and all East Asian clades) and includes primarily viruses isolated in the Northern Territory from 1979 to 1983, as well as a single virus isolated at Peachester in Queensland in 1980 (CS710/Peachester/1980). This suggests strong geographic clustering of AKAV M segments with largely (but not completely) separate virus populations in the northern and eastern states. The M segment of a single AKAV isolate from Africa (MP496/Kenya/1972), was more divergent than all other AKAV isolates,

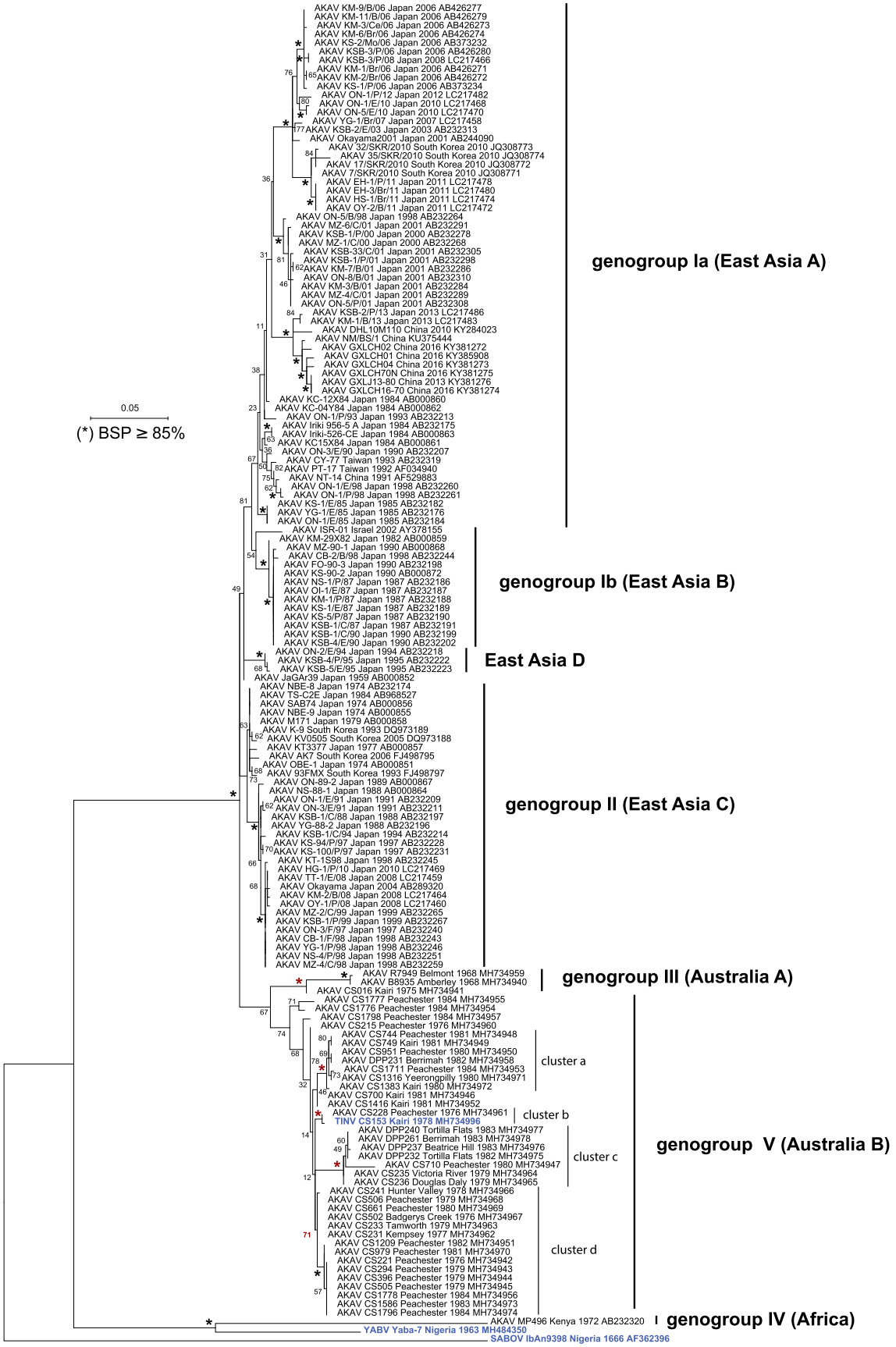


**Fig. 2.** (*continued*)

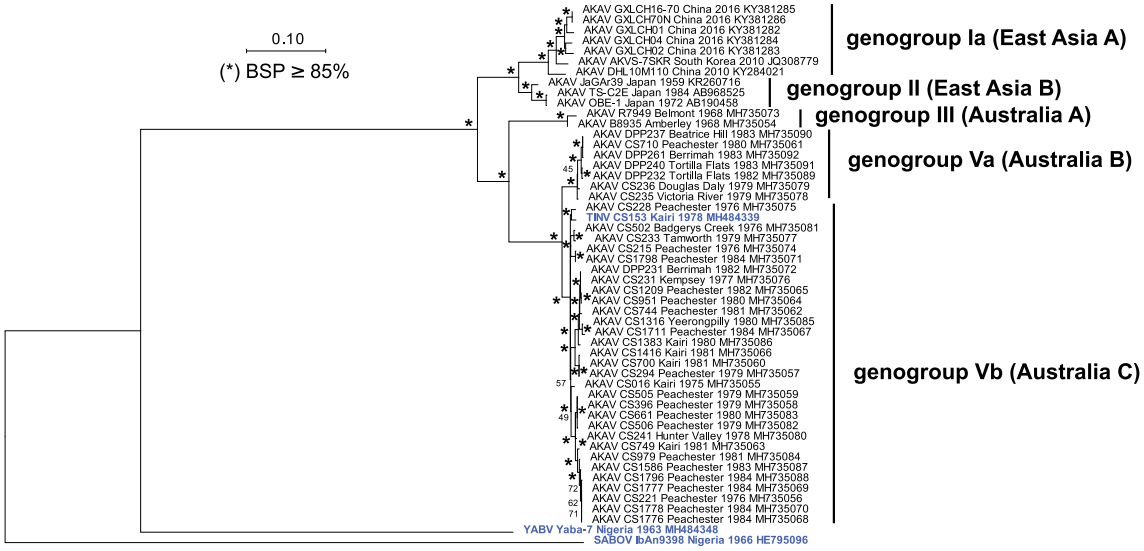


**Fig. 2.** (*continued*)

clustering with YABV, also from Africa (Yaba-7/Nigeria/1963). The African AKAV clade has been assigned previously as genogroup IV. The M segments of Australian TINV (CS153/Kairi/1978) and African SABOV (IbAn9398/Nigeria/1966) formed the most divergent group in the phylogeny.

Analysis of the S segments also revealed the existence of several AKAV clades in East Asia and Australia (Fig. 2B). The S segments of East Asian AKAV isolates clustered together into four distinct clades (A-D). East Asia clade A comprised isolates from Japan, mainland China and South Korea from 1984 to 2016, and included all isolates previously assigned to genogroup Ia. There appeared to be some degree of temporal progression of these isolates with one sub-clade including viruses isolates from 2000 to 2016 and viruses isolated from 1984 to 1990 forming a divergent sub-clade. East Asia clade B comprised viruses from Japan isolates between 1982 and 1998, including viruses that had previously been assigned as AKAV genogroup Ib. A single AKAV isolate from Israel in 2002 (ISR-01/Israel/2002) also fell within this clade. East Asia clade C comprised AKAV isolates from Japan and South Korea from 1974 to 2005, and included most isolates previously assigned to genogroup II. Finally, East Asia clade D included only three viruses isolated in Japan in 1994 and 1995, two of which were previously assigned to genogroup II. The original 1959 Japanese AKAV isolate (JaGAr39/Japan/1959), which also was previously assigned to genogroup II, did not fall within any of the other clades in our analysis. The S segments of Australian AKAV isolates clustered into two major clades. Australia clade A comprised only three isolates from Queensland, two of which were isolated in 1968 and one in 1975; the two 1968 isolates have previously been assigned as AKAV genogroup III. The major Australian clade (Australia clade B) included AKAV isolates from the Northern Territory, Queensland and New South Wales sampled from 1976 to 1984, as well as TINV isolated at Kairi in Queensland in 1978. Six AKAV isolates from the Northern Territory formed a separate subclade (cluster c in Fig. 2B) within clade B with the single Queensland isolate (CS710/Peachester/1980) with which they clustered in the M segment. As observed for the M segment, the seventh isolate from the Northern Territory (DPP231/Berrimah/1982) clustered with Queensland isolates (cluster a in Fig. 2B). There were no patterns suggestive of a temporal or geographic progression of the viruses as a population. Australian clade B is assigned here as AKAV genogroup V. The single AKAV isolate from Africa (MP496/Kenya/1972) again fell on a divergent branch with YABV from Nigeria.

Analysis of the L segments also identified several AKAV clades from East Asia and Australia (Fig. 2C). The small number of available complete L segment coding sequences from East Asia formed two clades (A and B) corresponding to M segment genogroups Ia and II (see above). East Asia clade A included AKAV isolates from China and South Korea from 2010 to 2016. East Asia clade B comprised only three isolates from Japan, including the original AKAV isolate from 1959. The L segments of Australian AKAV isolates clustered into three clades. Australia clade A comprised only two isolates from Queensland in 1968 which were assigned previously as AKAV genogroup III. Australia clade B (genogroup Va) included six isolates from the Northern Territory from 1979 to 1983 and the single isolate from Peachester in Queensland in 1980 with which they also clustered in M segment. Australia clade C (genogroup Vb) included AKAV isolates from Queensland and New South Wales from 1976 to 1984, a single isolate from the Northern Territory (DPP231/Berrimah/1982) and TINV isolated in Queensland in 1978. The L segments of African YABV (Yaba-7/Nigeria/1963) formed the most divergent lineage in the phylogeny.

### 3.3. Evidence of intratypic genome segment reassortment

The genogroup assignments of Australian AKAV isolates in phylogenetic trees inferred from the complete S, M and L coding sequences were compared to test for the presence of genome segment reassortment (Table 1). In addition to the major genogroup assignments, Cluster Picker (http://hiv.bio.ed.ac.uk/software.html) was used to identify three well supported clusters in M segment genogroup III (IIIa-IIIc; Fig. 2A) and four well supported isolate clusters in S segment genogroup V (Va-Vd; Fig. 2B). The S segments of six Australian isolates did not fall within the identified clusters and so were not assigned. Each of the earliest Australian AKAV isolates (R7949/Belmont/1968 and B8935/Amberley/1968) shared the same unique viral genotype (SIII/MIIIb/LIII). S segment genotype III occurred in only one other isolate (CS016/Kairi/1975) but with reassorted genotypes in the M and L segments (MIIIc and LVb). M segment genotype IIIb occurred in only three other isolates (CS215/Peachester/1976; CS228/Peachester/1976; CS1798/Peachester/1984) but in association with different S segments (unassigned and genotype Vb) and L segments (genotype Vb). L segment genotype Vb occurred in all other isolates from eastern Australia and one isolate from the Northern Territory (DPP231/Berrimah/1982) but in association with various other genotypes in the S segment (Va, Vd or unassigned) and the M segment (IIIa or IIIc). The data indicate that genome segment reassortment has occurred commonly amongst AKAV isolates in eastern Australia. As observed previously (Akashi et al., 1997; Kobayashi et al., 2007), the single TINV isolate (CS153/Kairi/1987) is also a reassortant virus, sharing the S segment genotype (Vb) and L segment genotype (Vb) with AKAV isolate CS228/

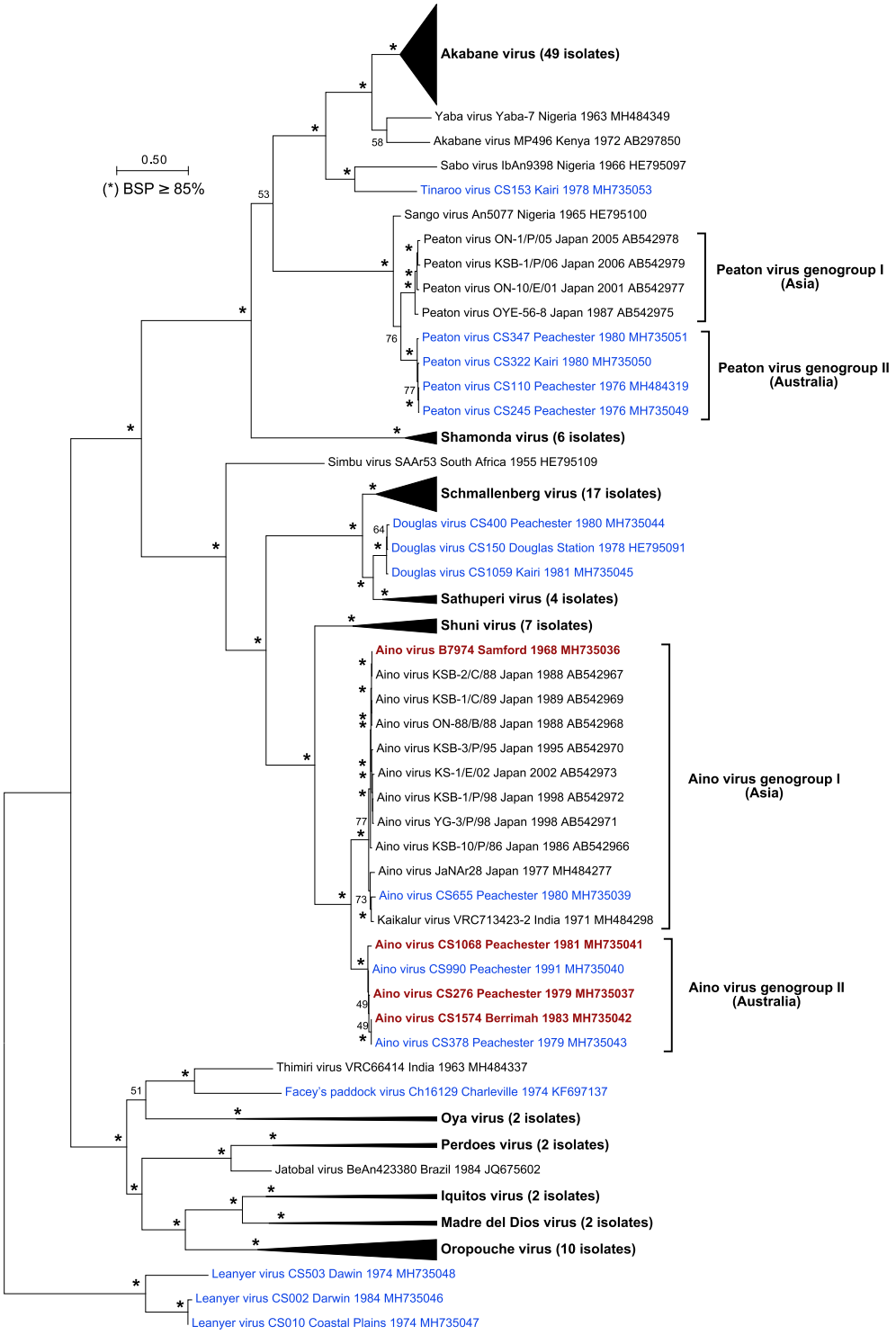


**Fig. 3.** Maximum likelihood phylogenetic trees of complete coding sequences of viruses assigned to the Simbu phylogroup. Rooting was determined by initially including other representative members of the genus *Orthobunyavirus* (La Crosse virus) or genus *Herbevirus* (Herbert virus). A) M segments (141 sequences). B) S segments (176 sequences). C) L segments (120 sequences). Branch lengths are proportional to the number of nucleotide substitutions per site. Values for bootstrap support proportion (BSP) were determined from 100 bootstrap iterations. Simbu phylogroup virus isolates from Australia are shown in blue. Virus isolates that have been identified as reassortants are indicated in magenta. Several clades have been collapsed for the purpose of illustration. Complete trees showing bootstrap values and the GenBank accession numbers of all sequences are presented in Suppl. Fig. 1-3.

Peachester/1976. In contrast, six AKAV isolates from the Northern Territory and a single isolate from Queensland (CS710/Peachester/1980) shared a unique genotype in each of the three segments (SVc/MV/LVa) with no evidence of reassortment with other genotypes present in Eastern Australia during the time interval of the study.

A similar analysis was conducted using genotype assignments of the S, M and L coding sequences of all East Asian AKAV isolates for which the complete sequence L and M segment sequences were available in GenBank (Table 2). As few complete L sequences were available, genogroups assigned in a previous study (using only partial L segment

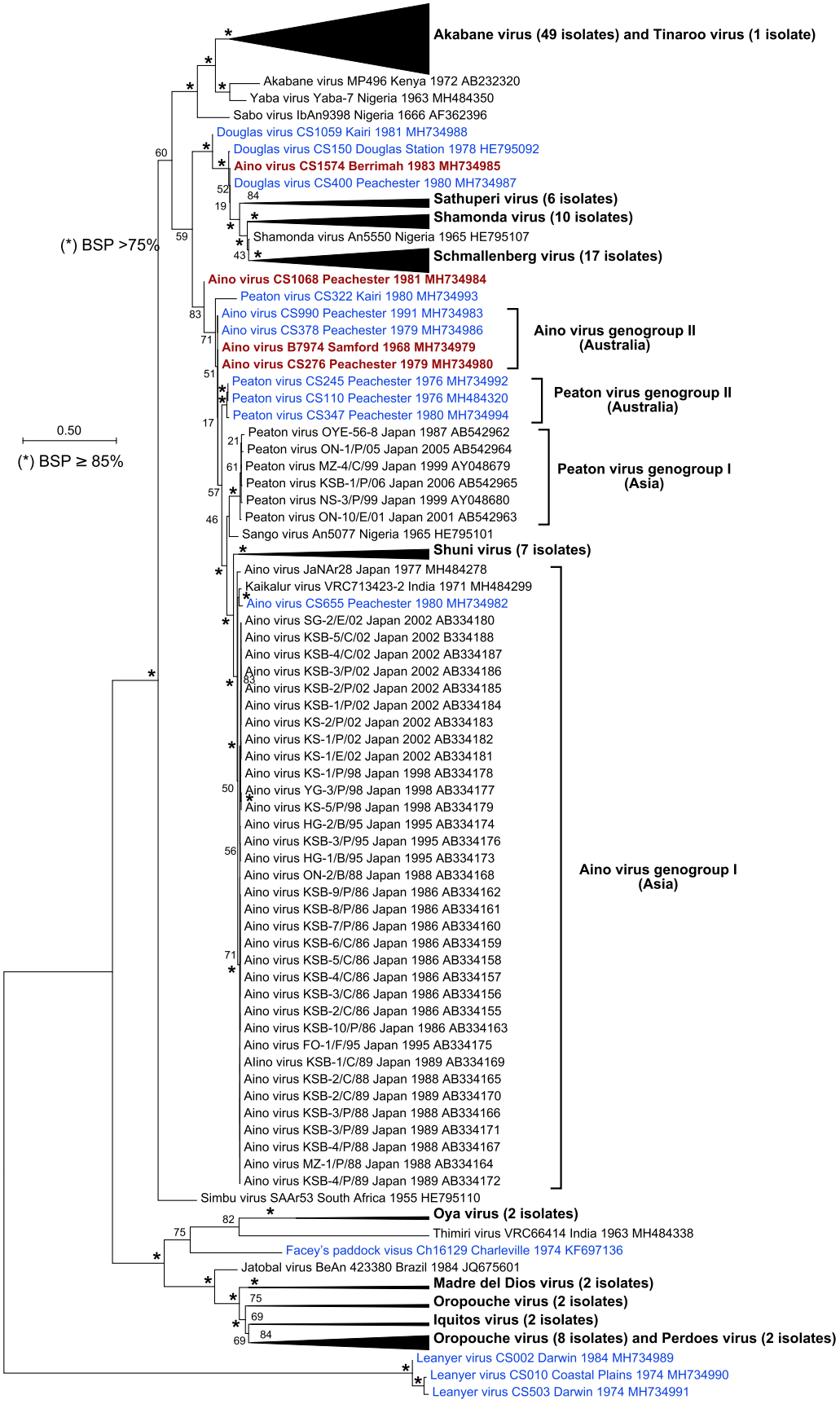


**Fig. 3.** (*continued*)

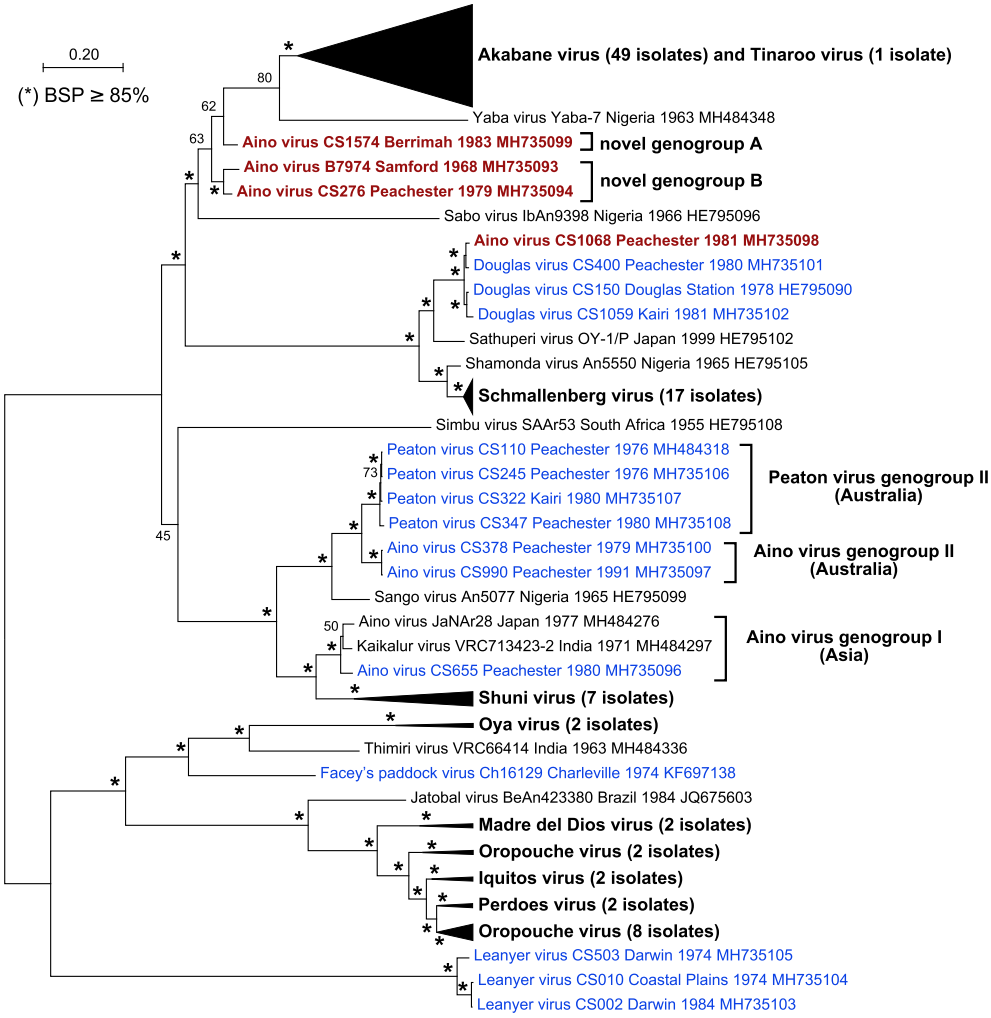


**Fig. 3.** (*continued*)

sequences) were also included in the analysis (Kobayashi et al., 2007). The data indicated that most available East Asian viruses corresponded to only three genotypes (SIa/MIa/LIa), (SIb/MIb/LIb) and (SII/MII/LII). Two viruses isolated in Japan (ON-2/E/Japan/1994 and KSB-4/P/Japan/1995) were distinct. For each virus, the S segment fell into the small East Asian clade D and, although the M segment sequences were assigned to genogroup II, they formed a distinct and separate sub-clade. A previous study using partial L segment sequences also recognised that they were distinct and did not assign these viruses to any genogroup. The complete S and M segment coding sequences were available for four Japanese isolates (NS-4/P/Japan/1998; MZ-2/C/Japan/1999; CB-1/F/Japan/1998; ON-3/F/Japan/1997) that had been reported previously to be likely reassortant genotypes (SII/MII/LIb) (Kobayashi et al., 2007). However, as no complete L segment coding sequences are yet available for these viruses, we could not confirm the genotype assignments.

### 3.4. Evidence of intertypic genome segment reassortment

Maximum likelihood phylogenetic trees were inferred from nucleotide sequence alignments of the complete M, S and L coding sequences of Australian isolates of AKAV (39 isolates), TINV (1 isolate), AINOV (7 isolates), DOUV (3 isolates), PEAV (4 isolates), FPV (1 isolate) and LEAV (3 isolates), as well as those of Simbu-serogroup orthobunyaviruses available in GenBank (Fig. 3). Initially, trees were inferred using sequences of La Crosse virus (LACV; California serogroup; genus *Orthobunyavirus*, family *Peribunyaviridae*) and Herbert virus (HERV; genus *Herbevirus*, family *Peribunyavirudae*) as outgroups to establish the correct rooting pattern. The final trees were then inferred using only sequences of viruses assigned to the Simbu genogroup and compared to identify evidence of intertypic genome segment reassortment.

The M segment tree (141 isolates) indicated clustering of sequences according to the antigenic assignment of each virus (Fig. 3A). PEAV isolates clustered into two genogroups comprising isolates from Japan (genogroup I) and Australia (genogroup II), and were most closely related to SANV. AINOV isolates also fell into two genogroups and were most closely related to SHUV. AINOV genogroup I comprised primarily Japanese isolates but also included two isolates from Australia (AINO/B7974/Samford/1968 and AINOV/CS655/Peachester/1980) as well as Kaikalur virus (KAIV) from India, which should be considered to be a variant of AINOV. AINOV genotype II comprised only isolates from Australia. As reported previously, DOUV isolates clustered in a complex with SATV and SBV (Yanase et al., 2012), FPV was most closely related to THIV, clustering with other viruses of Simbu group clade A including OROV. Finally, LEAV isolates clustered separately from other viruses in the set.

In the S segment tree (176 sequences), AINOV isolates from Japan and Australia did not form a single monophyletic group (Fig. 3B). As in the M tree (Fig. 3A), AINOV genogroup I (comprising all Japanese isolates, AINOV/CS655/Peachester/1980 from Australia and KAIV from India) clustered with SHUV. However, AINOV genogroup II, comprising most Australian AINOV isolates, were less closely related to the AINOV genogroup I than all PEAV isolates, all SHUV isolates and

**Table 1**
Genogroup and clade assignments of the S, M and L segments of Australian AKAV and TINV isolates (1969–1984) illustrating frequent intratypic and intertypic reassortment.

| AKAV isolate | Genogroup/cluster | | |
|---|---|---|---|
| | S RNA | M RNA | L RNA |
| R7949/Belmont/1968 | III | IIIb | III |
| B8935/Amberley/1968 | III | IIIb | III |
| CS016/Kairi/1975 | III | IIIc | Vb |
| CS153/Kairi/1978 | Vb | TINV | Vb |
| CS228/Peachester/1976 | Vb | IIIb | Vb |
| CS1798/Peachester/1984 | unassigned | IIIb | Vb |
| CS215/Peachester/1976 | unassigned | IIIb | Vb |
| CS502/Badgery's Creek/1976 | Vd | IIIc | Vb |
| CS231/Kempsey/1977 | Vd | IIIc | Vb |
| CS241/Hunter Valley/1978 | Vd | IIIc | Vb |
| CS233/Tamworth/1979 | Vd | IIIc | Vb |
| CS506/Peachester/1979 | Vd | IIIc | Vb |
| CS661/Peachester/1980 | Vd | IIIc | Vb |
| CS221/Peachester/1976 | Vd | IIIc | Vb |
| CS396/Peachester/1979 | Vd | IIIc | Vb |
| CS505/Peachester/1979 | Vd | IIIc | Vb |
| CS979/Peachester/1981 | Vd | IIIc | Vb |
| CS1209/Peachester/1982 | Vd | IIIc | Vb |
| CS1586/Peachester/1983 | Vd | IIIc | Vb |
| CS1796/Peachester/1984 | Vd | IIIc | Vb |
| CS1778/Peachester/1984 | Vd | IIIc | Vb |
| CS294/Peachester/1979 | Vd | IIIa | Vb |
| CS1777/Peachester/1984 | unassigned | IIIc | Vb |
| CS1776/Peachester/1984 | unassigned | IIIc | Vb |
| CS749/Kairi/1981 | Va | IIIc | Vb |
| CS1383/Kairi/1980 | Va | IIIa | Vb |
| CS951/Peachester/1980 | Va | IIIa | Vb |
| CS1316/Yeerongpilly/1980 | Va | IIIa | Vb |
| CS744/Peachester/1981 | Va | IIIa | Vb |
| DPP231/Berrimah/1982 | Va | IIIa | Vb |
| CS1711/Peachester/1984 | Va | IIIa | Vb |
| CS700/Kairi/1981 | unassigned | IIIa | Vb |
| CS1416/Kairi/1981 | unassigned | IIIa | Vb |
| CS710/Peachester/1980 | Vc | V | Va |
| CS235/Victoria River/1979 | Vc | V | Va |
| CS236/Douglas Daly/1979 | Vc | V | Va |
| DPP232/Tortilla Flats/1982 | Vc | V | Va |
| DPP240/Tortilla Flats/1983 | Vc | V | Va |
| DPP237/Beatrice Hill/1983 | Vc | V | Va |
| DPP261/Berrimah/1983 | Vc | V | Va |

**Table 2**
Genogroup and clade assignments of the S, M and L segments of East Asian AKAV isolates (1959–2016) illustrating intratypic genome segment reassortment.

| AKAV isolate | Genogroup/clade | | |
|---|---|---|---|
| | S RNA | M RNA | L RNA[b] |
| JaGAr39/Japan/1959 | unassigned | II | II |
| OBE-1/Japan/1974 | II | II | II |
| KT3377/Japan/1977 | II | II | (II) |
| TS-C2E/Japan/1984 | II | II | II |
| NS-88-1/Japan/1988 | II | II | (II) |
| YG-88-2/Japan/1988 | II | II | (II) |
| KSB-1/C/Japan/1988 | II | II | |
| ON-89-2/Japan/1989 | II | II | |
| 93FMX/South Korea/1993 | II | II | |
| K9/South Korea/1993 | II | II | |
| KSB-1/C/Japan/1994 | II | II | |
| NS-4/P/Japan/1998[a] | II | II | (Ib) |
| MZ-2/C/Japan/1999[a] | II | II | (Ib) |
| CB-1/F/Japan/1998[a] | II | II | (Ib) |
| ON-3/F/Japan/1997[a] | II | II | (Ib) |
| Okayama/Japan/2004 | II | II | |
| KV0505/South Korea/2005 | II | II | |
| AK7/South Korea/2006 | II | II | |
| OY-1/P/Japan/2008 | II | II | |
| TT-1/E/Japan/2008 | II | II | |
| HG-1/P/Japan/2010 | II | II | |
| ON-2/E/Japan/1994[a] | D | II | (unassigned) |
| KSB-4/P/Japan/1995[a] | D | II | (unassigned) |
| Iriki/Japan/1984 | Ia | Ia | (Ia) |
| KS-1/E/Japan/1985 | Ia | Ia | (Ia) |
| ON-1/P/Japan/1993 | Ia | Ia | (Ia) |
| CY-77/Taiwan/1993 | Ia | Ia | (Ia) |
| ON-5/B/Japan/1998 | Ia | Ia | (Ia) |
| ON-3/E/Japan/1990 | Ia | Ia | (Ia) |
| ON-1/E/Japan/1998 | Ia | Ia | (Ia) |
| MZ-1/C/Japan/2000 | Ia | Ia | (Ia) |
| Okayama/Japan/2001 | Ia | Ia | |
| KS-2/Mo/Japan/2006 | Ia | Ia | |
| KSB-3/P/Japan/2006 | Ia | Ia | |
| KM-2/Br/Japan/2006 | Ia | Ia | |
| KM-1/Br/Japan/2006 | Ia | Ia | |
| YG-1/Br/Japan/2007 | Ia | Ia | |
| ON-1/E/Japan/2010 | Ia | Ia | |
| DHL10M110/China/2010 | Ia | Ia | Ia |
| 7/SKR/South Korea/2010 | Ia | Ia | Ia |
| 17/SKR/South Korea/2010 | Ia | Ia | |
| 35/SKR/South Korea/2010 | Ia | Ia | |
| 32/SKR/South Korea/2010 | Ia | Ia | |
| EH-3/Br/Japan/2011 | Ia | Ia | |
| HS-1/Br/Japan/2011 | Ia | Ia | |
| ON-1/P/Japan/2012 | Ia | Ia | |
| KM-1/B/Japan/2013 | Ia | Ia | |
| KSB-2/P/Japan/2013 | Ia | Ia | |
| GXLCH02/China/2016 | Ia | Ia | Ia |
| GXLCH04/China/2016 | Ia | Ia | Ia |
| GXLCH01/China/2016 | Ia | Ia | Ia |
| GXLCH70N/China/2016 | Ia | Ia | Ia |
| GXLCH16-70/China/2016 | Ia | Ia | Ia |
| NM/BS/1/China | Ia | Ia | |
| FO-90-3/Japan/1990 | Ib | Ib | (Ib) |
| KSB-1/C/Japan/1987 | Ib | Ib | (Ib) |

[a] Reassortant genotypes.
[b] Bracketed genogroup assignments are those reported by Kobayashi et al. (2007) using partial L segment sequences and were not determined in this study.

the single available SANV isolate from Nigeria. Notably, AINOV genogroup II also included the original Australian isolate (AINOV/B7974/Samford/1968) which clustered with AINOV genogroup I in the M segment. Similarly, PEAV isolates did not form a single monophyletic group; PEAV genotype I (Asia) was most closely related to the SANV isolate from Nigeria, while PEAV genotype II (Australia) was more deeply rooted in the tree. As in the M segment tree, DOUV isolates fell in a complex with SATV (6 isolates) and SBV (17 isolates), as well as SHAV from Japan (10 isolates) and Nigeria (1 isolate). However, one Australian AINOV isolate (CS1574/Berrimah/1983) also grouped with DOUV.

In the L segment tree (120 sequences), AINOV isolates were again not monophyletic (Fig. 3C). Consistent with the S segment, Australian isolate AINOV/CS655/Peachester/1980 clustered with KAIV and the single available Japanese isolate (AINOV/JaNAr28/Japan/1977), representing AINOV genotype I. Two Australian AINOV isolates (CS378/Peachester/1978 and CS990/Peachester/1990) clustered with all available Australian PEAV isolates (PEAV genotype II); no L segment sequences are yet available for Japanese PEAV isolates. One Australian AINOV isolate (CS1068/Peachester/1981) fell with DOUV in a clade that also contained SBV, SATV and SHAV isolates. Three Australian AINOV isolates (CS1574/Berrimah/1983, CS276/Peachester/1979 and B7974/Samford/1968) formed novel clades (A and B) that were more closely related to AKAV, YABV and SABOV. As was observed for the M segment, the S and L segments of FPV were most closely related to THIV and OYAV (2 isolates), in turn clustering with OROV and other viruses of Simbu group clade A. In all segments, LEAV isolates fell as a distinct phylogenetic group.

The cluster patterns in the three phylogenetic trees were used to assign a genotype to each Australian isolate (Table 3). Uniquely in this

**Table 3**
Genotype assignments of the S, M and L segments of Australian Simbu-group virus isolates (1968–1991) illustrating frequent intertypic reassortment in Aino virus.

| Simbu serogroup virus isolate | genotype | | |
|---|---|---|---|
| | S RNA | M RNA | L RNA |
| AINOV_CS655_Peachester_1980_MH734982 | AINOV I | AINOV I | AINOV I |
| AINOV_CS378_Peachester_1979_MH734986 | AINOV II | AINOV II | AINOV II |
| AINOV_CS990_Peachester_1991_MH734983 | AINOV II | AINOV II | AINOV II |
| AINOV_B7974_Samford_1968_MH734979[a] | AINOV I | AINOV II | novel B |
| AINOV_CS1068_Peachester_1981_MH734984[a] | AINOV II | AINOV II | DOUV |
| AINOV_CS276_Peachester_1979_MH734980[a] | AINOV II | AINOV II | novel B |
| AINOV_CS1574_Berrimah_1983_MH734985[a] | DOUV | AINOV II | novel A |
| DOUV_CS150_Douglas_Station_1978_HE795092 | DOUV | DOUV | DOUV |
| DOUV_CS1059_Kairi_1981_MH734988 | DOUV | DOUV | DOUV |
| DOUV_CS400_Peachester_1980_MH734987 | DOUV | DOUV | DOUV |
| PEAV_CS110_Peachester_1976_MH484320 | PEAV II | PEAV II | PEAV II |
| PEAV_CS245_Peachester_1976_MH734992 | PEAV II | PEAV II | PEAV II |
| PEAV_CS322_Kairi_1980_MH734993 | PEAV II | PEAV II | PEAV II |
| PEAV_CS347_Peachester_1980_MH734994 | PEAV II | PEAV II | PEAV II |
| LEAV_CS002_Darwin_1984_MH734989 | LEAV | LEAV | LEAV |
| LEAV_CS010_Coastal_Plains_1974_MH734990 | LEAV | LEAV | LEAV |
| LEAV_CS503_Darwin_1974_MH734991 | LEAV | LEAV | LEAV |
| FPV_Ch16129_Charleville_1974_KF697136 | FPV | FPV | FPV |

[a] Reassortant genotypes.

data set, one Australian isolate (AINOV/CS655/Peachester/1980) was assigned in all segments to AINOV genogroup I, comprising AINOV isolates from Japan and KAIV from India. Four Australian AINOV isolates were assigned as reassortant genotypes. AINOV/B7974/Samford/1968 and AINOV/CS276/Peachester/1979 were each assigned to AINOV genogroup II in the S and M segments and to a novel and distinct AINOV genogroup (B) in the L segment. AINOV/CS1068/Peachester/1981 was also assigned to AINOV genogroup II in the S and M segments but was assigned to the DOUV genogroup in the L segment. AINOV/CS1574/Berrimah/1983 was assigned to AINOV genogroup II in the M segment but to the DOUV genogroup in the S segment and to a novel and distinct AINOV genogroup (A) in the L segment. All other Australian isolates of AINOV, DOUV, PEAV, LEAV and FPV were assigned to the same genogroups in all three segments.

## 4. Discussion

This study was conducted using a set of historical isolates of Simbu serogroup viruses which had been collected during a 17-year period between 1968 and 1984. At that time, a well-resourced arbovirus research program was active in Australia, allowing the isolation of a large number of viruses from cattle and insects collected at multiple locations. Unfortunately, it was not possible to obtain additional virus isolates from the period since these early studies were conducted as, although AKAV remains a significant animal health issue in Australia, virus isolation is no longer conducted routinely. Nevertheless, our study provides a useful window into the distribution and evolution of the Simbu serogroup viruses in Australia.

In total, 57 virus isolates were recovered from storage and sequenced in the study. One of the samples, CS296 collected in 1979 from a healthy bull at Peachester in Queensland, had been previously identified serologically as a mixed infection with AKAV and AINOV (Cybinski and Zakrzewski, 1983). This observation was confirmed in our analysis with sequences representing the S, M and L segments of AKAV but only the S and M segments of AINOV identified in the sample. It appears that this co-infection was present in the animal from which the sample was taken as neither AKAV or AINOV were being handled in the laboratory at the time of isolation (Cybinski and Zakrzewski, 1983). However, as there was no way to establish the segment combinations associated with each virus and clearly specify genotypes, the sample was excluded from further analysis.

Phylogenetic analysis of AKAV isolates from East Asia confirmed previous reports of the existence of three distinct genogroups (Ia, Ib and II) (Kobayashi et al., 2007; Yamakawa et al., 2006). However, our analysis of full-length S segment coding sequences excluded the original Japanese AKAV isolate (AKAV/JaGAr39/Japan/1959) from other clades. We also identified an additional distinct and well supported clade (East Asia clade D; Fig. 2B) that was distinct from genogroups Ia, Ib and II. The isolates in this clade had previously been assigned to East Asian genogroup II but with poor bootstrap support (Kobayashi et al., 2007; Yamakawa et al., 2006). Our results support other more recent studies of Korean, Chinese and Japanese AKAV isolates which also excluded the isolates from genogroup II (Oem et al., 2012; Tang et al., 2017).

Prior to this study, sequence data were available for only two Australian AKAV isolates (AKAV/R7949/Belmont/1969 and AKAV/B8935/Amberley/1968) and these had been assigned in phylogenetic studies as genogroup III, with the sole African AKAV isolate assigned as genogroup IV (Kobayashi et al., 2007; Yamakawa et al., 2006). We adopted these genogroup assignments here and assigned all viruses clustering with these two Australian isolates as AKAV genogroup III. However, our study also revealed the existence of other genogroups in Australia. In the M segment, novel genogroup V comprised a set of seven isolates, six of which were collected in the Northern Territory between 1979 and 1983, and a single isolate from Peachester in Queensland in 1980. These seven AKAV isolates also clustered separately in the S segment (genogroup V, cluster c) and L segment (genogroup Va) from the 32 other isolates, 31 of which were collected from locations on the east coast of Australia. This geographic clustering of isolates suggests the presence of two distinct AKAV episystems in Australia, one in the far north and one in the east, with constraints on the movement of viruses between the two regions. Phylogenetic studies of bluetongue virus (BTV) in Australia have also identified two distinct episystems with constrained, but not complete blockage, of movement of viruses between the far north and eastern states (Firth et al., 2017). Notably, AKAV and BTV are each transmitted by biting midges (*Culicoides* spp.). In the case of BTV, multiple species appear to be involved in transmission in the northern episystem whereas the principal vector in the east is *C. brevitasis*. AKAV is also transmitted by biting midges and has been isolated in Australia on many occasions from *C. brevitarsis*. Phylogenetic analysis of genome sequences using single nucleotide polymorphisms (SNPs) and mitochondrial DNA of *C. brevitarsis* samples

from northern and eastern Australia SNPs has also revealed the presence of a sub-population in the Northern Territory, suggesting that the movement of viruses across the continent is constrained by geographic barriers to the movement of the vector (Onyango et al., 2016). In contrast, bovine ephemeral fever virus (BEFV), which is transmitted in Australia by mosquitoes, appears to move easily and quite rapidly between the far north and the eastern regions (Trinidad et al., 2014).

Our phylogenetic analysis also provided evidence that both intratypic and intertypic genome segment reassortment contribute to the population structure and evolution of AKAV in Australia. As reported previously (Kobayashi et al., 2007), TINV (CS153/Kairi/1987) was observed to be a reassortant, with a unique M segment but sharing S and L segments with Australian AKAV isolates. We also observed multiple intratypic reassortment events amongst Australian AKAV isolates involving all three genome segments. Interestingly, all reassortant AKAV isolates were from the east coast episystem with isolates from the northern episystem displaying a stable genotype (Vc/V/Va). Intratypic segment reassortment has been reported previously amongst Japanese AKAV isolates with the L segments of four isolates clustering differently from the S and M segments (Kobayashi et al., 2007). In this study, we confirmed that observation and also identified two additional isolates (ON-2/E/Japan/1994 and KSB-4/P/Japan/1995) appear to be intratypic reassortants with the S segment representing a distinct new genogroup (D).

Phylogenetic analysis of other Simbu serogroup viruses indicated that, except for two isolates (AINOV/CS655/Peachester/1980 and AINOV/B7974/Samford/1968), all three segments of Australian AINOV and PEAV isolates represent lineages that are separate and distinct from those that have been reported from Japan. Uniquely, AINOV/CS655/Peachester/1980 was assigned in all three segments to AINOV genogroup I which comprises all Japanese isolates and, importantly, Kaikalur virus (KAIV) from India, suggesting that this genogroup may be distributed geographically over a wide expanse of South and East Asia. Interestingly, KAIV has recently been assigned to the virus species *Shuni orthobunyavirus* (Maes et al., 2018). However, our analysis indicates that this assignment is incorrect and that KAIV should be considered to be an isolate of AINOV (species *Aino orthobunyavirus*). AINOV/B7974/Samford/1968 (the earliest Australian AINOV isolate) displayed a unique reassortant genotype with the M segment assigned to AINOV genogroup I (Asia), the S segment assigned to AINOV genogroup II (Australia) and the L segment assigned to novel genogroup B. A previous report that AINOV/B7974/Samford/1968 derived the S and L segments from PEAV (Yanase et al., 2010) was not confirmed and appears to be an incorrect interpretation due to the limited availability of isolates for that analysis.

Our data also confirmed previous observations that DOUV is closely related to SHAV, SATV and SBV. However, we have also shown that several AINOV isolates are reassortants with the S segment (AINOV/CS1574/Berrimah/1983) or the L segment (AINOV/CS1068/Peachester/1981) derived from DOUV. Three of the four identified AINOV reassortant isolates (AINOV/CS1574/Berrimah/1983, AINOV/B7974/Samford/1968 and AINOV/CS276/Peachester/1979) derived L segments from novel unassigned genogroups A or B, suggesting the possible existence of a yet unidentified Simbu group virus.

It is not known whether the identified intertypic reassortment events occurred in Australia or at some time prior to the introduction of the viruses to the continent. The frequent arrival of new BTV serotypes in Australia is well documented (Boyle et al., 2012, 2014; Firth et al., 2017) and both phylogenetic studies and spatial modelling suggest that the wind-borne translocation of biting midges from Indonesian Archipelago and/or Papua New Guinea are the likely pathways of entry (Eagles et al., 2012, 2013, 2014; Firth et al., 2017). Previous studies have indicated high prevalence of neutralising antibodies to AINOV (99%), AKAV (80%), TINV (68%), PEAV (58%) and DOUV (12%) in Indonesian cattle (Daniels et al., 1995; Miura et al., 1982) and the isolation of AKAV (genogroup Ib) has recently been reported from West Java (Purnomo Edi et al., 2017). It is highly likely that Australian Simbu serogroup viruses also have their origins in Melanesia and that there is a dynamic relationship between the northern Australian and South-East Asian episystems.

The historical nature of this study limits interpretation with respect to the current epidemiological situation in Australia. Phylogenetic studies indicate that AKAV genogroups identified in Asia have remained extant and distinct over a long period at least from the 1980s until the present. We may therefore expect that the novel genogroups of AKAV, AINOV and PEAV identified in Australia may also remain extant. It is also possible that additional genogroups of Asian origin have since been introduced. As no vaccine is currently in use in Australia, AKAV remains a significant issue for farmers with previous disease cost estimates of several million dollars annually (Kirkland and Barry, 1986). Serological surveillance is conducted as a national program to support the live animal export trade but virus isolation is no longer resourced. As has been observed recently (Geoghegan et al., 2014), access to a data set of current virus isolates, both from within Australia and globally, would provide a more complete picture of the epidemiology and phylodynamics of Simbu serogroup viruses, and greatly facilitate risk management.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.013.

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