Virology 448 (2014) 146–158

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Enhanced arbovirus surveillance with deep sequencing: Identification of novel rhabdoviruses and bunyaviruses in Australian mosquitoes

Lark L. Coffey [a,b], Brady L. Page [a,b], Alexander L. Greninger [b], Belinda L. Herring [c], Richard C. Russell [d,e], Stephen L. Doggett [e], John Haniotis [e], Chunlin Wang [f], Xutao Deng [a,b], Eric L. Delwart [a,b,*]

[a] Blood Systems Research Institute, University of California, San Francisco, USA
[b] Department of Laboratory Medicine, University of California, 270 Masonic Avenue, San Francisco, CA 94118, USA
[c] Griffith University, Gold Coast Campus, Queensland, Australia
[d] Sydney Medical School, University of Sydney, Australia
[e] Centre for Infectious Diseases and Microbiology, Westmead Hospital, New South Wales, Australia
[f] Genome Technology Center, Stanford University School of Medicine, Stanford, CA, USA

ARTICLE INFO

Article history:
Received 20 August 2013
Returned to author for revisions
6 September 2013
Accepted 28 September 2013
Available online 25 October 2013

Keywords:
Virus discovery
Deep sequencing
Arbovirus surveillance
Bunyavirus
Rhabdovirus
Reovirus
Novel virus
Mosquito
Australia

ABSTRACT

Viral metagenomics characterizes known and identifies unknown viruses based on sequence similarities to any previously sequenced viral genomes. A metagenomics approach was used to identify virus sequences in Australian mosquitoes causing cytopathic effects in inoculated mammalian cell cultures. Sequence comparisons revealed strains of Liao Ning virus (*Reovirus*, *Seadornavirus*), previously detected only in China, livestock-infecting Stretch Lagoon virus (*Reovirus*, *Orbivirus*), two novel dimarhabdoviruses, named Beaumont and North Creek viruses, and two novel orthobunyaviruses, named Murrumbidgee and Salt Ash viruses. The novel virus proteomes diverged by $\geq$50% relative to their closest previously genetically characterized viral relatives. Deep sequencing also generated genomes of Warrego and Wallal viruses, orbiviruses linked to kangaroo blindness, whose genomes had not been fully characterized. This study highlights viral metagenomics in concert with traditional arbovirus surveillance to characterize known and new arboviruses in field-collected mosquitoes. Follow-up epidemiological studies are required to determine whether the novel viruses infect humans.

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Background

Numerous arboviruses that cause significant human disease are endemic to Australia. To address the risks posed by arboviruses to human and veterinary health in Australia, annual arbovirus surveillance is conducted by a network of regional diagnostic laboratories that trap mosquitoes and other insects and test them for known viruses. Viruses tested include members of the *Flaviviridae* and *Togaviridae* families-Barmah Forest virus (BFV), Edge Hill virus (EHV), Kunjin virus (KUNV), Kokobera virus (KOKV), Murray Valley encephalitis virus (MVEV), Ross River virus (RRV), Sindbis virus (SINV), and Stratford virus (STRV)-detected using antigenic tests on inoculated mosquito and vertebrate cell monocultures showing cytopathic effects (CPE) (Knope et al., 2013). Many of these

Abbreviations: ABLV, Australian bat lyssavirus; AHSV, African horse sickness virus; AINOV, Aino virus; AKAV, Akabane virus; ALFV, Alfuy virus; ALMV, Almpiwar virus; BANV, Banna virus; BASV, Bas-Congo virus; BEAUV, Beaumont virus; BEFV, bovine ephemeral fever virus; BFV, Barmah Forest virus; BYSM, barley yellow striate mosaic; CCHFV, Crimean-Congo hemorrhagic fever virus; CHIKV, chikungunya virus; CHVV, Charleville virus; CPV, Coastal Plains virus; DAff sigma virus, Drosophila affinis sigma virus; DENV, dengue virus; DMel sigma virus, Drosophila melanogaster sigma virus; DObs sigma virus, Drosophila obscura sigma virus; DOUV, Douglas virus; EBLV, European bat lyssavirus; EHDV, epizootic hemorrhagic disease virus; EHV, Edge Hill virus; EUBV, Eubenangee virus; FPV, Facey's Paddock virus; GGV, Gan Gan virus; HDOOV, Humpty Doo virus; IHNV, infectious hematopoietic necrosis virus; JEV, Japanese encephalitis virus; KDV, Kadiporo virus; KOKV, Kokobera virus; KUNV, Kunjin virus; LEAV, Leanyer virus; LNV, Liao Ning virus; LYMoV, lettuce yellow mottle virus; LNYV, lettuce necrotic yellows virus; MAPV, Mapputta virus; MFSV, maize fine streak virus; MURBV, Murrumbidgee virus; MVEV, Murray Valley encephalitis virus; NCMV, Northern cereal mosaic virus; NORCV, North Creek virus; PEAV, Peaton virus; PHSV, Peruvian horse sickness virus; RRV, Ross River virus; RVFV, Rift Valley fever virus; SASHV, Salt Ash virus; SFTSV, severe fever with thrombocytopenia syndrome virus; SINV, Sindbis virus; SLOV, Stretch Lagoon orbivirus; STRV, Stratford virus; SVCV, spring viremia of carp virus; TIBV, Tibrogargan virus; TILV, Tilligerry virus; TINV, Tinaroo virus; THIV, Thimiri virus; TRUV, Trubanaman virus; VHSV, viral hemorrhagic septicemia virus; VSV, vesicular stomatitis virus; WALV, Wallal virus; WARV, Warrego virus; WNV, West Nile virus

* Corresponding author at: Department of Laboratory Medicine, University of California, 270 Masonic Avenue, San Francisco, CA 94118, USA. Fax: +1 419 791 4220.
E-mail address: delwarte@medicine.ucsf.edu (E.L. Delwart).

0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.09.026

arboviruses cause human disease, ranging from mild febrile illness to encephalitis and death. Identifying these circulating viruses, as well as other novel viruses, in anthropophilic mosquitoes, is therefore important to implement strategies to detect and mitigate arbovirus transmission to humans and other animals. However, despite extensive diagnostic testing, some CPE-causing viral isolates cannot be identified using current assays specific for known arboviruses.

An increasing number of viruses are being discovered in arthropods (reviewed in (Junglen and Drosten 2013)). Viral metagenomics, so-called 'deep sequencing', has been used in mosquitoes to survey viral diversity (Ng et al., 2011) and to sequence arboviruses previously broadly classified using antigenic cross-reactivity (Bishop-Lilly et al., 2010; Chowdhary et al., 2012; Hall-Mendelin et al., 2013; Quan et al., 2010; Swei et al., 2013; Vasilakis et al., 2013). However, its use for identifying viruses in surveillance settings has so far been limited to detection of an insect-restricted densovirus in Chinese mosquitoes (Ma et al., 2011).

In this study, deep sequencing was used to non-specifically amplify and sequence enriched viral nucleic acids from CPE-positive supernatants in which arboviruses were not identified by conventional antigenic tests. Viral sequences were identified by similarities of their *in silico* translated protein sequences with all previously sequenced viral proteins. We detected sequences of known reoviruses including Liao Ning virus (LNV), Stretch Lagoon orbivirus (SLOV), Wallal virus (WALV), and Warrego virus (WARV), as well as two novel rhabdoviruses, and two novel bunyaviruses. RRV, EHV and KOKV sequences were also identified in some pools. Detection of novel and known arboviruses not previously recognized in Australia highlights the use of complementing conventional arbovirus surveillance with viral metagenomics approaches.

Results

Detection of virus genomes and sequence determinations

Virus genomes were detected by deep sequencing in 21 of the 47 culture supernatants from insect pools (Table 1, Table S1, only pools with identified virus genomes are listed; raw sequence reads are available upon request). Pools contained from 0 to 3 viruses. At least 17 pools contained sequences that matched with $\geq 90\%$ nucleotide identity to known arboviruses in GenBank. These viruses included EHV, KOKV, LNV, RRV, SLOV, WALV and WARV. Since these viral sequences were highly similar to published sequences, we consider that they represent strains of known viruses. Since only partial genome data was available in GenBank for WALV and WARV, we sequenced the genomes from 1 pool each for both viruses.

Five pools contained sequences with $\leq 50\%$ amino acid (aa) identity to different rhabdovirus or bunyavirus species in GenBank. Two of these pools (932 and 934) contained sequences that were $\geq 99\%$ identical, reflecting variants of the same viral species. Sequences from the other 3 pools were dissimilar. Complete genomes of 2 novel bunyaviruses were obtained. Partial genome sequences comprising 75% of the L gene for one virus and 90% of the complete genome of the other virus for 2 novel rhabdoviruses were obtained.

Novel rhabdoviruses

Two mosquito pools, 6 and 954, contained genomes consistent with rhabdovirus genome organization (Kuzmin et al., 2009). Sample 6 (GenBank accession number KF310911) was isolated from *Anopheles annulipes* in Griffith, NSW near Beaumont; we propose the name 'Beaumont virus' (BEAUV) for this rhabdovirus. Sample 954 (GenBank accession numbers KF360970-3) was isolated from *Culex sitiens* in Ballina, NSW near North Creek. We propose the name 'North Creek virus' (NORCV) for this rhabdovirus. BEAUV and NORCV both exhibit similar genome organization, genome length and %GC in the regions studied to representative rhabdovirus species (Table 2 and Fig. S2). A maximum likelihood phylogeny (Fig. 1) was constructed using L gene, the RNA dependent RNA polymerase (RdRp), aa sequences of BEAUV and NORCV and other rhabdoviruses, including members of all *Rhabdoviridae* genera. GenBank sequences for selected viruses, including most Australian

Table 1
Virus genomes detected by deep sequencing insect pools passaged in cell culture. Bold denotes novel genomes detected for the first time.

Sample	Collection year	Collection source	Collection location in New South Wales, Australia	Passage history	Virus genome detected		
					Virus 1	Virus 2	Virus 3
1	2004	*Anopheles amictus*	Bourke	ppcc	LNV	RRV	
2	1998	biting midges	Wentworth	bbc	LNV	RRV	
3	1996	*Aedes camptorhynchus*	Sydney	bbcc	RRV		
4	1995	*Culex australicus*, *Culex molestus*	Griffith	bbbc	SLOV	LNV	RRV
5	2005	*Anopheles amictus*	Boggabilla	bbc	LNV	RRV	
6	2005	*Anopheles annulipes*	Griffith	ppp	**BEAUV (*Rhabdoviridae*)**	LNV	RRV
7	1995	*Culex quinquefasciatus*	Sydney	ppcc	RRV		
8	1997	*Culex sitiens*	Ballina	pppcc	LNV	RRV	
9	1995	*Aedes vigilax*	Batemans Bay	pppcc	LNV	RRV	
10	1995	*Culex sitiens*	Tweed Heeds	ppcc	LNV	RRV	
922	2005	*Culex annulirostris*	Hawkesbury	ppcc	WALV		
927	2010	*Anopheles annulipes*	Griffith	ppc	WALV		
929	2006	*Culex annulirostris*	Griffith	ppc	SLOV		
931	1992	*Aedes vigilax*	Port Stephens	bbc	**SASHV (*Bunyaviridae*)**		
932	1996	*Anopheles annulipes*	Griffith	bb	**MURBV (*Bunyaviridae*)**		
933	1999	*Culex annulirostris*	Griffith	ppppcc	KOKV		
934	1997	*Anopheles annulipes*	Griffith	none	**MURBV (*Bunyaviridae*)**		
935	1998	*Aedes vigilax*	Batemans Bay	pppcc	EHV		
948	1995	*Culex annulirostris*	Griffith	bbbc	WARV		
949	1995	*Anopheles annulipes*	Griffith	bbc	WARV		
954	1997	*Culex sitiens*	Ballina	pppcc	**NORCV (*Rhabdoviridae*)**		
955	n/a	hamster cell culture	n/a	b			

Abbreviations: b: baby hamster kidney cell passage, p: porcine stable equine kidney cell passage, c: C6/36 *Aedes albopictus* larval cell passage, BEAUV: Beaumont virus, EHV: Edge Hill virus, KOKV: Kokobera virus, LNV: Liao Ning virus, MURBV: Murrumbidgee virus, NORCV: North Creek virus, RRV: Ross River virus, SASH: Salt Ash virus, SLOV: Stretch Lagoon orbivirus, WALV: Wallal virus, and WARV: Warrego virus.

Table 2
Open reading frame lengths (Leng, amino acid) and percent GC content of rhabdoviruses, bunyaviruses and reoviruses sequenced in this study (bold) compared to representative related species.

Virus Family	Species	GenBank accession numbers	Genome organization	Open Reading Frame									
				Length N	%GC N	Length P	%GC P	Length M	%GC M	Length G	%GC G	Length L	%GC L
Rhabdoviridae	**Beaumont virus**	KF310911	ns	ns	ns	ns	ns	ns	ns	ns	ns	1568[a]	41
	North Creek virus	KF360970-3	3′N-P-M-G-L5′	430	47	331	48	ns	ns	522	39	2120	44
	Tibrogargan virus	NC_020804	3′N-P-M-G-L5′	428	37	308	36	253	35	662	35	2119	34
	Wongabel virus	EF612701	3′N-P-M-G-L5′	440	36	245	39	198	34	623	36	2118	34
	Chandipura virus	NC_020805	3′N-P-M-G-L5′	422	46	293	46	229	43	530	46	2092	41
	Bas Congo virus	JX297815	3′N-P-M-G-L5′	407	40	215	40	218	41	629	39	1954	38
				Open reading frame									
				Length S	**%GC S**	**Length M**	**%GC M**	**Length L**	**%GC L**				
Bunyaviridae	**Murrumbidgee virus**	KF234253-5	3 segments: S, M, L	237	41	1371	32	2242	31				
	Salt Ash virus	KF234256-8	3 segments: S, M, L	237	39	1377	33	2212[a]	32				
	Oropouche virus	KC759122-4	3 segments: S, M, L	231	47	1420	35	2250	34				
	Guaroa virus	JN801039, AY38058, X73466	3 segments: S, M, L	233	38	1418	34	2231	33				
	Jamestown Canyon virus	HM007356-8	3 segments: S, M, L	235	44	1444	36	2263	33				
	Bunyamwera virus	NC001925-7	3 segments: S, M, L	233	42	1433	37	2238	33				
	Wyeomyia virus	JN801036-8	3 segments: S, M, L	233	39	1419	32	2236	31				
				Open reading frame									
				Length 1	**%GC 1**	**Length 2**	**%GC 2**	**Length 3**	**%GC 3**	**Length 4**	**%GC 4**	**Length 5**	**%GC 5**
Reoviridae	**Wallal virus**	KF234259-68	10 segments	1306	37	984	37	899	38	644	40	557	40
	Warrego virus	KF310902-10	10 segments	1128[a]	36	ns	ns	900	39	641	37	549	39
	Eubenangee virus	JQ070376-85	10 segments	1308	42	969	42	901	43	645	44	557	47
	Tilligerry virus	JQ070366-75	10 segments	1308	42	976	44	901	43	645	45	557	46

Virus Family	Species	Length 6	%GC 6	Length 7	%GC 7	Length 8	%GC 8	Length 9	%GC 9	Length 10	%GC 10
Reoviridae	**Wallal virus**	530	39	350	44	341	40	329	40	246	40
	Warrego virus	528	39	351	44	378	39	330	41	238	41
	Eubenangee virus	530	43	351	48	352	47	329	46	243	46
	Tilligerry virus	530	43	351	47	352	48	330	49	243	46

ns: Not sequenced.

[a] Incomplete gene.

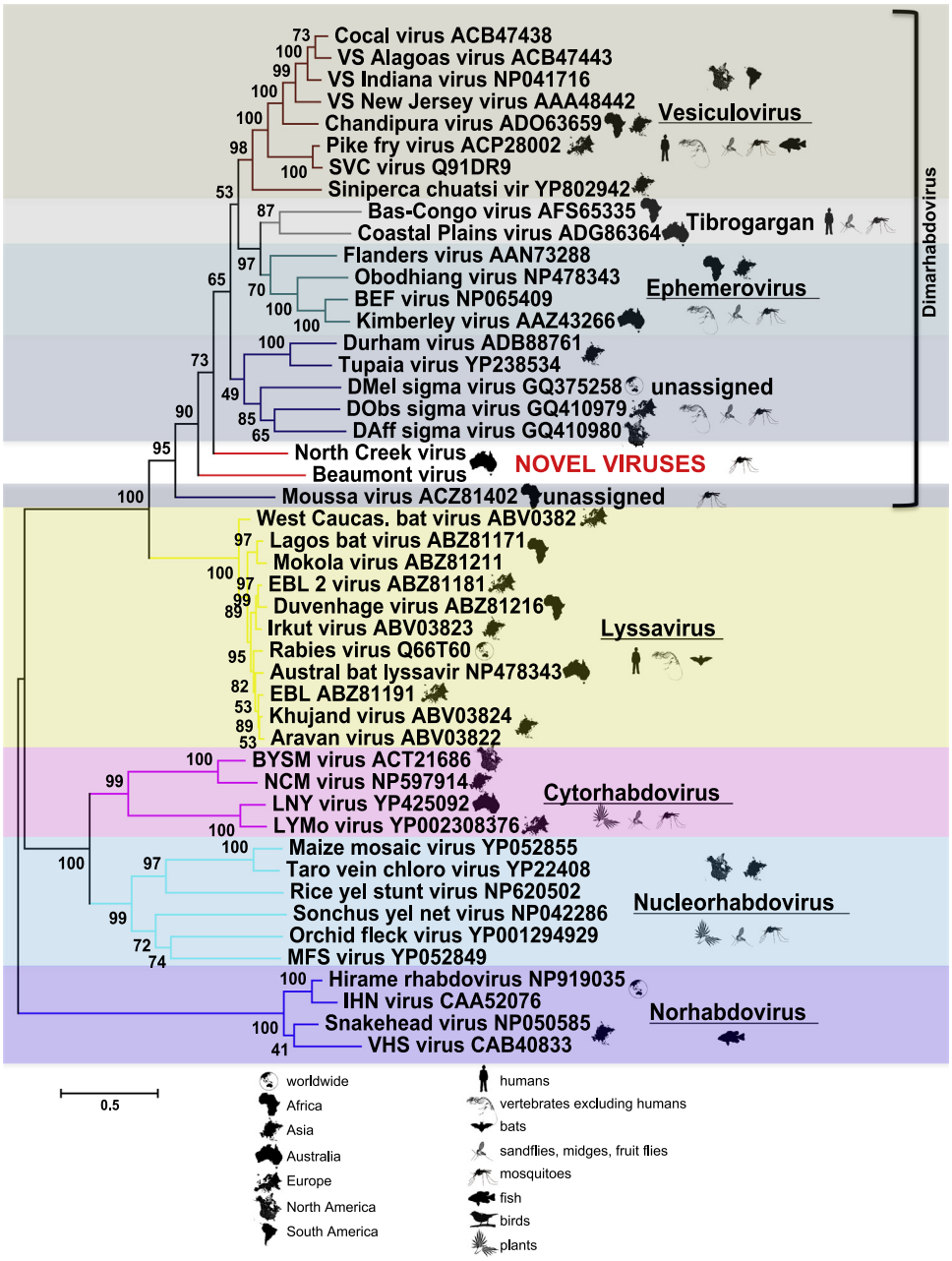

Fig. 1. *Rhabdovirus* maximum likelihood L gene open reading frame phylogeny with novel viruses highlighted in red. Numbers left of branches show statistical significance of tree topologies based on 1000 bootstrap re-sampling iterations. Symbols indicate geographic distributions and organism pictures show host ranges. Colored boxes show clade groupings; underlined groups denote established genera. L gene sequences used were 1568 aa (BEAUV), 2120 aa (NORCV, complete gene), and ≈2100 aa (complete gene) for GenBank sequences.

rhabdoviruses, were often short (133 aa, e.g.), and were therefore excluded from phylogenies. Despite the exclusion of these sequences from trees, BEAUV and NORCV shared <70% aa identity in the 133 aa region available for the Australian rhabdoviruses in GenBank, indicating that the novel viruses do not constitute known Australian rhabdovirus species that are poorly genetically characterized. Both novel viruses are located in the dipteran-mammal associated rhabdovirus (dimarhabdovirus) supergroup that includes several unassigned clades, tentative groups Tibrogargan (as well as Almpiwar, Hart Park, and Le Dantec that are not included in Fig. 1 since no full L gene sequence data is available in GenBank), and two established genera, Vesiculovirus and Ephemerovirus. BEAUV and NORCV are positioned at two deeply rooted branches in the dimarhabdovirus supergroup and neither virus clusters with any other known viral species, including any rhabdoviruses previously identified in Australia. BEAUV and NORCV share 37% aa identity in the L gene, a level similar to their identities with representative species in other dimarhabdovirus clades (Table 3). Nucleotide similarity plots (Fig. S2) show that both novel viruses share ≈65% L gene nucleotide (nt) identity with species from different dimarhabdovirus clades, supporting the placement of both viruses outside of established dimarhabdovirus clades. Compared to other species that cluster together (shaded boxes in Table 3), BEAUV and NORCV are less similar to each other (Table 3), supporting their placement on two separate deeply rooted dimarhabdovirus branches.

Novel bunyaviruses

Three mosquito pools, 931, 932, and 934, contained genomes consistent with orthobunyavirus genome organization (Walter and

Table 3
Percent L gene amino acid identities for selected dimarhabdoviruses. with GenBank accession numbers noted. Colors denote species that cluster (Fig. 1) and shaded boxes highlight identities for species in the same group.

	Beaumont virus	North Creek virus	Tupaia virus YP238534	DMel sigma virus GQ375258	Cocal virus ACB47438	Chandipura virus ADO63659	Bas-Congo virus AFS65335	Coastal Plains virus ADG86364	Obodhiang virus NP478343	Kimberley virus AAZ43266	Moussa virus ACZ81402
Beaumont virus	100										
North Creek virus	37	100									
Tupaia virus YP238534	33	37	100								
DMel sigma virus GQ375258	31	37	41	100							
Cocal virus ACB47438	34	39	45	42	100						
Chandipura virus ADO63659	35	39	44	41	61	100					
Bas-Congo virus AFS65335	39	34	36	34	38	39	100				
Coastal Plains virus ADG86364	31	35	38	36	41	40	42	100			
Obodhiang virus NP478343	33	35	37	34	39	39	38	40	100		
Kimberley virus AAZ43266	31	35	37	36	41	40	39	42	50	100	
Moussa virus ACZ81402	27	31	33	30	33	33	31	31	30	30	100

Table 4
L (A) and M (B) segment amino acid identities for the novel Murrumbidgee and Salt Ash viruses versus representative orthobunyavirus species with GenBank accession numbers noted. Colors denote orthobunyavirus groups and correspond to patterns in Fig. 2. Shaded boxes highlight identities for species in the same group.

A

L segment

	Murrumbidgee virus	Salt Ash virus	Oropouche virus AAQ04607	Peaton virus CCG93476	Guaroa virus JN801039	Jamestown Canyon ADK3667	La Crosse virus ADH04704	Bunyamwera virus AAA42777	Wyeomyia virus JN572081
Murrumbidgee virus	100								
Salt Ash virus	61	100							
Oropouche virus AAQ04607	46	47	100						
Peaton virus CCG93476	45	48	59	100					
Guaroa virus JN801039	47	47	47	46	100				
Jamestown Canyon ADK3667	50	51	51	50	49	100			
La Crosse virus ADH04704	50	50	51	49	49	83	100		
Bunyamwera virus AAA42777	49	49	50	48	48	55	56	100	
Wyeomyia virus JN572081	48	50	49	47	48	55	55	54	100

B

M segment

	Murrumbidgee virus	Salt Ash virus	Oropouche virus AAQ04607	Peaton virus CCG93476	Guaroa virus JN801039	Jamestown Canyon ADK3667	La Crosse virus ADH04704	Bunyamwera virus AAA42777	Wyeomyia virus JN572081
Murrumbidgee virus	100								
Salt Ash virus	52	100							
Oropouche virus AAQ04607	29	30	100						
Peaton virus CCG93476	30	31	40	100					
Guaroa virus JN801039	33	33	32	30	100				
Jamestown Canyon ADK3667	32	33	33	33	32	100			
La Crosse virus ADH04704	32	33	33	33	32	75	100		
Bunyamwera virus AAA42777	32	33	35	32	32	44	44	100	
Wyeomyia virus JN572081	33	33	34	32	33	44	44	54	100

Barr 2011) including three RNA segments: a large (L) segment that encodes the RdRp, a medium (M) segment that encodes a polyprotein, and a small (S) segment that encodes a nucleocapsid protein (NP) and a non-structural protein (NSs). Samples 934 (GenBank accession numbers KF234253-5) and 932 contained sequences that were $\geq$99% identical to each other. Both originated from *A. annulipes* mosquitoes from Griffith, NSW, near the Murrumbidgee River. We therefore propose the name 'Murrumbidgee virus' (MURBV) for the novel virus. Sample 931 (GenBank accession numbers KF234256-8) was isolated from a pool of *Aedes vigilax* from Port Stephens, NSW, near the town of Salt Ash. We propose the name Salt Ash virus (SASHV) for this virus. MURBV and SASHV both exhibit similar genome organization, gene length and %GC to other orthobunyavirus species (Table 2). Examining

nucleotide similarity plots and segment-wide identity with representative orthobunyaviruses from different clades, SASHV exhibits 54–56% nt identity and MURBV shows 53–63% nt identity (L segment, Fig. S3). The two viruses are more similar to each other (63% in L, 57% nt identity in M, 74% in S, Fig. S3) than they are to other orthobunyaviruses. Considering aa identity, the two novel viruses exhibit $\leq$51% aa identity (L segment) and $\leq$33% (M) with representative members of the orthobunyavirus genus (Table 4A and B), consistent with similarities observed between orthobunyavirus species that belong to different antigenic groups (Savji et al., 2011). Phylogenetic comparisons based on maximum-likelihood algorithms of MURBV and SASHV with L (Fig. 2A) and M (Fig. 2B) segments of published bunyavirus genomes show that, despite their relatively low aa identity to each other, the two viruses group together in a deeply rooted branch that is distinct from any of the established orthobunyavirus groups (Bunyamwera, Wyeomyia, California Encephalitis, Simbu). The M segment phylogeny (Fig. 2B) places MURBV and SASHV in a sister clade to the orthobunyaviruses and the L segment phylogeny (Fig. 2A) positions the novel viruses within the orthobunyavirus clade. Neighbor joining algorithms and phylogenies based on S segments (data not shown) yielded similar tree topologies. Maximum likelihood trees containing all *Bunyavirus* genera (Supplemental Fig. 1A and B) also show that MURBV and SASHV represent a distinct and deeply rooted orthobunyavirus clade. Notably, MURBV and SASHV cluster separately from the Simbu group viruses, many of which are borne by midges in Australia.

Reoviruses

Four reovirus genomes were detected in mosquito pools. LNV (*Seadornavirus* genus) was present in 8 pools from multiple species, and SLOV, WALV and WARV (*Orbivirus* genus) were detected in 2 pools each (pools 4 and 929, 922 and 927, 948 and 949, respectively). Reovirus genomes consist of 10–12 double-stranded RNA segments. The same viral species in different pools shared $\geq$95% nt identity. While WALV and WARV have been detected in NSW previously, only a portion of one genome segment is available in GenBank. We therefore assembled complete sequences for each of the 10 segments for WALV (GenBank accession numbers KF23459-68) and for 9 of 10 segments for WARV (GenBank accession numbers KF310902-10). The genome organization, segment lengths and GC% for each segment are similar to related orbiviruses (Table 2). A phylogenetic tree based on segment 1, the RdRp gene (Fig. 3) shows the genetic relatedness of genomes detected here to other reoviruses. Although all 4 detected viruses have been isolated from mosquitoes previously, LNV has only been reported in China. The 8 LNV isolates sequenced here were between ~1% and 4% different from each other (nt level) in the RdRp gene and were at least 5% different from previously published Chinese LNV (data not shown).

Togaviruses and flaviviruses

In addition to our detection of novel viruses and known viruses not previously observed in NSW, deep sequencing also detected the *Togavirus* RRV in 10 pools and the flaviviruses KOKV and EHV in one pool each. Virus sequences from all of these pools shared ~95% aa identity to published sequences from Australian isolates in GenBank (data not shown). Based on this high identity, these sequences represent strains of known flaviviruses and togaviruses endemic to Australia that were undetected during antigen testing, possibly due to low viral loads.

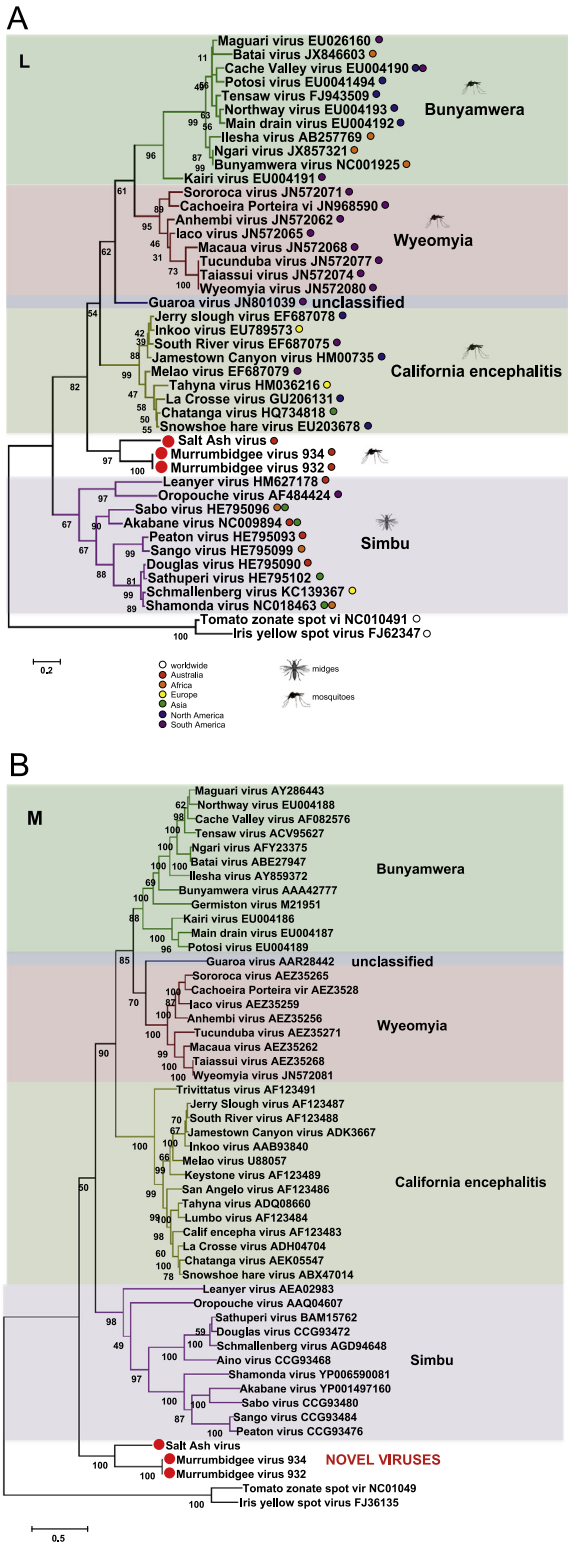

Fig. 2. (A and B) Orthobunyavirus maximum likelihood phylogenies of the L (A) and M (B) segment open reading frames with novel viruses highlighted in red. Sequences for M and L from GenBank were derived from the same isolate, when possible. Numbers to the left of branches show statistical significance of tree topologies based on 1000 bootstrap re-sampling iterations. Neighbor joining trees (not shown) exhibited similar topologies. Colored boxes show established orthobunyavirus groups defined based on genetic or serologic relatedness. Insect pictures show primary vectors. Gene sequences used were L: 2212 aa for SASHV, complete 2242 aa for MURBV, and complete $\approx$2200 aa for GenBank sequences; M: complete 1377 aa for SASHV, complete 1371 aa for MURBV, and complete $\approx$1400 aa for GenBank sequences.

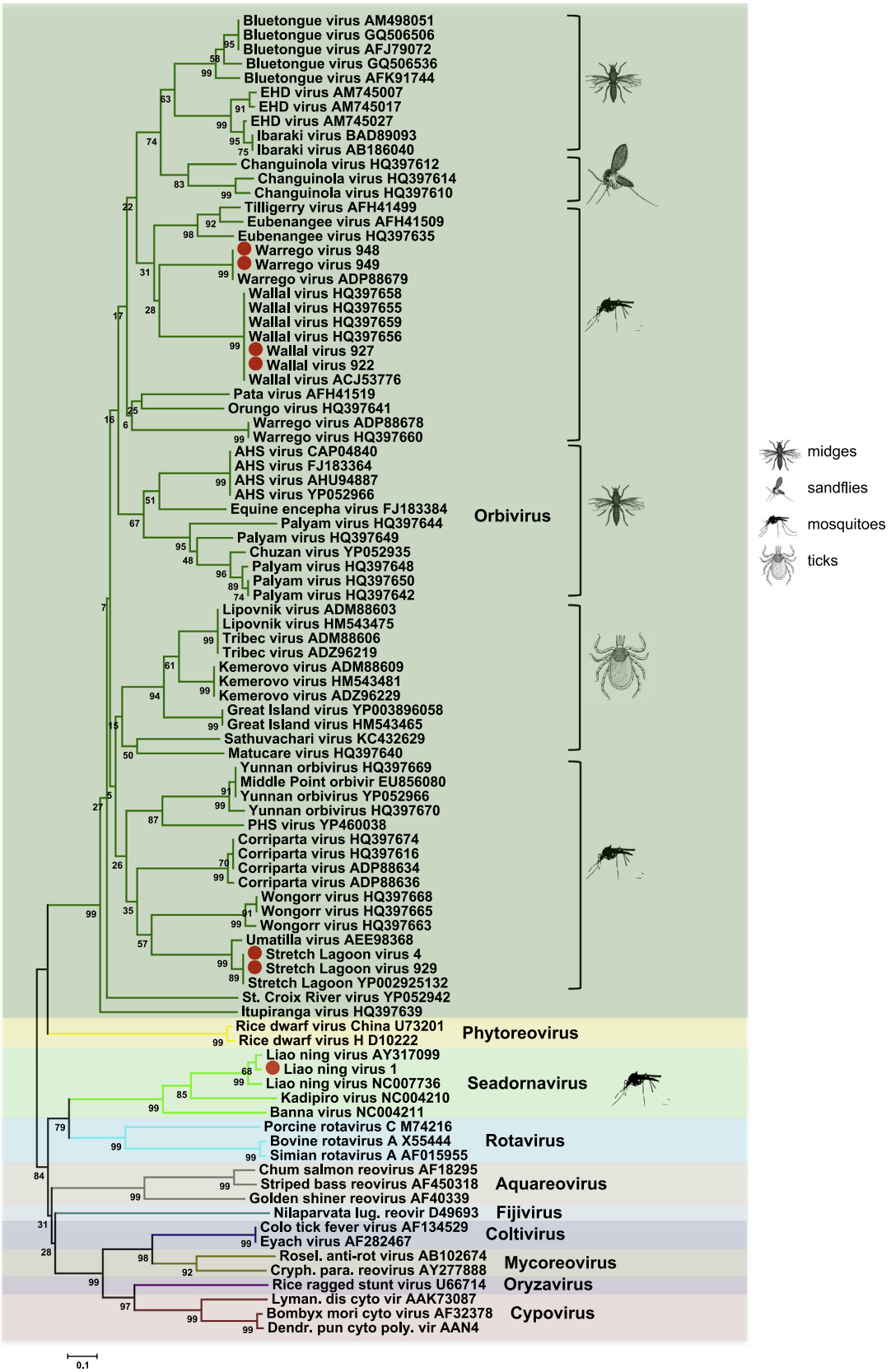

Fig. 3. *Reoviridae* RdRp gene maximum likelihood phylogeny with viruses detected here highlighted in red. Numbers to the left of branches show statistical significance of tree topologies based on 1000 bootstrap re-sampling iterations. Colored boxes show established reovirus genera. Pictures show primary vector types. Full ≈1250 aa RdRp gene sequences were used for viruses sequenced in this study, some shorter ≈130 aa sequences from GenBank were used when full sequences were not available.

Discussion

Summary

We used deep sequencing to detect viral sequences in mammalian cell culture supernatants from insect pools from NSW, Australia. This approach identified 4 novel virus genomes and 4 genomes of known viruses that had not been observed extensively in NSW, recognized in Australia, or fully sequenced. These findings highlight the utility of deep sequencing for identifying viruses that would otherwise be overlooked by conventional serologic testing and virus-specific RT-PCR, although until recently molecular viral detection was not standard in diagnostic platforms employed in NSW. Identification of these virus genomes in anthropophilic mosquitoes collected in NSW, coupled with their ability to replicate in mammalian cells, indicates that they may be capable of infecting humans or other vertebrates. The absence of viral sequences in 26 of the CPE-positive insect pools tested here indicates that some viral sequences went undetected, perhaps because of distant homology to viruses in the GenBank database, or that other microbes, such as bacteria or fungi, could have caused the CPE but were excluded from deep sequencing results via our selective filtration and bioinformatics steps.

Geographic distributions of Australian arboviruses

The locations of arbovirus isolations in this study, as well as areas where closely related bunyaviruses, rhabdoviruses, and reoviruses have been isolated previously, are shown in Fig. 4. The alphavirus and flaviviruses RRV, EHV and KOKV are not shown since they exhibit diffuse continental distributions and are not in the same families as the viruses detected here. We detected LNV at multiple sites in NSW. To our knowledge, this is the first detection of this virus outside China (Lu et al., 2011; Lv et al., 2012). Although both WALV and WARV have been isolated in NSW as recently as 1995–1996 (Hooper et al., 1999; Hooper 1999), detection of these viruses in Griffith and Hawkesbury (near Sydney) extends their distributions farther south. None of the Almpiwar group Australian rhaboviruses (Almpiwar (ALMV), Charleville (CHVV) and Humpty doo (HDOOV) viruses) have been detected in NSW; however, knowledge of their distributions relies on data based solely on single isolates from the Northern Territory and Queensland. The Australian orthobunyavirus AKAV exhibits a diffuse distribution along the northern and eastern coasts and overlaps with GGV, including in the Port Stephens area where SASHV was recovered. BEAUV and MURBV were both isolated in Griffith, in a region where other orthobunyaviruses have not been previously isolated. NORCV was isolated in Ballina near Port Stephens at the northern coastal edge of NSW, outside the known distribution of Australian rhabdoviruses. SLOV has previously been isolated in Western Australia and the Northern Territory, and once in Queensland and Sydney. Here we detected SLOV for the first time in Griffith, NSW.

Novel rhabdoviruses

Rhabdoviruses are single-stranded, negative-sense RNA enveloped viruses that comprise 6 recognized genera and more than 130 unassigned viruses with a worldwide distribution (Kuzmin et al., 2009) that impose a significant cost to human and veterinary health and to the agriculture industry. In Australia, there are at least 12 known arthropod-borne rhabdoviruses, including Adelaide river, Kimberley and Berrimah viruses (genus *Ephemerovirus*) that circulate among livestock and dipterans, Parry Creek and Wongabel viruses (Hart Park group) that use mosquitoes and midges as vectors, ALMV, HDOOV, and CHVV (Almpiwar group), Tibrogargan (TIBV) and Coastal Plains (CPV) viruses (Tibrogargan group) that use lizards, mosquitoes and midges as hosts, as well as the mosquito-borne Oak Vale virus (OVRV) and midge-borne Ngaingan virus (unassigned groups) (reviewed in (Kuzmin et al.,

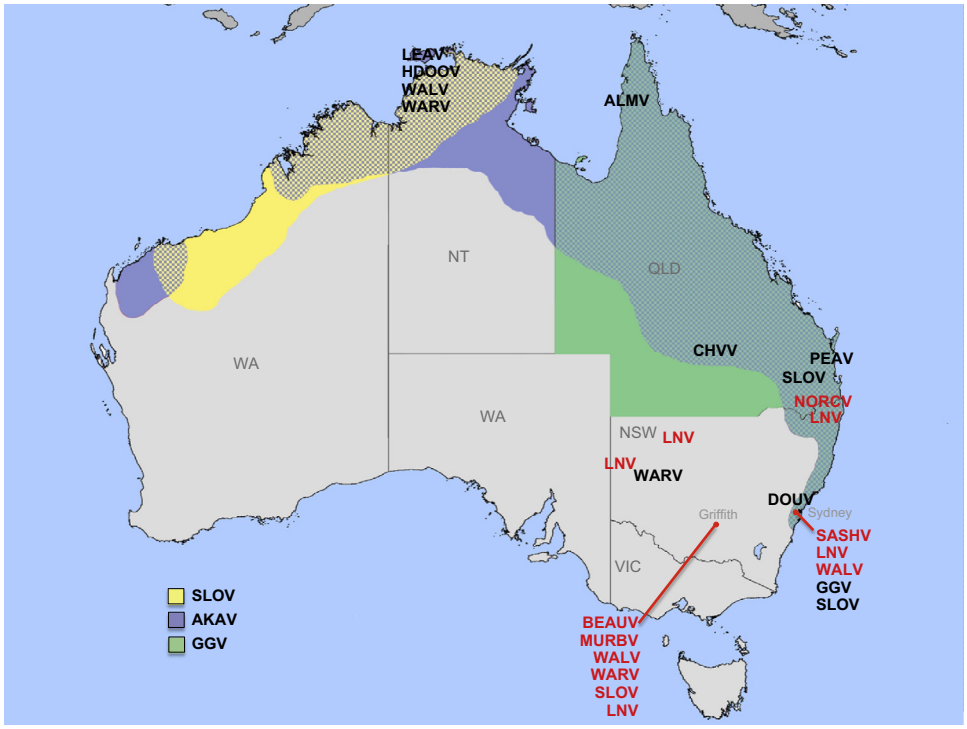

Fig. 4. Map of Australia showing mosquito collection locations for arboviruses detected in this study (red) as well as sites where other Australian arboviruses have been isolated previously (black and shaded regions) Monath et al. 1979; St George, 1980; Murray and Kirkland, 1995; Boughton et al., 1990; Akabane virus (AKAV, purple) Animal Health Australia, 2000, SLOV (yellow) Savji et al., 2011; Boughton et al., 1990, and GGV (green) Boughton et al., 1990. EHV, KOKV, and RRV are not shown since they exhibit diffuse continental distributions. LNV has not been previously isolated in Australia. NSW, New South Wales; NT, Northern Territory; QLD, Queensland; SA, South Australia; VIC, Victoria, WA, Western Australia.

2009)). None of these Australian rhabdoviruses have been linked to human disease; this may reflect their inability or lack of present ecological opportunities to infect humans or infrequent attempts at detection in patients. Here, we describe two novel rhabdovirus genomes in mosquito pools, BEAUV and NORCV, which diverge by ≈60% at the amino acid level in the RdRp from other rhabdoviruses. Strong bootstrap support leads us to classify these viruses in 2 deeply rooted branches in the dimarhabdovirus supergroup. Although rhabdoviruses in the same genera and dimarhabdovirus subgroups occur across different continents, genetically related virus species share similar hosts (Fig. 1). Most rhabdoviruses, lyssaviruses excluded, cycle between vertebrate and arthropod hosts (Kuzmin et al., 2009). To date, rhabdoviruses from Australia all belong to the Tibrogargan, Hart Park and Ampliwar groups. Given that BEAUV and NORCV do not cluster with any of these groups, they may constitute 2 novel mosquito-borne Australian dimarhabdovirus clades.

Novel bunyaviruses

Bunyaviruses comprise >350 known segmented negative-stranded RNA viruses that infect a variety of plants and animals sometimes causing hemorrhagic fever (Walter and Barr 2011). Of the five known *Bunyaviridae* genera (*Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus*), all but the hantaviruses are vectored by mosquitoes, midges, sandflies, ticks and thrips. The largest genus is the *Orthobunyavirus*, which contains >220 virus species historically differentiated by serologic relatedness (Mores et al., 2009). Although many orthobunyaviruses cause disease in humans and animals, molecular characterization has been limited to selected species (Savji et al., 2011; Mores et al., 2009). In Australia, all known orthobunyaviruses are members of the Simbu group and include Aino (AINOV), Akabane (AKAV), Douglas (DOUV), Facey's Paddock (FPV), GGV, Peaton (PEAV), Tinaroo (TINV), TRUV, and Thimiri (THIV) viruses (Blacksell et al., 1997), as well as recently identified Leanyer virus (LEAV) that may represent a distinct genetic and antigenic complex (Savji et al., 2011). Here we characterize the genomes of two novel orthobunyaviruses, MURBV and SASHV, that form a deeply rooted clade separate from any of the established orthobunyavirus groups (Fig. 2A, B and S1A, B), possibly reflecting a sixth group. Orthobunyaviruses show little geographic clustering (Fig. 2A), excepting Wyeomyia group species that have been reported exclusively in South America. Here we identify a new viral clade in Australia comprising MURBV and SASHV that likely use mosquitoes as vectors.

Novel detection of reoviruses in Australia

The family *Reoviridae* contains 9 genera including the arthropod-borne seadornaviruses and orbiviruses. Seadornaviruses are transmitted by anopheline and culicine mosquitoes and their genomes consist of 12 enveloped double-stranded RNA segments. There are three species of seadornaviruses: Banna virus (BAV), Kadiporo virus (KDV) and LNV. BAV has been isolated from encephalitic humans (Li, 1992; Xu et al., 1990), pigs, cattle (Liu et al., 2010), and mosquitoes in China (Chen and Tao 1996; Liting et al., 1995), Indonesia (Brown et al., 1993) and Vietnam (Nabeshima et al., 2008). Serosurveys of patients with encephalitis across China revealed a BAV seroprevalence of about 10% (Tao and Chen 2005). Unlike BAV, KDV and LNV have not been associated with human disease, although LNV replicates in vertebrate cells and produces viremia and nasal hemorrhages in mice (Attoui et al., 2006). LNV was first isolated in *Aedes dorsalis* mosquitoes from Liao Ning province in northeastern China in 2006 (Attoui et al., 2006) and has subsequently been isolated from *Culex* spp. and *A. dorsalis* throughout northern China (Lu et al., 2011; Lv et al., 2012; Li et al., 2010). Here we report the first detection of LNV in multiple pools of different species of mosquitoes and midges from NSW collected from 1995–2005 (Table 1) indicating that LNV is geographically diffuse in NSW, Australia, and may use multiple vector species there, similar to LNV circulation in China. Detection of LNV in pools of mosquitoes collected in 1995 indicates that the virus was present in Australia before it was recognized in China in 2006. No samples from China were ever present in our laboratories in Australia or the United States, and the level of sequence divergence (ca. 5%) between Australian LNV and Chinese LNV from GenBank is higher than would be expected if the sequences detected here represent contamination by Chinese LNV. Furthermore, a negative control hamster kidney cell culture that was passaged with insect pool extracts did not contain any arbovirus genome segments (Table 1), confirming the lack of cross-contamination between virus isolates or passages.

Orbiviruses, a genus within the Reovirus family, consist of 22 recognized viral species and 13 unassigned viruses, each with ≥10 double stranded RNA segments (Mertens et al., 2005). Many are transmitted by ticks, *Culicoides*, mosquitoes, and sand flies, and use cows, goats, sheep, equids, camelids, marsupials, sloths, bats, large carnivores, and humans as hosts (Belaganahalli et al., 2011). Among these, SLOV was first isolated in 2002 from a pool of *Culex annulirostris* mosquitoes collected in Kimberley, Western Australia, and a subsequent serologic survey revealed SLOV antibody in horses, goats and donkeys in the Northern Territory (Belaganahalli et al., 2011). The potential for human infection with SLOV is unknown, and SLOV has only once been isolated outside of Queensland and the Northern Territory, in Sydney (Fig. 4) (Jansen et al., 2009). Here we report the detection of SLOV genomes in two pools from Griffith, NSW: *C. annulirostris* collected in 2006 and a mixed pool of *C. australicus* and *C. molestus* collected in 1995. These detections extend the distribution of SLOV in NSW and also indicate that SLOV was probably circulating in mosquitoes in Australia for at least 6 years before it was first isolated in 2002 (Cowled et al., 2009).

Midge- and mosquito-borne orbiviruses WALV and WARV are associated with epidemics of blindness in kangaroos and WALV-inoculated kangaroos developed chorioretinitis (Hooper, 1999; Reddacliff et al., 1999; Standfast et al., 1984). Full genome sequences of these 2 viruses have not been previously published. WALV and WARV were first isolated in the 1970s in the Northern Territory from the two vector species implicated here, *C. annulirostris* and *A. annulipes* (Standfast et al., 1984). Standfast et al., (1984) also detected these two viruses in other insects, including *Anopheles farauti* and various *Culicoides spp*., suggesting that WALV and WARV exhibit broad vector ranges. Nine park workers sampled after a 1994–1995 outbreak of kangaroo blindness lacked neutralizing antibody to WALV (Tallis et al., 1998); in the absence of widespread serosurveys or clinical surveillance of patients, the potential for either of these viruses to cause human infections and disease remains unknown.

Vector use patterns

Two of the viruses characterized here, BEAUV and MURBV, were isolated from freshwater *A. annulipes* s.l. mosquitoes, members of the Australasian *Annulipes* complex that comprises at least 15 sibling species, some of which vector malaria and myxomatosis in rabbits (Foley et al., 2007). *A. annulipes* exhibits a diffuse distribution throughout Australia and predominantly lays its eggs in freshwater streams, marshes and lakes (Cooper et al., 1996; Russell et al., 1991). The arboviruses OVRV (*Rhabdoviridae*, unassigned), (Quan et al., 2011) RRV (*Togaviridae*, alphavirus) and the bunyavirus Mapputta virus (MAPV, unassigned) (Standfast et al., 1984) have all been isolated from *A. annulipes.* SASHV, by contrast,

was isolated from *A. vigilax*, a significant vector of RRV, BFV and GGV (Azuolas et al., 2003; Harley et al., 2001), that is highly abundant in salt marshes (Russell et al., 1991). Blood meal identification studies show that *A. vigilax* exhibits broad host-feeding patterns, imbibing from dogs, birds, humans, horses and possums in Brisbane (Kay et al., 2007). *C. sitiens*, a mosquito that oviposits in brackish or saline coastal lagoons (Clements, 2000), from which NORCV was isolated, has been implicated as a vector of numerous alphaviruses and flaviviruses in Australia, including RRV, BFV, SINV, MVEV, KUNV, KOKV, ALFV, and Japanese encephalitis virus (JEV) (Van Den Hurk et al., 2006; Johansen et al., 2004; Johansen et al., 2003). The 4 newly genetically characterized viruses reported here therefore appear to use the same highly abundant vectors as other arboviruses known to pose significant threats to human health in Australia.

Future directions and use of deep sequencing in arbovirus surveillance

Serologic surveys using either neutralization of viral isolates or detection of antibody to viral antigens will be required to determine whether the 4 novel viruses identified here, as well as LNV and SLOV, infect humans or other animals in Australia. Serologic cross-reactivity of these novel viruses with lesser-described rhabdoviruses and bunyaviruses for which little or no sequence data is available (e.g. MAPV, GGV and TRUV,) while unlikely due to large expected genetic distances, may also help define their serologic relatedness to other members of the rhabdovirus and bunyavirus families.

Recent outbreaks of pathogenic arboviruses belonging to numerous viral families including the *Togaviridae* (chikungunya virus (CHIKV)) (Powers and Logue, 2007), *Bunyaviridae* (severe fever with thrombocytopenia syndrome virus (SFTSV)) (Yu et al., 2011), *Flaviviridae* (West Nile virus (WNV) (Suthar et al., 2013), dengue virus (DENV)) (Simmons et al., 2012), *Rhabdoviridae* (Bas-Congo virus (BASV)) (Grard et al., 2012), and *Reoviridae* (BANV) (Li, 1992) attest to their potential to cause significant human disease. Arbovirus epidemics may be potentiated by changing viral ecology and point mutations that enhance transmission. Arbovirus surveillance therefore plays an important role as an early warning system to mitigate consequences of infections that cause human and animal disease. This study provides an important step by identifying circulating viruses of potential concern in Australian mosquitoes. Future studies are warranted to address the possibility of human or animal infection by the novel viruses detected here, as well as LNV. This study also highlights the use of deep sequencing for identifying arboviruses in mosquitoes. Most vector surveillance programs screen pools only for known endemic and enzootic viruses, precluding detection of novel or newly emerging viruses. Here we show that deep sequencing can identify novel viruses without prior knowledge of virus genomes present, making this approach a valuable alternative to designing and optimizing family- or genus-level degenerate primers for PCR-based detection. Deep sequencing is rapidly becoming cheaper and widespread, and barcoding many samples for pooling in a single run can further reduce cost without significantly compromising data quantity, providing a possible new tool for virus detection and characterization in arbovirus surveillance settings. The focus of this study was to detect viruses that caused CPE but which were not identified by conventional serology or antigen tests used in arbovirus surveillance. For this reason, we did not test pools where an arbovirus was identified by serology, potentially precluding the detection of co-infecting viruses. Furthermore, serologic tests were not 100% sensitive, as evidenced by detection of tested viruses RRV, EHV and KOKV in selected pools. Some pools also contained multiple viruses (Table 1). These results suggest that if surveillance platforms begin using deep sequencing for virus detection, testing of some known arbovirus-positive samples may be warranted, even at added expense. Although the majority of the viruses detected in this study were from 3rd or 4th passages, we also detected MURBV and sequenced its entire genome directly from a homogenized pool of mosquitoes (sample 934), indicating that this approach will also work directly on unpassaged arthropod pools.

Conclusions

We used viral metagenomics in concert with traditional arbovirus surveillance to characterize known and new arboviruses in field-collected mosquitoes. This study exemplifies the application of deep sequencing technologies for the genetic characterization of arboviruses following their amplification in cell culture, as this both demonstrates their ability to replicate in mammalian and insect cells, and increases viral titer to facilitate full genome sequencing. We identified 2 novel rhabdoviruses, 2 novel bunyaviruses, and we detected the arbovirus LNV not previously recognized in Australia. Further deployment of deep sequencing to characterize arboviruses in anthropophilic vectors will enable a more complete description of potential arbovirus threats to human and animal health. Whether the novel and newly recognized arboviruses detected here infect animals or humans and cause disease will require serological studies and the further analysis of unexplained disease in exposed animal and human populations.

Materials and methods

Insect collection and virus culture

Mosquitoes and midges were collected in dry-ice baited traps at various sites in NSW, Australia between 1992 and 2010 (Table 1). As part of routine arbovirus surveillance in the region, individual mosquitoes and midges were identified using morphological criteria (Russell, 1996) and pooled into groups of up to 50 insects. Pools were mechanically homogenized with glass beads and inoculated onto porcine stable equine kidney (PSEK) or baby hamster kidney (BHK) cells that are highly susceptible to flavivirus and alphavirus infections, respectively, and monitored by microscopic examination for CPE, including cell rounding and death characteristic of arbovirus infections. CPE positive supernatants were transferred to new cultures of PSEK, BHK or *Aedes albopictus* larval (C6/36) cells for a total of 2–6 passages (only sample 934 represented unpassaged mosquito homogenate) until a strong CPE was elicited, suggesting virus infection. The lowest remaining available passage was used in this study. Virus identification using supernatants from passages was attempted by enzyme immunoassay with a panel of monoclonal antibodies to Australian arboviruses including BFV, EHV, KOKV, KUNV, MVEV, RRV, SINV, and STRV. Prior to 1994, viruses were identified by micro-neutralization tests using specific antisera to the same viruses as well as Alfuy (ALFV), Gan Gan (GGV), and Trubanaman viruses (TRUV) (Russell et al., 2001).

Viral particle purification

Supernatants from low passage insect pools were clarified from cell debris by 12,000*g* centrifugation for 2 min and then passed through a 0.45-μm filter (Millipore) to remove large particulates including bacteria. The filtrate was treated with a nuclease cocktail consisting of DNAses (Turbo DNAse from Ambion, Baseline-Zero from Epicentre and Benzonase from Novagen) and with RNAse (Fermentas) to digest host nucleic acids and non-encapsulated viral nucleic acids. Nucleic acids protected from nuclease digestion were then extracted using the QIAamp viral RNA mini kit (Qiagen)

according to manufacturer's recommendations with a 5 min incubation step at room temperature prior to elution from the column.

Sequence-independent nucleic acid amplification for deep sequencing

Deep sequencing was performed using Roche 454 and Illumina MiSeq platforms. For Roche 454 libraries, RNA-only and DNA-plus-RNA sequence-independent amplifications were combined before sequencing. For RNA-only amplification, 10 μl of extracted nucleic acids were treated with DNAse (Ambion) and were used as a template for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen). cDNAs were primed with an arbitrary sequence and eight fixed nucleotides at the 3′ end and six fixed nucleotides at the 5′ end that served as a barcode (Victoria et al., 2009). For the DNA-plus-RNA amplification, the DNAse step prior to reverse transcription was omitted. After reverse transcription, cDNAs were heat denatured and the primers were allowed to re-anneal for a single round of second strand DNA synthesis using Klenow DNA polymerase (New England Biolabs). Thirty-five PCR cycles were performed on double stranded cDNAs using primers consisting of arbitrary sequences also used as molecular tags. Each PCR reaction was performed in duplicate to increase sampling of viral nucleic acids, and then the four resulting PCR products were pooled and purified using the QIAquick PCR purification kit (Qiagen). Randomly amplified nucleic acids from each insect pool derived culture supernatant were normalized using Nanodrop (Thermo Scientific) and mixed at equimolar ratios to obtain 2.6 μg of DNA/sample. Amplicons from 300 to 1000 base pairs in this mixture were size selected by extraction from a 2% agarose gel and purified using the QIAquick gel extraction kit (Qiagen). PCR ends were polished using T4 polynucleotide kinase (New England Biolabs) and Roche 454 adapters were ligated to the ends. Small fragments were removed using AmpPure XP beads (Agencourt Biosciences) according to the manufacturer's protocol (GS FLX Titanium Library Preparation Kit, Roche).

To characterize sequences in selected samples more deeply to fill in gaps in genomes, libraries were also made and sequenced using MiSeq (Illumina). Nucleic acids were extracted from the same filtered and nuclease treated culture supernatants and the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre) was used following the manufacturer's protocol with 1X purifications performed using AmpPure XP beads (Agencourt Biosciences). Up to 35 PCR cycles were used when amplicons were not observed in 1% agarose gels after fewer cycles. Size distributions of libraries were assessed using an Agilent 2100 Bioanalyzer and concentrations of cluster-competent DNAs were determined using the SYBR FAST LightCycler 480 qPCR Kit (Kapa) according to manufacturer's instructions. Libraries were diluted to 7 pM for sequencing on the MiSeq.

For both sequencing platforms, barcode sequences were included in primers during non-specific amplifications to multiplex multiple insect pool derived culture supernatants in the same run.

Deep sequencing data processing and sequence assembly

Sequences were binned according to barcode and then the barcode and primer were removed. Clonal reads were eliminated and sequences were de novo assembled into contigs using Mira (Roche 454), SOAP (Illumina), PRICE (Ruby et al., 2013) or manually using Sequencher (Gene Codes; where alignments were made when 90% of at least 10 overlapping base pair reads were identical). Assembled contigs and single sequences that did not collapse into contigs were then analyzed by tBLASTx, querying the translated nucleotide database using a translated nucleotide query. To measure sequence similarity, expectation (*E*) scores were used, with a cutoff of $E < 10^{-5}$. *E* scores as high as 10^{-3} are used for virus discovery (Victoria et al., 2009); here 10^{-5} was selected to increase stringency but still allowed for detection of divergent sequences. Contigs and singlets were classified as eukaryotic, bacterial, phage, or viral based on best *E* score matches; sequences with tBLASTx *E* scores $> 10^{-5}$ were deemed unclassifiable. Contigs and singlets with best matches to annotated viruses in GenBank were then sorted by virus taxon.

Genome acquisitions by reverse transcription-polymerase chain reactions

Contigs and singlets showing strong tBLASTx *E* scores to viruses in GenBank were joined together by RT-PCRs using specific primers designed from deep sequencing reads and directly Sanger sequenced. Areas where deep sequencing coverage was lower than 5× or regions with sequencing discrepancies were also re-sequenced using Sanger technology directly on PCR products. The Titan One Tube RT-PCR kit (Roche) was used according to the manufacturer's protocol and cycling conditions were: cDNA synthesis at 50 °C for 30 m followed by denaturation at 94 °C for 2 min, then 35 cycles of: 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 2 m, followed by a final extension of 68 °C for 10 m. Full genomes, when obtained, were assembled using Sequencher (Gene Codes).

Phylogenetic analyses, similarity and identity comparisons

Reference viral amino acid sequences representing bunyavirus, reovirus and rhabdovirus families were obtained from GenBank. Sequence alignments were performed using CLUSTALX (Thompson et al., 1994) on the Mobyle portal website (http://mobyle.pasteur.fr). Phylogenies were generated using the maximum likelihood (Jones–Taylor–Thornton model) and neighbor-joining (maximum composite-likelihood) methods with MEGA software (Tamura et al., 2011). Statistical significance of tree topologies was evaluated by 1000 bootstrap re-sampling iterations. Sequence identities between species were calculated using the SIAS server (http://imed.med.ucm.es/Tools/sias.html) and the equation: percent identity = 100* (identical positions/length of alignment) where gaps in alignments were included in analyses. Similarity plots, calculated in % identities, were generated by aligning nucleotide sequences and calculating scanning pairwise identities using a window size of 100 bp using mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml).

Competing interests

None.

Authors' contributions

LLC conducted project, interpreted results and wrote the paper. BLP constructed libraries for deep sequencing. ALG, CW and XD performed bioinformatics and contig assembly. BLH, RCR, SLD, and JH collected mosquito pools, screened samples for known arboviruses, and provided samples to LLC and ELD for deep sequencing. ELD designed the project and edited the manuscript.

Acknowledgments

The NSW Arbovirus Surveillance Program was funded by the NSW Ministry of Health. The sequencing work was supported by the Blood Systems Research Institute and National Institutes of Health R01 HL105770.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.09.026.

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