RESEARCH ARTICLE

Seasonal activity, vector relationships and genetic analysis of mosquito-borne Stratford virus

Cheryl S. Toi[1]*, Cameron E. Webb[1,2], John Haniotis[1], John Clancy[1], Stephen L. Doggett[1]

1 Department of Medical Entomology, Centre for Infectious Diseases and Microbiology Laboratory Services, NSW Health Pathology, Westmead Hospital, Westmead, New South Wales, Australia, **2** Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney, New South Wales, Australia

* cheryl.toi@health.nsw.gov.au

Abstract

There are many gaps to be filled in our understanding of mosquito-borne viruses, their relationships with vectors and reservoir hosts, and the environmental drivers of seasonal activity. Stratford virus (STRV) belongs to the genus *Flavivirus* and has been isolated from mosquitoes and infected humans in Australia but little is known of its vector and reservoir host associations. A total of 43 isolates of STRV from mosquitoes collected in New South Wales between 1995 and 2013 was examined to determine the genetic diversity between virus isolates and their relationship with mosquito species. The virus was isolated from six mosquito species; *Aedes aculeatus*, *Aedes alternans*, *Aedes notoscriptus*, *Aedes procax*, *Aedes vigilax*, and *Anopheles annulipes*. While there were distinct differences in temporal and spatial activity of STRV, with peaks of activity in 2006, 2010 and 2013, a sequence homology of 95.9%–98.4% was found between isolates and the 1961 STRV prototype with 96.2%–100% identified among isolates. Temporal differences but no apparent nucleotide divergence by mosquito species or geographic location was evident. The result suggests the virus is geographically widespread in NSW (albeit only from coastal regions) and increased local STRV activity is likely to be driven by reservoir host factors and local environmental conditions influencing vector abundance. While STRV may not currently be associated with major outbreaks of human disease, with the potential for urbanisation and climate change to increase mosquito-borne disease risks, and the possibility of genomic changes which could produce pathogenic strains, understanding the drivers of STRV activity may assist the development of strategic response to public health risks posed by zoonotic flaviviruses in Australia.

Introduction

The strategic response of local authorities to the risk of mosquito-borne disease requires an understanding of the environmental, entomological, and ecological drivers of virus activity [1]. While there are over 70 arboviruses known to occur in the Australian region [2, 3],

KU059151, KU059152). Accession numbers are listed in Table 2 of the manuscript.

relatively few pose serious human health risks [4]. The alphaviruses, Ross River virus (RRV) and Barmah Forest virus (BFV) are the most common mosquito-borne pathogens causing human disease with around 6,000 cases officially notified across Australia each year [4]. However, endemic mosquito-borne flaviviruses including Murray Valley encephalitis virus (MVEV) and the Kunjin strain of West Nile virus (WNV$_{KUN}$) have the potential to cause serious human and veterinary health risks [5, 6]. Amid possible increases in future mosquito-borne disease risk associated with a changing climate, anthropogenic effects that result in increased vector/human contact, potential genomic changes that can increase the virulence of viruses, and the likely introduction of exotic vectors [7, 8], it is important to fill the gaps in our understanding of relationships between pathogens, vectors, reservoir hosts and environmental conditions to determine the key drivers of public health risks.

Stratford virus (STRV) belongs to the Kokobera subgroup of the genus *Flavivirus* and the family Flaviviridae [9]. It was first isolated in 1961 from *Aedes vigilax* Skuse collected in Cairns, Far North Queensland [10] and is endemic in Australia. The clinical role and importance of STRV to human health has not been fully defined, although serological evidence of past human infections have been identified during a serosurvey conducted in south-eastern Australia in 1985/86 that reported serum antibodies to STRV [11, 12]. At the time of this serosurvey, no isolations of STRV from mosquitoes were reported because no mosquito or mosquito-borne pathogen surveillance program existed. Subsequently, STRV has been isolated from the *Aedes* species of mosquitoes from a number of locations in south east Australia [2, 13]. There is no information available on reservoir hosts [2], other than the *Aedes* species display a wide host-feeding preference [13, 14].

Aedes vigilax is one of the most important pest and vector mosquitoes in Australia and commonly associated with tidally influenced estuarine wetlands in all but the most southern coastal regions of Australia [4]. Populations of this mosquito can be extraordinarily abundant and, notwithstanding the nuisance-biting impacts on coastal communities, there is strong evidence that this mosquito plays an important role in the transmission of arboviruses within these regions. *Aedes procax*, however, is closely associated with freshwater to brackish water habitats, particularly coastal swamp forests [15] and can be locally abundant. It has also been shown to transmit a range of arboviruses in the laboratory [16, 17]. *Aedes notoscriptus* is closely associated with natural and artificial water-holding containers and is widespread across urban areas of Australia [18]. It is a nuisance-biting pest and arboviruses have been isolated from field collected specimens [13]. This mosquito is shown to be an efficient vector of RRV and BFV in laboratory studies [19–21] but does not disperse widely from larval habitats [22].

Study on the geographic variation in pathogen genotype provide insights into the reservoir hosts of arboviruses and subsequently, may assist in the development of surveillance programs and epidemic early warnings systems. Phylogenetic studies of two endemic flaviviruses, MVEV and WNV$_{KUN}$, have already shown relative homogeneity within the viral type and a close association with mobile reservoir hosts such as birds. However, the endemic alphavirus RRV has shown distinct geographic variation with greater genetic variability. Subsequently, two topotypes have been identified from macropods which are relatively sedentary reservoir hosts [23]. The role of macropods as important reservoir hosts has been implicated through serological surveys [24]. This trend is not consistent across other Australian alphaviruses studied to date with the genetic variability of Sindbis virus (SINV) found to vary temporally, rather than geographically across 40 isolates studied; it was proposed that this temporal clustering suggests migratory avian hosts and that they may rapidly disperse the virus from enzootic focal points [25]. Similarly, BFV displays a high degree of sequence homology suggesting little geographic or temporal divergence, consistent with avian reservoir hosts [26]. However, while genetic analysis of mosquito-borne viruses may suggest mobile reservoirs hosts, it is important

to note that as well as avian hosts, flying mammals (e.g. bats) may also play a role in geographic dispersal of pathogens [6, 27].

Hence, the aim of this study was to investigate the genetic diversity between isolates of STRV derived from field collected mosquito specimens. This will determine if there are any temporal, geographic, or mosquito-specific relationships that may suggest entomological or wildlife drivers of arbovirus activity.

Materials and methods

Mosquito collection

All isolates of STRV were cultured from field collected mosquito specimens. These collections were undertaken between 1995 and 2013 as part of the NSW Arbovirus Surveillance and Mosquito Monitoring program that runs on a weekly basis between November and April at various locations throughout NSW [13]. Adult mosquitoes are collected using dry-ice baited encephalitis virus surveillance (EVS) traps [28] with specimens identified to species using the taxonomic keys of Russell (1993) [29].

Virus isolation and identification

Viruses were isolated via cell culture and identified using a Fixed-Cell ELISA [30]. Briefly, pools of up to 25 mosquitoes of the same species were placed into tubes containing 2 mL of RPMI 1640 (Roswell Park Memorial Institute medium), antibiotics, foetal calf serum and glass beads and shaken for 20 minutes at 4˚C until ground. The samples were clarified at 4000 rpm for 20 minutes at 4˚C and the supernatants inoculated onto C6/36 insect cells and incubated for 3–4 days for the initial replication of virus. An inoculum was transferred onto C6/36 and baby hamster kidney (BHK) cells and incubated for a further 3–4 days. The purpose was to increase the infectious titres of slower growing flaviviruses in C6/36 cells and to observe cytopathic effect (CPE) in mammalian cells. Culture supernatant from infected mammalian cells showing virus CPE were processed for sequencing. Positive isolates were identified by FC ELISA using a panel of flavivirus-specific MAbs, 1E3 and 2E5 that differentiates between Stratford virus from KOKV and other Kokobera-like viruses [31].

RT-PCR and DNA sequencing

Viral RNA was extracted from 200 μL of cell culture supernatant using the EZ1$^{®}$ Virus Mini Kit v2.0 on the BioRobot$^{®}$ (Qiagen, Limburg, Netherlands) in accordance with the manufacturers' instruction. A two-step RT-PCR was performed using Tetro Reverse Transcriptase (Bioline, Sydney, Australia) and random hexamer primers (Roche, Mannheim, Germany) for cDNA synthesis. The following cycling conditions were applied: 22˚C for 5 min, 50˚C for 35 min and 70˚C for 7 min on the Veriti$^{®}$ Thermal Cycler (Applied Biosystems, California, USA).

Universal primers spanning an 802 bp region encoding part of the methyltransferase and the start of the region encoding RNA-dependent-RNA polymerase in the flavivirus non- structural protein 5 (NS5) gene sequence were selected for PCR [32]. These comprised of consensus degenerate bases; Flav100F (5'-AAYTCNACNCANGARATGTAYT-3') and reverse primer Flav200R (5'-CCNARCCACATRWACCA-3'). The primer sequences and their positions (8276–9078) are relative to the Yellow fever virus genome, GenBank reference NC_002031.

The 802 bp fragment was amplified by real-time PCR on the Rotogene 6000 (Qiagen, Victoria, Australia) in a total volume of 20 μL containing 1.8 μL of cDNA template, 300 nM of each primer, 1 × of 20-fold EvaGreen (Biotium, CA, USA), 1 × My Taq reaction buffer, a proprietary formulation containing dNTPs, $MgCl_2$ and enhancers, 1U of MyTaq™ HS DNA

polymerase (Bioline, Sydney, Australia) and RNase- and DNAse-free water (Sigma, St. Louis, USA). The thermal cycling commenced with enzyme activation (95˚C for 1 min), followed by 40 cycles of denaturation (95˚C for 20 s), annealing (51˚C for 30 s), extension (72˚C for 40 s), and a final extension at 72˚C for 2 min. A pre-hold cycle was set at 50˚C for 30 s followed by a melt cycle with ramping temperatures between 75˚C and 95˚C to serve as a check on purity of the amplified product. The amplicons were verified on a 1% agarose gel and examined for non-specific banding.

The PCR amplicons were pre-treated with illustra™ ExoProStar 1-Step (GE Healthcare, Buckinghamshire, UK). Ten microlitres of the PCR product together with 2 µL of enzyme made up to a total volume of 14 µL with nuclease free water were incubated at 37˚C for 20 min and inactivated for 20 min at 80˚C. A total volume of 12 µL containing 10.8 µL of the cleaned amplicon and 1.2 µL of the Flav 100F forward primer (20 µM) were sent to the Australian Genome Research Facility (AGRF), Sydney, for Sanger sequencing using the ABI BigDye® Terminator chemistry Version 3.1.

Sequence analysis and phylogenetic tree construction

The partial sequences of the STRV isolates were compared against the NS5 gene region of the STRV prototype that was first isolated in 1961, GenBank reference STRV C338 KF917540 and KM225263. For completeness of study, comparisons were also made against the Kokobera virus (KOKV) AUS MRM32 (GenBank AY632541.4), Torres virus strain TS5273 (GenBank KC788513.1), a STRV-like virus; Bainyik virus strain MK7979 (GenBank KM225264.1) and New Mapoon virus strain CY1014 (KC788512.1). Data were subjected to the jModelTest 2.1.4 [33] to determine the appropriate mode of analysis. Phylogenetic analyses were conducted using Geneious 6.0.6 and *MEGA* version 5.10 [34]. The sequenced gene targets were aligned by the ClustalW Multiple Alignment Tool and edited to a final alignment of 556bp. A Bayesian Markov chain Monte Carlo (MCMC) analysis using MrBayes was used for phylogenetic inference [35]. The analysis was performed using a molecular clock probability distribution on branch lengths with gamma distributed rate variation among sites. MCMC analysis with four chains and temperature set to 0.2 were run for 1100000 cycles. The phylogeny construction of translated protein sequences were analysed by the ML method using the Jones-Taylor-Thornton (JTT) model for amino acid substitution [36]. Confidence limits were set on 1000 bootstrap replicates. A pairwise distance matrix was used to display distances between alignments. All STRV NS5 partial sequences in this study were deposited in GenBank (Accession no. KU059110—KU059152) (Table 1).

Results

A total of 43 isolates of STRV was detected in mosquitoes collected in coastal NSW between 1995 and 2013 (Table 1). The virus was isolated from mosquitoes collected in the far north coast (Byron Bay), mid-north coast and Hunter region (Port Stephens, Lake Macquarie, and Central Coast), Sydney metropolitan region (Blacktown, Georges River, Hawkesbury, Parramatta, Penrith, Sydney Olympic Park and West Pennant Hills) and south coast (Batemans Bay). No STRV was detected in mosquitoes collected at sampling sites west of the Great Dividing Range. There was a marked difference in the seasonal activity of STRV across the study period with sporadic detection of the virus from 1995 until 2006, when the first major season of STRV activity was recorded (n = 14), followed by notable peaks in 2010 (n = 11) and 2013 (n = 11) Table 2.

The majority of STRV isolates were from separate pools of *Ae. vigilax* (46.5%), *Aedes procax* (Skuse) (34.9%) and *Aedes notoscriptus* (Skuse) (11.6%), and these were across a range of

Table 1. Coordinates of mosquito collection sites on the NSW coast where Stratford virus was isolated, 1995–2013. Locations are listed in Table 2.

Collection Site	Coordinates [37]	
Byron Bay	28˚ 38' 29.080"S	153˚ 36' 43.026"E
Port Stephens	32˚ 42' 47.303"S	152˚ 03' 56.872"E
Parramatta	33˚ 47' 58.341"S	151˚ 00' 22.415"E
Lake Macquarie	33˚ 03' 42.281"S	151˚ 36' 05.239"E
Central Coast	33˚ 25' 30.728"S	151˚ 22' 46.844"E
Batemans Bay	35˚ 41' 25.072"S	150˚ 09' 34.965"E
Hawkesbury	33˚ 20' 25.760"S	150˚ 46' 58.488"E
West Pennant Hills	33˚ 38' 02.331"S	150˚ 58' 50.711"E
Penrith	33˚ 44' 59.987"S	150˚ 42' 01.544"E
Blacktown	33˚ 46' 00.002"S	150˚ 55' 00.935"E
Sydney Olympic Park	33˚ 50' 52.721"S	151˚ 04' 05.431"E
Georges River	33˚ 59' 15.243"S	151˚ 01' 30.671"E

doi:10.1371/journal.pone.0173105.t001

geographic locations (Table 1). Three of the STRV isolates were also detected in *Aedes aculeatus* (Theobald), *Aedes alternans* (Westwood), and *Anopheles annulipes* Skuse. There was no discernible trend in the temporal association between mosquito species and STRV detection.

Examination of 43 sequenced isolates covered a total of 556 bp positions in the final data set. Temporal clustering between groups of some isolates collected from 2006, 2010 and 2013 are presented by clades A, B, C, D and E (Fig 1A) with posterior probability ≥ 0.96 for clustered nodes. Comparison between the STRV isolates and the 1961 STRV prototypes (GenBank KM225623; KF917540) showed a high level of support (0.99) with the nucleotide sequence of isolate 33016 showing the most number of nucleotide inconsistencies (n = 10–22). Excepting for Cluster B, no discernible clustering by geographic location or by mosquito species was evident.

The relationship between STRV isolates, STRV prototypes, Torres virus STRV-like strain TS5273, KOKV AusMRM32-AY632541.4 and other viruses in the KOKV group (Bainyik virus strain MK7979, KM225264.1 and New Mapoon virus strain CY1014, KC788512.1) is presented in Fig 1B. A sequence homology of 0.64 was evident between the STRV isolates, STRV C338 and the STRV-like TS5273.

A pairwise comparison between test sequences and the STRV prototypes showed between 95.9% and 98.4% homology. The percentage identity between the STRV isolates, TS5273 and the KOKV AY632541.4 prototype was 76.4%– 78.3% and 74.4% -77.5%, respectively. Between 96.2%–100% genetic similarity was shown between STRV isolate sequences with up to 22 observed oligonucleotide differences. Comparison of the deduced amino acid sequences are shown in Fig 2. There were a total of 175 positions in the final dataset. No distinctive difference between STRV isolates was evident with 99% homology shown between STRV isolates and the STRV prototypes. However, 50% similarity was apparent between STRV isolates, the TS5273, KOKV AusMRM32 and the KOKV group of viruses. A paired comparison of amino acids between the STRV isolates alone was 95.8% with a total of eight variances shown, whereas 100% identity was evident between STRV isolates and STRV C388.

Discussion

This study represents one of the first genetic analyses of STRV isolates distributed over a wide spatial and temporal scale in New South Wales. Surveillance of mosquito-borne pathogens has been underway across Australia for many decades but the isolation of STRV from field

Table 2. The total number (n = 43) of Stratford virus isolates recorded by location and mosquito species from 1995 through 2013.

Isolate No.	Year Collected	Site of virus isolation	Mosquito species	GenBank Accession No.
23769	1995	Batemans Bay	*Aedes vigilax*	KU059132
23770	1995	Batemans Bay	*Aedes vigilax*	KU059133
33016	1996	Port Stephens	*Aedes vigilax*	KU059134
54892	1999	Parramatta	*Aedes notoscriptus*	KU059135
57653	1999	West Pennant Hills	*Aedes notoscriptus*	KU059136
92B0060	2002	Penrith	*Anopheles annulipes*	KU059152
72083	2005	Hawkesbury	*Aedes procax*	KU059137
76859	2006	Port Stephens	*Aedes procax*	KU059138
77538	2006	Port Stephens	*Aedes procax*	KU059139
78596	2006	Port Stephens	*Aedes procax*	KU059146
78618	2006	Port Stephens	*Aedes vigilax*	KU059147
78621	2006	Port Stephens	*Aedes vigilax*	KU059148
78563	2006	Port Stephens	*Aedes vigilax*	KU059145
78776	2006	Port Stephens	*Aedes vigilax*	KU059149
78202	2006	Lake Macquarie	*Aedes procax*	KU059142
77883	2006	Lake Macquarie	*Aedes procax*	KU059140
78224	2006	Lake Macquarie	*Aedes vigilax*	KU059143
78240	2006	Lake Macquarie	*Aedes vigilax*	KU059144
81272	2006	Lake Macquarie	*Aedes aculeatus*	KU059150
79463	2006	Lake Macquarie	*Aedes alternans*	KU059151
77970	2006	Port Stephens	*Aedes vigilax*	KU059141
160783	2010	Byron Bay	*Aedes notoscriptus*	KU059112
160375	2010	Byron Bay	*Aedes notoscriptus*	KU059110
162702	2010	Byron Bay	*Aedes notoscriptus*	KU059120
161325	2010	Port Stephens	*Aedes vigilax*	KU059114
161383	2010	Port Stephens	*Aedes vigilax*	KU059115
161114	2010	Port Stephens	*Aedes vigilax*	KU059113
162539	2010	Georges River	*Aedes vigilax*	KU059119
160641	2010	Georges River	*Aedes vigilax*	KU059111
161640	2010	Lake Macquarie	*Aedes vigilax*	KU059118
161572	2010	Batemans Bay	*Aedes vigilax*	KU059116
161575	2010	Batemans Bay	*Aedes vigilax*	KU059117
178964	2013	Central Coast	*Aedes procax*	KU059127
178003	2013	Central Coast	*Aedes vigilax*	KU059124
177701	2013	Blacktown	*Aedes procax*	KU059123
177238	2013	Blacktown	*Aedes procax*	KU059121
177296	2013	Blacktown	*Aedes procax*	KU059122
179388	2013	Penrith	*Aedes procax*	KU059131
178816	2013	Byron Bay	*Aedes procax*	KU059126
178803	2013	Byron Bay	*Aedes procax*	KU059125
179211	2013	Lake Macquarie	*Aedes procax*	KU059129
179071	2013	Lake Macquarie	*Aedes procax*	KU059128
179347	2013	Sydney Olympic Park	*Aedes procax*	KU059130

doi:10.1371/journal.pone.0173105.t002
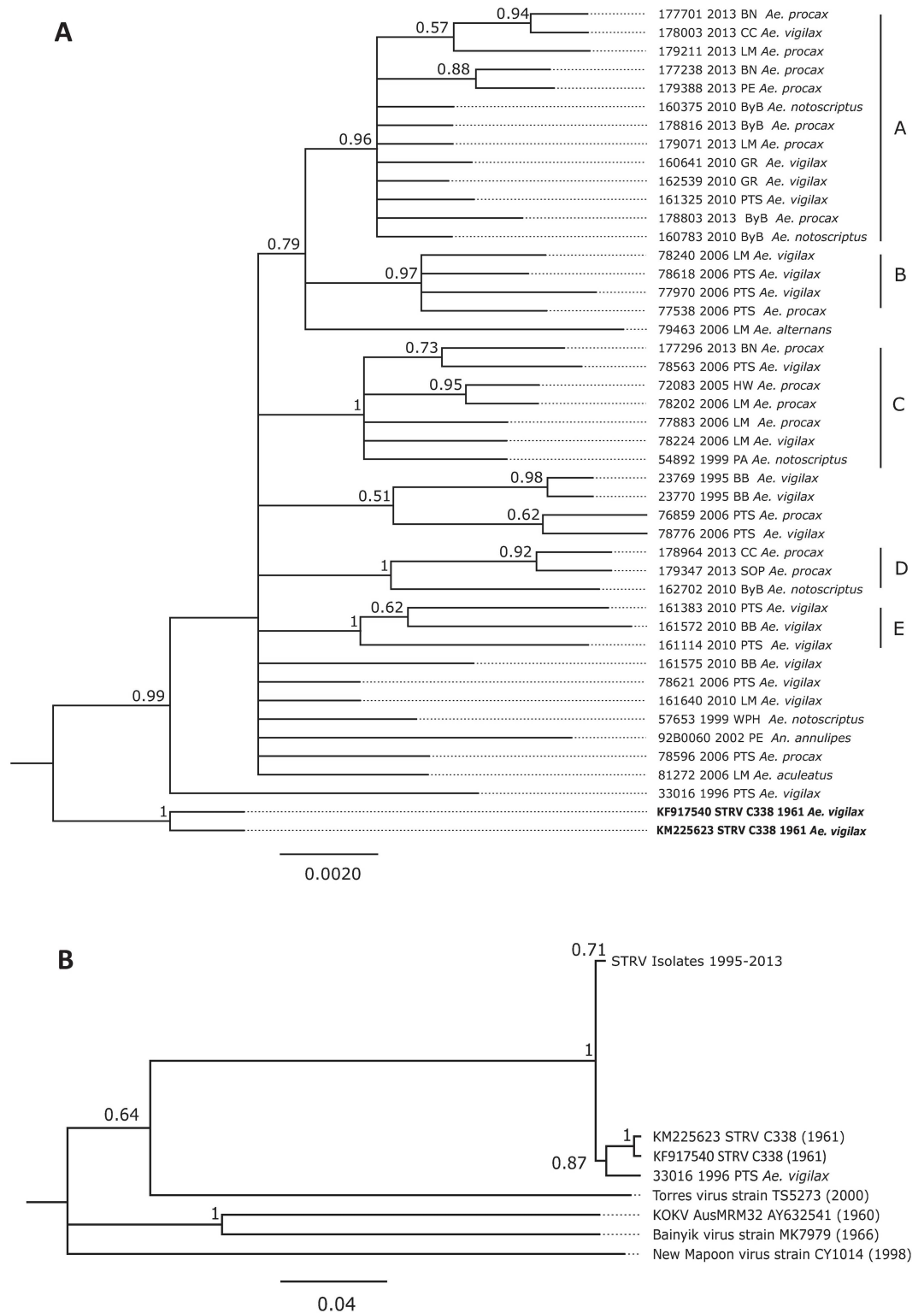
Fig 1. Phylogenetic association of STRV isolates (n = 43) and STRV C388 GenBank prototypes (A) and the association between STRV isolates, TS5273, KOKV AusMRM32, Banyik MK7979 and the New Mapoon CY1014 virus strain (B). The trees are based on the nucleic acid partial sequence of the NS5 gene region (556 bp). The posterior probability values are shown at the nodes with the branch lengths measured in the number of substitutions per site indicated by the scale bar. Abbreviations used: BN = Blacktown; CC = Central Coast;

LM = Lake Macquarie; ByB = Byron Bay; PE = Penrith; PTS = Port Stephens; GR = Georges River; BB = Batemans Bay; SOP = Sydney Olympic Park; HW = Hawkesbury; PA = Parramatta; WPH = West Pennant Hills.

collected mosquitoes is not commonly reported. Isolations of STRV in this study were mainly from the *Aedes* species of mosquito. Similarly, Johansen and co-workers [38] detected a total of eight STRV isolates from *Aedes camptorhynchus* (Thomson) and *Aedes ratcliffei* (Marks) during arbovirus surveillance in 2002–2003 in Western Australia. In 2014, a surveillance program in South Australia detected only three isolates of STRV from one mosquito species, *Ae. notoscriptus* [39].

Sequence analysis of the 556 bp region encoding part of the methyltransferase and the start of the region encoding RNA-dependent-RNA polymerase in the NS5 gene sequence showed a sequence homology of 95.9%–98.4% between STRV isolates and STRV C338. In comparison, studies on isolates collected in Western Australia, and on isolates collected from NSW in 1995 (23759) and 1981(CS946) showed 97%–99% [38] and >97% [40] sequence homology, respectively.

Clustering of STRV isolates from 2010 and 2013 (Clusters A,D,E) was apparent, with collection sites spread over a wide area from the far north coast (Byron Bay), the mid-north coast and Hunter region (Lake Macquarie, Port Stephens and Central Coast) to the Sydney metropolitan area (Georges River and Blacktown). Other than Cluster B where three of the four isolates were collected within the same year (2006) from Port Stephens, there was no relationship between the geographic location and the temporal clustering of STRV sequences. This is unlike KOKV where three topotypes with both temporal and geographic clustering was established [41].

An overall divergence of 2% was shown between the STRV isolates and the C388 strain. This indicates a slow evolutionary change within the 43 STRV isolates collected over a period of 18 years in NSW from the first isolation of the virus in Cairns, Queensland, from *Ae. vigilax* in 1961[10]. However, the divergence shown between the STRV test isolates was higher at 4% with the greatest differences shown between the 1996 isolate and 2005, 2010 and 2013 isolates with distances of 13.00, 12.82 and 12.55, respectively. Isolate 33016, cultured from *Ae. vigilax* in 1996 from Port Stephens presented the least nucleotide similarity (96%). Consequently, eight of the STRV isolations from Port Stephens that were detected 10 years later (2006) showed sequence similarity in three isolates only (Cluster B). However, translation of the nucleic acids into proteins showed virtually no difference suggesting that there was no character change in phenotype.

The percentage identity shown between the STRV isolates, TS5273, and KOKV AusMRM32 was comparable to Nisbet et al. [31]. They found the nucleotide sequence of STRV to be closely related to TS5273 that was first isolated in 2000 from the Saibai Island and KOKVAusMRM32 with 74%–80% and 75% –76% nucleotide similarity, respectively. Subsequently, a species level cut-off of between 85% and 90% nucleotide identity has been proposed for species within the KOKV group [40]. Hence, this re-classifies STRV as a separate species. Overall, reports show little genetic change over a period of 34 years in the NS5/3' UTR region from the original C338 STRV isolated in Cairns in 1961 [40]. This similarity, often attributable to a common ancestor suggests that there is likely to be a single population of virus that may circulate within the study region, and across Australia, with annual activity driven by as yet undetermined environmental, entomological, or zoonotic factors. Moreover, although the variability between isolates was small in this study, genetic variability has the potential to drive activity. Furthermore, the NS5/3' UTR region is more conserved and therefore is likely to underestimate the variability in other regions.
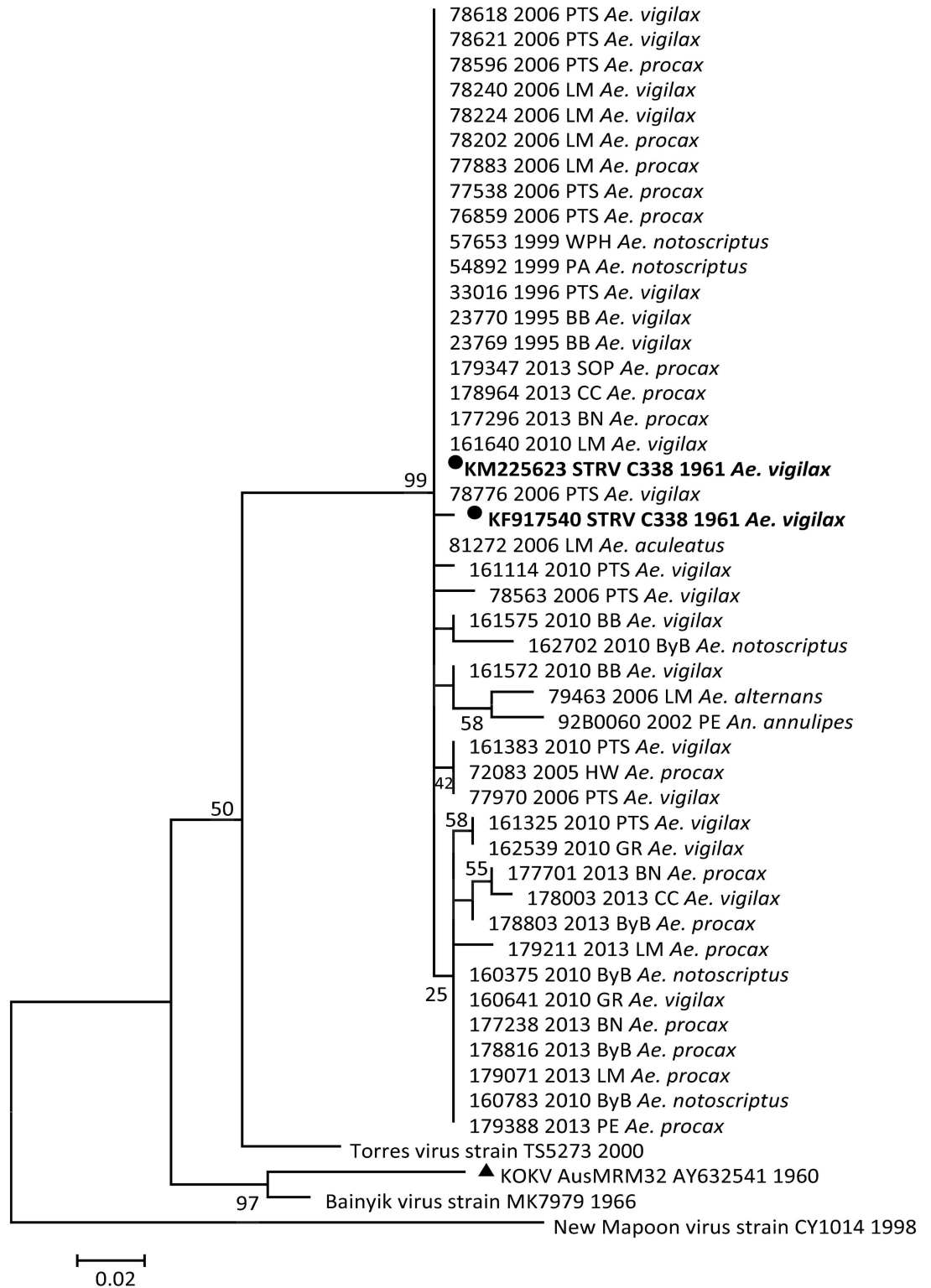
Fig 2. Phylogenetic association of deduced amino acids of the partial sequence of the NS5 gene region of STRV isolates (n = 43), STRV C338 ●, TS5273, KOKV AusMRM32 ▲, Bainyik MK7979 and New Mapoon CY1014 virus strain. The percentage association is shown above the branches with branch lengths measured in the number of substitutions per site indicated by the scale bar.

The results of this investigation again identify mosquito species associated with coastal wetlands as playing a potentially important role in the transmission of mosquito-borne arboviruses [4]. The importance of STRV to public health has not been fully defined, yet serum antibody in Kokobera-like human infections have been recorded [11, 12], and the virus has been regularly isolated from mosquito species known to bite humans found in close association with urban habitats. The impact of STRV is not unlike Zika virus from its first identification in 1947 where initially, very little clinical importance was placed on the virus until the first recognized outbreak in 2007 from the island of Yap [42, 43], followed by the 2013–2014 and the 2015 outbreak in Brazil [44]. STRV was detected in only six mosquito species and little is known about its capacity to infect different mosquito species, or if it has any vector preferences. The ecology of the *Aedes* mosquitoes predisposes them to transmitting STRV to humans. The identification of reservoir hosts via blood meals has provided evidence that *Ae. notoscriptus* and *Ae. vigilax* occasionally feed on birds, although feeding on mammals is preferred [14, 45].

Notably, there were no isolates from *Culex annulirostris* Skuse during the study. This mosquito associated with freshwater habitats is considered the most important vector of endemic flaviviruses [4] and is generally thought to feed commonly on avian hosts [46]. The lack of STRV isolates from this mosquito species could be in part attributed to vector competence phenotype that is influenced by genetic factors and the environment [47]. Although the abundance of this mosquito is generally greater across inland regions, *Cx. annulirostris* was still common in coastal regions of NSW during the study period when favourable environmental conditions occurred, particularly during periods of above average rainfall and also in locations where STRV was isolated from other mosquito species [13].

Identifying host-seeking preferences of mosquitoes can potentially yield insights into important reservoir hosts of arboviruses. The mosquitoes from which STRV was isolated in this study have been identified as feeding on a wide range of mammal and avian hosts in urban environments [46, 48]. However, this investigation identifies the *Aedes* species of mosquitoes as being the main vector of Stratford virus with the first detection of the STRV prototype C338 in *Ae. vigilax* in 1961. Molecular studies have shown that there may be genetic differences between populations of *Ae. vigilax*, *Ae.notoscriptus* and *Cx. annulirostris* from different and potentially overlapping regions in Australia [49–52]. These genetic differences have been proposed as explanations of variable intra-specific vector competencies [50, 53] but may also influence other drivers in vector-pathogen-host transmission cycles such as host-seeking behaviour.

More research is required to determine if STRV exists in small cryptic foci across NSW or is introduced from elsewhere in the country by the movement of insects or vertebrate hosts. The genetic homology of STRV isolates suggests regular movement into coastal regions rather than isolated clusters of local activity. Notwithstanding the hosts, routes and potential environmental drivers of movement, drivers of local mosquito population abundance probably also play an important role in the activity of STRV. Population abundance of *Ae. vigilax* is dependent on the environmental drivers of temperature, rainfall and tides between seasons and regions across Australia [54–58] and is therefore difficult to predict. However, this mosquito is common during warmer months in many coastal regions with peak numbers differing between seasons. The environmental conditions for *Ae. procax* differ in that the availability of suitable habitats and seasonal distribution of rainfall primarily drives abundance. Consequently, numbers of this mosquito will vary between seasons, whereas the abundance of *Ae. notoscriptus* being primarily associated with urban habitats is generally consistent between seasons. Overall, while local environmental conditions may play a role in the activity of the identified vectors of STRV, there is currently no evidence that these conditions drive activity of the virus.

In the case of mosquito-borne viruses such as STRV that may not currently be associated with a significant public or veterinary health risk, understanding their genetic variability can

provide insights into their vector and host associations, particularly as small genetic changes in the arboviruses can result in significantly enhanced virulence [59]. Future genetic studies should encompass a larger and less conserved region of the STRV virus genome such as the pre-membrane (prM) and Envelope (Env) genes that could be more informative in the study of evolutionary change and more meaningful in human infection. Viruses that are well adapted to a particular set of environmental conditions can perpetuate genetic qualities that reduce the genetic diversity within a population. Also, similarity between isolates can be attributed to constraints of the virus cycling between the mosquito vector and the natural host population [60–62]. In the absence of localized serological surveys of wildlife, it is difficult to identify the reservoir hosts of critical importance. The feeding habits of the *Aedes* species of mosquitoes suggests numerous host possibilities, from that of a mobile host, perhaps a bird or bat and mammals such as macropods may play a role in the dispersal of the virus into coastal regions of NSW.

Stratford virus activity was limited to the coastal regions of NSW and was primary vectored by the *Aedes* mosquito. The limited activity of STRV from inland regions of NSW and the absence of detection in the known flaviviruses vector *Cx. annulirostris* suggests that the virus may be generally limited in its distribution from coastal regions, differing to MVEV and WNV_{KUN} which are generally not associated with coastal regions [5, 63]. More research in tracking STRV is required to understand the evolution and host cycling of this endemic Australian arbovirus.

Acknowledgments

Author Contributions

Conceptualization: CEW CST.

Data curation: CST CEW.

Formal analysis: CST.

Funding acquisition: CEW.

Investigation: CST CEW JH JC SLD.

Methodology: CST JH JC CEW SLD.

Project administration: CST CEW.

Resources: CST.

Software: CST.

Supervision: CST CEW.

Validation: CST CEW.

Visualization: CST CEW SLD.

Writing – original draft: CST CEW SLD.

Writing – review & editing: CST.

References

1. Jacups SP, Whelan PI, Harley D. Arbovirus models to provide practical management tools for mosquito control and disease prevention in the Northern Territory, Australia. J Med Entomol. 2011; 48(2):453–60. Epub 2011/04/13. PMID: 21485389

2. Russell RC. Arboviruses and their vectors in Australia: an update on the ecology and epidemiology of some mosquito-borne arboviruses. Rev Med Vet Entomol. 1995; 83:141–58.

3. Mackenzie JS, Lindsay MD, Coelen RJ, Broom AK, Hall RA, Smith DW. Arboviruses causing human disease in the Australasian zoogeographic region. Arch Virol. 1994; 136:447–67. PMID: 8031248

4. Russell RC, Kay BH. Medical Entomology: changes in the spectrum of mosquito-borne disease in Australia and other vector threats and risks, 1972–2004 Aust Entomol. 2004; 43:271–82.

5. Roche SE, Wicks R, Garner MG, East IJ, Paskin R, Moloney BJ, et al. Descriptive overview of the 2011 epidemic of arboviral disease in horses in Australia. Aust Vet J. 2013; 91(1–2):5–13. Epub 2013/01/30. doi: 10.1111/avj.12018 PMID: 23356366

6. van den Hurk AF, Smith CS, Field HE, Smith IL, Northill JA, Taylor CT, et al. Transmission of Japanese Encephalitis virus from the black flying fox, *Pteropus alecto*, to *Culex annulirostris* mosquitoes, despite the absence of detectable viremia. Am J Trop Med Hyg. 2009; 81(3):457–62. Epub 2009/08/27. PMID: 19706915

7. Russell RC. Mosquito-borne disease and climate change in Australia: time for a reality check Aust Entomol. 2009; 48:1–7.

8. Harley D, Sleigh A, Ritchie S. Ross River virus transmission, infection, and disease: a cross-disciplinary review. Clin Microbiol Rev. 2001; 14(4):909–32, table of contents. Epub 2001/10/05. doi: 10.1128/CMR.14.4.909-932.2001 PMID: 11585790

9. Warrilow D, Hall-Mendelin S, Hobson-Peters J, Prow NA, Allcock R, Hall RA. Complete coding sequences of three members of the kokobera group of flaviviruses. Genome Announc. 2014; 2(5). Epub 2014/09/23.

10. Doherty RL, Carley JG, Mackerras MJ, Marks EN. Studies of arthropod-borne virus infections in Queensland. III. Isolation and characterization of virus strains from wild-caught mosquitoes in North Queensland. Aust J Exp Biol Med Sci. 1963; 41:17–39. Epub 1963/02/01. PMID: 14028387

11. Boughton CR, Hawkes RA, Naim HM. Illness caused by a Kokobera-like virus in south-eastern Australia. Med J Aust. 1986; 145(2):90–2. Epub 1986/07/21. PMID: 3016489

12. Hawkes RA, Boughton CR, Naim HM, Wild J, Chapman B. Arbovirus infections of humans in New South Wales. Seroepidemiology of the flavivirus group of togaviruses. Med J Aust. 1985; 143(12–13):555–61. Epub 1985/12/09. PMID: 3007952

13. Doggett SL, Clancy J, Haniotis J, Webb CE, Hueston L, Marchetti M, et al. Arbovirus and vector surveillance in New South Wales, 2004/5-2007/8. Arbovirus Res Aust. 2009; 10:28–37.

14. Jansen CC, Webb CE, Graham GC, Craig SB, Zborowski P, Ritchie SA, et al. Blood sources of mosquitoes collected from urban and peri-urban environments in eastern Australia with species-specific molecular analysis of avian blood meals. Am J Trop Med Hyg. 2009; 81(5):849–57. Epub 2009/10/29. doi: 10.4269/ajtmh.2009.09-0008 PMID: 19861621

15. Ryan PA, Kay BH. Emergence trapping of mosquitoes (Diptera: Culicidae) in brackish forest habitats in Maroochy Shire, south-east Queensland, Australia, and a management option for *Verrallina funerea* (Theobald) and *Aedes procax* (Skuse). Aust Entomol. 2000; 39:212–8.

16. Boyd AM, Kay BH. Experimental infection and transmission of Barmah Forest virus by *Aedes vigilax* (Diptera: Culicidae). J Med Entomol. 1999; 36(2):186–9. Epub 1999/03/20. PMID: 10083756

17. Ryan PA, Kay BH. Vector competence of mosquitoes (Diptera: Culicidae) from Maroochy Shire, Australia, for Barmah Forest virus. J Med Entomol. 1999; 36(6):856–60. Epub 1999/12/11. PMID: 10593091

18. Kay BH, Watson TM, Ryan PA. Definition of productive Aedes notoscriptus (Diptera: Culicidae) habitats in western Brisbane, and a strategy for their control Aust J Entomol. 2008; 47:142–8.

19. Doggett SL, Russell RC. *Aedes notoscriptus* can transmit inland and coastal isolates of Ross River and Barmah Forest viruses from New South Wales! Arbovirus Res Aust. 1997; 7:79–81.

20. Watson TM, Kay BH. Vector competence of *Aedes notoscriptus* (Diptera: Culicidae) for Ross River virus in Queensland, Australia. J Med Entomol. 1998; 35(2):104–6. Epub 1998/04/16. PMID: 9538569

21. Watson TM, Kay BH. Vector competence of *Aedes notoscriptus* (Diptera: Culicidae) for Barmah Forest virus and of this species and *Aedes aegypti* (Diptera: Culicidae) for dengue 1–4 viruses in Queensland, Australia. J Med Entomol. 1999; 36(4):508–14. Epub 1999/09/01. PMID: 10467781

22. Watson TM, Saul A, Kay BH. *Aedes notoscriptus* (Diptera: Culicidae) survival and dispersal estimated by mark-release-recapture in Brisbane, Queensland, Australia. J Med Entomol. 2000; 37(3):380–4. Epub 2004/11/13. PMID: 15535581

23. Lindsay MD, Coelen RJ, Mackenzie JS. Genetic heterogeneity among isolates of Ross River virus from different geographical regions. J Virol. 1993; 67(6):3576–85. PMID: 8497065

24. Potter A, Johansen CA, Fenwick S, Reid SA, Lindsay MD. The seroprevalence and factors associated with Ross river virus infection in western grey kangaroos (*Macropus fuliginosus*) in Western Australia. Vector Borne Zoonotic Dis. 2014; 14(10):740–5. Epub 2014/10/18. doi: 10.1089/vbz.2014.1617 PMID: 25325318

25. Sammels LM, Lindsay MD, Poidinger M, Coelen RJ, Mackenzie JS. Geographic distribution and evolution of Sindbis virus in Australia. J Gen Virol. 1999; 80 (Pt 3):739–48. Epub 1999/03/26.

26. Poidinger M, Roy S, Hall RA, Turley PJ, Scherret JH, Lindsay MD, et al. Genetic stability among temporally and geographically diverse isolates of Barmah Forest virus. Am J Trop Med Hyg. 1997; 57(2):230–4. Epub 1997/08/01. PMID: 9288821

27. Ryan PA, Martin L, Mackenzie JS, Kay BH. Investigation of Gray-Headed Flying Foxes (*Pteropus poliocephalus*) (Megachiroptera: Pteropodidae) and Mosquitoes in the Ecology of Ross River Virus in Australia. Am J Trop Med Hyg. 1997; 57(4):476–82. PMID: 9347967

28. Rohe DL, Fall RP. A miniature battery powered CO2 baited light trap for mosquito borne encephalitis surveillance. B Soc Vector Ecol. 1979; 4:24–7.

29. Russell RC. Mosquitoes and mosquito-borne disease in South Eastern Australia. Sydney: University of Sydney Printing Service, NSW; 1993. 310 p.

30. Broom AK, Hall RA, Johansen CA, Oliveira N, Howard MA, Lindsay MD, et al. Identification of Australian arboviruses in inoculated cell cultures using monoclonal antibodies in ELISA. Pathology. 1998; 30 (3):286–8. Epub 1998/10/14. PMID: 9770194

31. Nisbet DJ, Lee KJ, van den Hurk AF, Johansen CA, Kuno G, Chang GJ, et al. Identification of new flaviviruses in the Kokobera virus complex. J Gen Virol. 2005; 86(Pt 1):121–4. doi: 10.1099/vir.0.80381-0 PMID: 15604438

32. Maher-Sturgess SL, Forrester NL, Wayper PJ, Gould EA, Hall RA, Barnard RT, et al. Universal primers that amplify RNA from all three flavivirus subgroups. Virol J. 2008; 5:16. Epub 2008/01/26. doi: 10.1186/1743-422X-5-16 PMID: 18218114

33. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012; 9(8):772. Epub 2012/08/01.

34. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28(10):2731–9. Epub 2011/05/07. doi: 10.1093/molbev/msr121 PMID: 21546353

35. Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. Bayesian inference of phylogeny and its impact on evolutionary biology. Science. 2001; 294(5550):2310–4. doi: 10.1126/science.1065889 PMID: 11743192

36. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences: CABIOS. 1992; 8(3):275–82. PMID: 1633570

37. Map UTN. USGS National Map Viewer. http://viewer.nationalmap.gov/viewer/ 2016 [cited 4 January 2017]. http://viewer.nationalmap.gov/viewer/.

38. Johansen C, Maley F, Broom AK. Isolation of Stratford virus from mosquitoes collected in the southwest of Western Australia. Arbovirus Research in Australia. 2005; 9:164–6.

39. Flies EJ, Toi C, Weinstein P, Doggett SL, Williams CR. Converting mosquito surveillance to arbovirus surveillance with honey-baited nucleic acid preservation cards. Vector Borne Zoonotic Dis. 2015; 15 (7):397–403. doi: 10.1089/vbz.2014.1759 PMID: 26186511

40. May FJ, Clark DC, Kim P, Diviney SM, Williams DT, Field EJ, et al. Genetic divergence among members of the Kokobera group of flaviviruses supports their separation into distinct species. Journal of General Virology. 2013; 94(7):1462–7.

41. Poidinger M, Hall RA, Lindsay MD, Broom AK, Mackenzie JS. The molecular epidemiology of Kokobera virus. Virus Res. 2000; 68(1):7–13. PMID: 10930658

42. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. The New England journal of medicine. 2009; 360(24):2536–43. doi: 10.1056/NEJMoa0805715 PMID: 19516034

43. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008; 14(8):1232–9. doi: 10.3201/eid1408.080287 PMID: 18680646

44. Imperato PJ. The Convergence of a Virus, Mosquitoes, and Human Travel in Globalizing the Zika Epidemic. Journal of community health. 2016; 41(3):674–9. doi: 10.1007/s10900-016-0177-7 PMID: 26969497

45. Kent RJ, Norris DE. Identification of mammalian blood meals in mosquitoes by a multiplexed polymerase chain reaction targeting cytochome B. The American journal of tropical medicine and hygiene. 2005; 73(2):336–42. PMID: 16103600

46. Jansen CC, Prow NA, Webb CE, Hall RA, Pyke AT, Harrower BJ, et al. Arboviruses isolated from mosquitoes collected from urban and peri-urban areas of eastern Australia. J Am Mosq Control Assoc. 2009; 25(3):272–8. Epub 2009/10/27. doi: 10.2987/09-5908.1 PMID: 19852216

47. Tabachnick WJ. Nature, nurture and evolution of intra-species variation in mosquito arbovirus transmission competence. Int J Environ Res Public Health. 2013; 10(1):249–77. Epub 2013/01/25. doi: 10.3390/ijerph10010249 PMID: 23343982

48. Kay BH, Boyd AM, Ryan PA, Hall RA. Mosquito feeding patterns and natural infection of vertebrates with Ross River and Barmah Forest viruses in Brisbane, Australia. Am J Trop Med Hyg. 2007; 76 (3):417–23. Epub 2007/03/16. PMID: 17360861

49. Puslednik L, Russell RC, Ballard JWO. Phylogeography of the medically important mosquito *Aedes* (*Ochlerotatus*) *vigilax* (Dipter: Culicidae) in Australasia. J Biogeogr. 2012; 39:1333–46.

50. Endersby NM, White VL, Chan J, Hurst T, Rasic G, Miller A, et al. Evidence of cryptic genetic lineages within *Aedes notoscriptus* (Skuse). Infect Genet Evol. 2013; 18:191–201. Epub 2013/05/18. doi: 10.1016/j.meegid.2013.04.035 PMID: 23681021

51. Hardy CM, Court LN, Morgan MJ, Webb CE. The complete mitochondrial DNA genomes for two lineages of *Aedes notoscriptus* (Diptera: Culicidae). Mitochondrial DNA. 2014:1–2. Epub 2014/10/29.

52. Hardy CM, Court LN, Morgan MJ. The complete mitochondrial DNA genome of *Aedes vigilax* (Diptera: Culicidae). Mitochondrial DNA. 2015:1–2. Epub 2015/06/24.

53. Hemmerter S, Slapeta J, Beebe NW. Resolving genetic diversity in Australasian Culex mosquitoes: incongruence between the mitochondrial cytochrome c oxidase I and nuclear acetylcholine esterase 2. Mol Phylogenet Evol. 2009; 50(2):317–25. Epub 2008/12/09. doi: 10.1016/j.ympev.2008.11.016 PMID: 19059488

54. Webb CE, Russell RC. Towards management of mosquitoes at Homebush Bay, Sydney, Australia. I. Seasonal activity and relative abundance of adults of *Aedes vigilax*, *Culex sitiens*, and other salt-marsh species, 1993–94 through 1997–98. J Am Mosq Control Assoc. 1999; 15(2):242–9. Epub 1999/07/21. PMID: 10412120

55. Jacups SP, Whelan PI, Markey PG, Cleland SJ, Williamson GJ, Currie BJ. Predictive indicators for Ross River virus infection in the Darwin area of tropical northern Australia, using long-term mosquito trapping data. Trop Med Int Health. 2008; 13(7):943–52. Epub 2008/05/17. doi: 10.1111/j.1365-3156.2008.02095.x PMID: 18482196

56. Yang GJ, Brook BW, Whelan PI, Cleland S, Bradshaw CJ. Endogenous and exogenous factors controlling temporal abundance patterns of tropical mosquitoes. Ecol Appl. 2008; 18(8):2028–40. Epub 2009/03/07. PMID: 19263895

57. Jacups SP, Kurucz N, Whelan PI, Carter JM. A comparison of *Aedes vigilax* larval population densities and associated vegetation categories in a coastal wetland, Northern Territory, Australia. J Vector Ecol. 2009; 34(2):311–6. Epub 2010/09/15. doi: 10.1111/j.1948-7134.2009.00039.x PMID: 20836834

58. Hu W, Mengersen K, Dale P, Tong S. Difference in mosquito species (Diptera: Culicidae) and the transmission of Ross River virus between coastline and inland areas in Brisbane, Australia. Environ Entomol. 2010; 39(1):88–97. Epub 2010/02/12. doi: 10.1603/EN07037 PMID: 20146843

59. Stapleford KA, Moratorio G, Henningsson R, Chen R, Matheus S, Enfissi A, et al. Whole-Genome Sequencing Analysis from the Chikungunya Virus Caribbean Outbreak Reveals Novel Evolutionary Genomic Elements. PLoS neglected tropical diseases. 2016; 10(1):e0004402. doi: 10.1371/journal.pntd.0004402 PMID: 26807575

60. Coffey LL, Vasilakis N, Brault AC, Powers AM, Tripet F, Weaver SC. Arbovirus evolution in vivo is constrained by host alternation. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105(19):6970–5. doi: 10.1073/pnas.0712130105 PMID: 18458341

61. Weaver SC, Barrett ADT. Transmission cycles, host range, evolution and emergence of arboviral disease. Nat Rev Micro. 2004; 2(10):789–801.

62. Ciota AT, Kramer LD. Insights into Arbovirus Evolution and Adaptation from Experimental Studies. Viruses. 2010; 2(12):2594–617. doi: 10.3390/v2122594 PMID: 21994633

63. Knox J, Cowan RU, Doyle JS, Ligtermoet MK, Archer JS, Burrow JN, et al. Murray Valley encephalitis: a review of clinical features, diagnosis and treatment. Med J Aust. 2012; 196(5):322–6. Epub 2012/03/22. PMID: 22432670