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# Genetic and epidemiological characterization of Stretch Lagoon orbivirus, a novel orbivirus isolated from *Culex* and *Aedes* mosquitoes in northern Australia

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Stretch Lagoon orbivirus (SLOV) was isolated in 2002 from pooled *Culex annulirostris* mosquitoes collected at Stretch Lagoon, near the Wolfe Creek national park in the Kimberley region of Western Australia. Conventional serological tests were unable to identify the isolate, and electron microscopy indicated a virus of the genus *Orbivirus*, family *Reoviridae*. Here, a cDNA subtraction method was used to obtain approximately one-third of the viral genome, and further sequencing was performed to complete the sequences of segment 1 (viral polymerase) and segment 2 (conserved inner-core protein). Phylogenetic analysis showed that SLOV should be considered a new species within the genus *Orbivirus*. A real-time RT-PCR test was designed to study the epidemiology of SLOV in the field. Six additional isolates of SLOV were identified, including isolates from four additional locations and two additional mosquito species. Horses, donkeys and goats were implicated as potential vertebrate hosts in a serological survey.

## INTRODUCTION

Northern Australia has long been recognized as a hotspot for arbovirus activity. The region is prone to regular cyclones that can carry insects over large distances, potentially providing a route for exotic arbovirus incursions (St George, 1992; St George *et al.*, 2001). To address the risks posed by arboviruses to human and animal health in Australia, arbovirus surveillance is carried out by a network of regional diagnostic laboratories using approaches that include insect trapping and maintenance of sentinel animals for virus isolation and serology (National Arbovirus Monitoring Program, 2008). Viruses from several recognized insect-transmitted virus families are regularly isolated in northern Australia. These are members of the families *Togaviridae* (genus *Alphavirus*), *Flaviviridae*, *Bunyaviridae*, *Rhabdoviridae* and *Reoviridae* (genus *Orbivirus*). Since arbovirus monitoring began in Australia, a large collection of unidentified isolates has accumulated, despite extensive diagnostic testing. The majority of unidentified isolates examined to date have had features consistent with members of the family *Reoviridae* (genus *Orbivirus*). One of these, K49460, was isolated in 2002 from a pool of 25 *Culex annulirostris* mosquitoes collected at Stretch Lagoon (19° 40′ S, 127° 35′ E) near the Wolfe Creek national park in the Kimberley region of Western Australia. K49460 was initially examined at the Arbovirus Surveillance and Research Laboratory at The University of Western Australia, using a panel of antibodies raised against all known Australian members of the genera *Alphavirus* and *Flavivirus* (Broom *et al.*, 1998). It was then examined further at the Berrimah Veterinary Laboratories in the Northern Territory using a large panel of antisera raised against veterinary arboviruses, including all of the recognized Australian orbiviruses, as well as the Changuinola, Kemerovo and Japanaut orbiviruses. No identification was obtained in any of these tests.

The most recent longitudinal study of arboviruses in mosquitoes in the Northern Territory covered the period 1982–1992 (Weir *et al.*, 1997). Of the nine species of orbivirus that were known in Australia during that study,

The GenBank/EMBL/DDBJ accession numbers for the sequences of SLOV segments 1 and 2 are EU718676 and EU718677.

members of the orbivirus species *Wallal virus*, *Warrego virus*, *Wongorr virus*, *Eubenangee virus*, *Corriparta virus* and *Bluetongue virus* were isolated from mosquitoes on multiple occasions, and CSIRO Village virus, a strain of the species *Palyam virus* was isolated on one occasion. The tentative orbivirus Lake Clarendon virus has not been isolated at all since its initial discovery in 1984, when it was isolated from a bird-feeding tick in southeast Queensland (St George *et al.*, 1984). In summary, the only orbivirus known to be in circulation in northern Australia during the study period that was not isolated from a mosquito species was epizootic hemorrhagic disease virus (EHDV) (Weir *et al.*, 1997). Two other Australian orbiviruses have not been isolated from mosquitoes or any other arthropod to date: Middle Point orbivirus [MPOV; Cowled *et al.*, 2007; related to the tentative orbivirus Yunnan orbivirus (YUOV), isolated in China] and Elsey virus [related to the tentative orbivirus Peruvian horse sickness virus (PHSV), isolated in Peru]. However, these viruses have only been described recently, and YUOV and PHSV were isolated from mosquitoes in China and Peru, respectively (Attoui *et al.*, 2005; GenBank accession nos NC_007748–NC_007757). *Culicoides* species are the main vectors for many of the above-mentioned orbiviruses [including bluetongue virus (BTV)], but the combined evidence suggests that the majority of Australian orbiviruses can also infect mosquitoes. In the absence of vector-competency studies, it is not known whether mosquitoes are true vectors of many of these orbiviruses (i.e. capable of transmitting viruses to new vertebrate hosts) or dead-end hosts.

The International Committee for Virus Taxonomy (ICTV) list of criteria for defining orbivirus species is polythetic, meaning that some but not all of the criteria need to be fulfilled in order for an isolate to qualify as a new species (Mertens *et al.*, 2005). Attoui *et al.* (2001) found that all orbiviruses had >30 % identity in the amino acid sequence of the viral RNA polymerase (VP1). They also found that orbiviruses within a single species group have >91 % identity in the amino acid sequence of the inner core protein (T2). The genetic sequences of VP1 and T2 can therefore be used to define a new species within the genus *Orbivirus*. In several orbivirus species (including *Bluetongue virus*), T2 is encoded on genome segment 3 and is called VP3. In other species, it is encoded on segment 2 and is called VP2. Based on the ICTV criteria, there are 22 recognized species and ten tentative species of orbiviruses (Mertens *et al.*, 2005). Of these, genetic sequence data are available from 14 species. Full genome sequences are available for only six of them. For the orbiviruses that have been only partially sequenced, data are mainly from the central region of the conserved T2 gene.

K49460 could not be identified by extensive serological testing, and appears to be only distantly related to sequenced orbiviruses at the genetic level. Based on these findings, we suggest that K49460 represents a new species within the genus *Orbivirus* and suggest the name Stretch Lagoon orbivirus, bringing the total number of orbivirus species described in Australia to 12.

## METHODS

**Virus isolation and culture.** The majority of viruses examined in this study were isolated by staff at the Arbovirus Surveillance and Research Laboratory at The University of Western Australia using published methods (Broom *et al.*, 1989; Johansen *et al.*, 2000; Lindsay *et al.*, 1993). DPP5368 was isolated at the Berrimah Veterinary Laboratories (Northern Territory), and CSIRO1747 was isolated at CSIRO, Brisbane. Viruses were cultivated *in vitro* using BSR cells (a subclone of the hamster cell line BHK-21), which were grown at 37 °C in cell-culture flasks containing Eagle's basal medium (pH 7.2), supplemented with 10 mM HEPES, 6.7 mM $NaHCO_3$, 2 mM L-glutamine, 80 U penicillin $ml^{-1}$, 137 μM streptomycin and 5 % fetal calf serum.

**Electron microscopy.** Infected BSR cells were scraped from the culture surface and pelleted in a bench-top centrifuge (2000 ***g***) for 5 min. The supernatant was adsorbed onto Parlodion-filmed, carbon-coated copper grids for 5 min and stained with 2 % phosphotungstic acid for 1 min for negative-contrast electron microscopy. The cell pellet was processed for thin-section electron microscopy as described previously (Cowled *et al.*, 2007). Cell pellet thin sections and supernatants were examined under a Hitachi H7000 transmission electron microscope at 75 kV.

**RNA extraction.** Total RNA was purified from tissue culture supernatant harvested during the late stage of cytopathic effect. Supernatants were decanted from flasks and centrifuged at 300 ***g*** for 10 min at 4 °C to pellet cell debris. Clarified supernatants were centrifuged at 100 000 ***g*** for 90 min at 4 °C to pellet virions. RNA was then extracted using an RNeasy Mini kit (Qiagen) following the manufacturer's instructions and quantified using a GeneQuant II DNA/RNA Calculator (Pharmacia).

**PCR-Select suppression subtractive hybridization** Total Stretch Lagoon orbivirus (SLOV) RNA from tissue culture supernatant (the 'tester') was analysed using a PCR-Select kit (Clontech) following the manufacturer's instructions. For the 'driver', we used total RNA from BTV-1 (Australia) cultivated in BSR cells. Briefly, RNA from tester and driver samples was converted into double-stranded cDNA and digested with *Rsa*I. The tester cDNA was divided into two portions and each portion was 5′-labelled with one of two oligonucleotide adaptors. A sequence of hybridizations was then performed, yielding a mixture in which fragments of sequence unique to the tester formed double-stranded cDNA hybrids with a different adaptor at each end, permitting PCR amplification using the Advantage II kit (Clontech) to generate a cDNA library highly enriched for fragments of the SLOV genome (Diatchenko *et al.*, 1996). The cDNA synthesis procedure was modified for double-stranded RNA as follows: 2 μg RNA was combined with 0.5 μl random-hexamer oligonucleotides (1.5 μg $ml^{-1}$; GeneWorks) and 0.5 μl formamide in a total volume of 5 μl. This mixture was denatured at 100 °C for 1 min in a heat block, chilled rapidly on ice and then used in a 10 μl reverse transcription reaction following the PCR-Select kit instructions.

**Cloning.** The enriched cDNA library generated by cDNA subtraction was treated with DNA polymerase I – Klenow large fragment (New England Biolabs) to generate blunt ends and then ligated into the pCR-Blunt-II-TOPO vector and electroporated into OneShot TOP-10 *Escherichia coli* using a Zero-Blunt-II-TOPO PCR cloning kit (Invitrogen). Transformants were grown on Luria–Bertani agar plates supplemented with 50 μg kanamycin $ml^{-1}$ (Gibco). Colonies were

picked at random and grown overnight in 5 ml cultures for plasmid DNA isolation using a Qiagen Spin Miniprep kit.

**5′–3′ ligation rapid amplification of cDNA ends (RACE).** 5′–3′ Ligation RACE was carried out following the method of Mandl *et al.* (1991) to obtain the terminal nucleotide sequences of SLOV genome segments. Tobacco acid pyrophosphatase (Epicentre Biotechnologies) was used to remove the 5′-cap structure from 2 μg RNA, and T4 RNA ligase (New England Biolabs) was used to circularize the RNA. The reaction was carried out overnight at 4 °C, followed by phenol/chloroform extraction and ethanol precipitation. Circular RNA was resuspended in 2 μl RNase-free $H_2O$, combined with 1 μl formamide, denatured at 100 °C for 1 min in a heated block and then chilled on ice. The denatured RNA was then used in a 20 μl reverse transcription reaction containing 200 U StrataScript reverse transcriptase (Stratagene) and 20 U RNase inhibitor (Invitrogen). This was followed by two rounds of PCR with nested primers designed using sequence obtained through the PCR-Select cDNA subtraction method. Secondary PCR products were cloned and sequenced with a vector-specific primer.

**Sequencing and sequence analysis.** PCR products and plasmids were sequenced using ABI Big Dye Terminator sequencing reagents and an ABI Prism 3130xl Genetic Analyzer. Sequencing primers were obtained from GeneWorks. DNA sequences were trimmed and assembled using Seqman version 7 (Lasergene; DNASTAR). Sequences were identified using the online BLAST search engine (http://www.ncbi.nlm.nih.gov/BLAST/). PCR primers and sequencing primers were designed using Clone Manager version 8 (SciEd Software). Multiple sequence alignments were created using CLUSTAL W (Thompson *et al.*, 1994) and phylogenetic trees were prepared using a distance matrix and Fitch, PHYLIP software and BioManager by ANGIS (http://www.angis.org.au), and TreeView (Page, 1996). Amino acid sequences were analysed using PredictProtein (http://www.predictprotein.org/) (Rost *et al.*, 2004).

**RT-PCR test for SLOV.** Real-time RT-PCR was carried out using a Corbett Rotor-Gene 6000 real-time PCR machine and a SYBR Green one-step RT-PCR kit (Invitrogen), targeting a 251 bp region of the conserved T2 gene sequence (segment 2, nt 602–852). HPLC-pure SLOV-specific primers were obtained from GeneWorks (forward primer: 5′-TGAACCGGCCGATACAGAAT-3′; reverse: 5′-TGAGGGATTTGGTGGTAATGTG-3′) and used at a final concentration of 250 nM each. A master mix was prepared on ice containing (per reaction) 0.4 μl Superscript III RT/Platinum *Taq*, 9.6 μl SYBR Green reagent and 2.5 μl of each primer (2 μM stocks). For each sample, total RNA was extracted from 100 μl of infected tissue culture supernatant using a Qiagen RNeasy Mini kit and eluted in 30 μl $H_2O$. RNA (5 μl) was denatured at 100 °C for 1 min in a heat block, rapidly chilled on ice and then combined with 15 μl master mix to give a total reaction volume of 20 μl. Cycling parameters were: 50 °C for 3 min, 95 °C for 5 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 30 s. Melting-curve analysis was performed including a 90 s pre-melt step at 37 °C.

**Virus neutralization test.** Serum samples were initially diluted 1 : 4 in cell-culture medium. Aliquots (50 μl) of diluted serum were placed into duplicate wells of 96-well, flat-bottomed tissue culture plates and combined with 50 μl virus pre-titrated to contain 100 $TCID_{50}$ or culture medium only (serum control). Plates were then incubated at 37 °C and 5 % $CO_2$ for 1 h. A BSR cell suspension (100 μl) containing $2 \times 10^5$ cells $ml^{-1}$ was added to each well and the plates returned to the incubator. Back titration of the pre-titrated virus was performed on a control plate and the test was read when the control plate read 100 $TCID_{50}$ at 5 days post-infection (p.i.). Positive sera were titrated in duplicate using serial twofold dilutions and titres were defined as the reciprocal of the highest dilution causing complete inhibition of the cytopathic effect.

## RESULTS

### Electron microscopy

Viral particles and putative viral tubules were observed in the supernatant of SLOV-infected BSR cells at 5 days p.i. by negative-contrast electron microscopy (Fig. 1a, b). The particles appeared relatively smooth and almost spherical with electron-dense cores and were approximately 70 nm in diameter. Putative tubules ranged from approximately 17 to 34 nm in diameter. Viral particles and putative tubules were also observed in the cytoplasm of infected cells at 5 days p.i. by thin-section electron microscopy (Fig. 1c). These features are consistent with viruses of the family *Reoviridae*, genus *Orbivirus*.

### Genetic sequencing

SLOV was subjected to PCR-Select cDNA subtraction, using BTV-1-infected tissue culture supernatant as the driver. Of the 49 clones that were sequenced, inserts from 25 (15 unique) returned BLAST hits to known viruses. When assembled on a genome scaffold (based on the structure of YUOV), the sequenced fragments were distributed randomly across six of the ten gene segments and amounted to 7129 bp (36.6 %) of the estimated 19 500 bp genome (Fig. 2). The segments encoding the viral polymerase

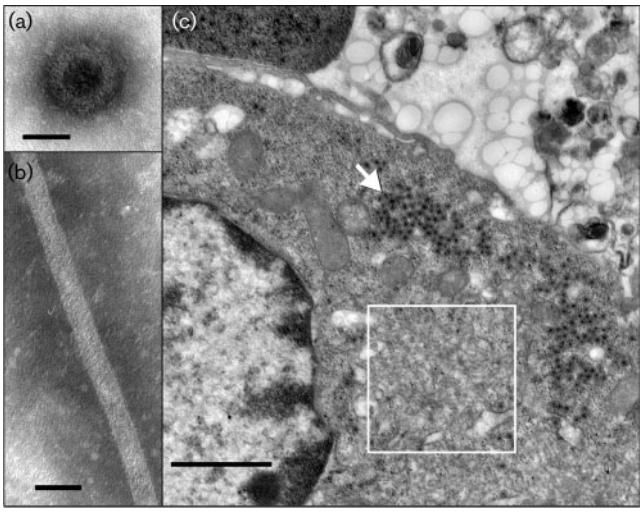


**Fig. 1.** (a, b) Negative-contrast electron micrographs of a virion and putative viral tubule in the supernatant of SLOV-infected BSR cells at 5 days p.i. Bars, 50 nm. (c) Thin-section electron micrograph of a SLOV-infected BSR cell at 5 days p.i. featuring abundant virions (e.g. arrow) and putative viral tubules located to the right of the nucleus in an irregular arrangement (boxed). Bar, 1 μm.

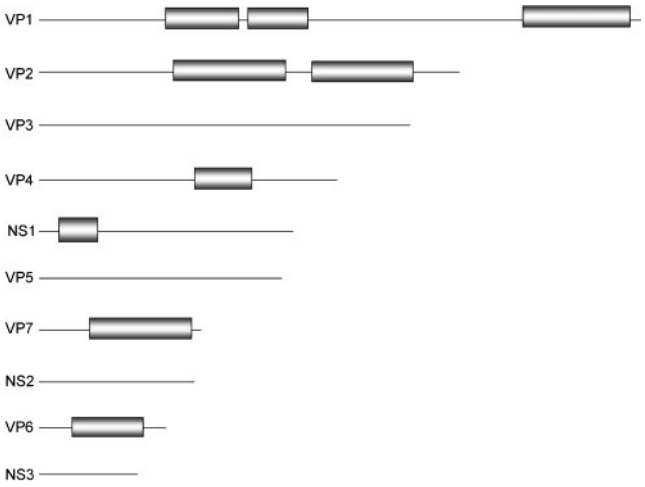


**Fig. 2.** Predicted structure of the SLOV genome based on the structure of the YUOV genome. The line lengths and protein assignments represent predicted size and coding potential of the gene segments of SLOV. Shaded bars indicate the fragments of SLOV sequence positioned relative to homologous sequences in YUOV.

(VP1) and T2 (VP2) were then selected for full sequencing. PCR was used to fill the internal gaps and the 5′-3′ ligation RACE method of Mandl *et al.* (1991) was used to obtain the end sequences of both segments.

## Sequence analysis

SLOV segment 1 (VP1) was 3933 bp in length. It comprised a 5′ untranslated region (UTR) of 12 nt, a 3′ UTR of 21 nt, one large open reading frame (ORF) encoding a protein of 1299 aa in the coding strand and six smaller, additional ORFs in the complementary strand corresponding to potential polypeptides ranging in size from 121 to 178 aa, although these are unlikely to be translated. The 3900 nt ORF featured a Kozak consensus sequence for strong eukaryotic translation [(A/G)XXAUGG; Kozak, 1997], but the smaller ORFs did not. SLOV RNA polymerase (VP1) has a predicted molecular mass of 148.3 kDa, a predicted pI of 8.55 and contains 11 cysteine residues, none of which are predicted to participate in disulphide bonding.

A nucleotide–nucleotide BLAST search of SLOV segment 1 returned no hits, but a translated BLAST search returned hits to the RNA-dependent RNA polymerases of other orbiviruses (VP1). The protein identity of these alignments ranged from 40 % for St. Croix River virus (SCRV) to 51 % for PHSV. These figures are above the 30 % minimum similarity suggested by Attoui *et al.* (2001) for membership of the genus *Orbivirus*. Avian rotavirus VP1 (GenBank protein no. BAA24146) aligned with 21 % amino acid identity to SLOV VP1.

SLOV segment 2 (T2) was 2792 bp in length. The 5′ UTR was 18 nt, whilst the 3′ UTR was 56 nt. One large ORF of 2715 nt encoding a protein of 905 aa was identified in the coding strand. Three additional ORFs encoding polypeptides of 63, 64 and 87 aa were also observed in the coding strand. The 2715 nt ORF featured a Kozak consensus sequence for strong eukaryotic translation, but the smaller ORFs did not. SLOV T2 has a predicted molecular mass of 103.3 kDa, a predicted pI of 6.51 and contains seven cysteine residues, none of which is predicted to form disulphide bonds.

A nucleotide–nucleotide BLAST search of segment 2 returned no hits, but a translated BLAST search returned 95 hits, including 84 to orbivirus inner core protein (T2) sequences, confirming that SLOV segment 2 encodes T2 (VP2). The protein identity of these alignments ranged from 23 % for SCRV to 44 % for PHSV. These figures are well below the 91 % threshold for species demarcation within the genus *Orbivirus*, indicating that SLOV represents a new orbivirus species.

The first four and last five nucleotides (5′ and 3′ ends, respectively) were conserved between segments 1 and 2 of SLOV (Fig. 3). The terminal nucleotides (GUU...UAC) were identical to those found in most other orbiviruses. Phylogenetic trees generated from the amino acid sequences of VP1 and T2 placed SLOV on a distinct branch, distant from other orbivirus species but within a cluster that includes YUOV, PHSV, Corriparta virus, Broadhaven virus (BRDV) and Wongorr virus (Figs 4 and 5).

## Identification of additional isolates of SLOV

In total, 300 unidentified viral isolates from the Northern Territory and Western Australia were screened for SLOV isolates using a rapid molecular diagnostic test (RT-PCR). The collection consisted of 190 isolates from sentinel cattle and 110 from insects. From these, six additional isolates of SLOV were identified, four of which were isolated from pooled *C. annulirostris* mosquitoes (Table 1). K23270 was collected near Broome in 1995, P5224 was collected from Newman in 1997, and K49472 was collected from Stretch Lagoon in 2002, all in Western Australia. DPP5368 was collected near Oenpelli, Northern Territory, in 2001. K50877 was isolated from pooled *Aedes normanensis* mosquitoes caught at Stretch Lagoon in 2003. CSIRO1747 was isolated from *Culex orbostensis* mosquitoes caught near Peachester, Queensland, in 1984. A map showing these locations is given in Fig. 6.

## Multiple genotypes of SLOV

PCR primers designed from sequences obtained from the prototype isolate of SLOV (K49460) were used to obtain fragments of sequence data from additional isolates of SLOV to look for evidence of genetic variation within the species. Sequences obtained from isolate K50877 revealed substantial nucleotide differences relative to the prototype within the gene encoding VP4 (helicase). Over a 540 nt region of VP4, K50877 was 13 % different at the nucleotide

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    5′UTR                                                                  3′UTR
VP1 GUUUUAGUUGCCAUG.....................................UAGUCGAGUGGCAACAAAGGAUAC
VP2 GUUUAAGAUGACUCAGCAAUG...UGAGCGAGGGACUUCAGCCACCCCACGUUUCUCCUAGAAGGAGGGUCAUCAAAAGAUAC
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**Fig. 3.** Alignment of the terminal UTRs of SLOV segments encoding VP1 and VP2. Conserved terminal nucleotides are highlighted in bold. Start and stop codons are underlined.

level and 3 % different at the amino acid level. In contrast, VP1, VP2 (T2), VP6 and VP7 showed only 1–2 % difference at the nucleotide level and 0–2 % difference at the amino acid level.

CSIRO1747 was substantially different from the prototype isolate of SLOV (K49460) within the genes encoding VP1 (polymerase), VP2 (T2) and VP7 (core surface protein). Sequences obtained from these genes had a significant degree of difference at the nucleotide level (9, 8 and 14 %, respectively). VP7 was 5 % different at the amino acid level, whereas VP1 and VP2 were less than 1 % different from the prototype at the amino acid level.

### Serological survey

A collection of sera from livestock and wildlife living in the Northern Territory was screened by a virus neutralization test to detect neutralizing antibodies to SLOV. The serum panel consisted of samples from 670 cattle, 109 goats, 107 horses, 96 crocodiles, 80 bats, 60 dogs, 32 donkeys, 24 camels, 23 banteng (*Bos javanicus*, Indonesian cattle), 15 kangaroos, 13 birds and small numbers (<10) from antelope, cane toads, cats and deer. Sera showing evidence of neutralization at an initial low dilution were then titrated in a confirmatory test. A notable number of sera from horses (19/107), donkeys (15/32) and goats (6/109) were able to neutralize SLOV (Table 2). A small number of sera from other species (cattle, camel, bat and antelope) had weak or incomplete neutralization titres. To control for non-specific neutralization effects, all positive serum samples were also screened against a distantly related orbivirus (MPOV) and found to be negative (not shown). Although all of the observed positive titres were low, the fact that sera from multiple individuals of the same species tested positive and performed correctly in the controls suggests that the results were genuine cases of SLOV-specific neutralization.

## DISCUSSION

Orbivirus species are genetically defined by the presence of 91 % homology in the T2 amino acid sequence (Attoui *et al.*, 2001). The observation that SLOV T2 aligned with a maximum of 44 % identity to only one sequenced orbivirus, PHSV, and did not react with sera against other orbiviruses, suggests that SLOV represents a new orbivirus

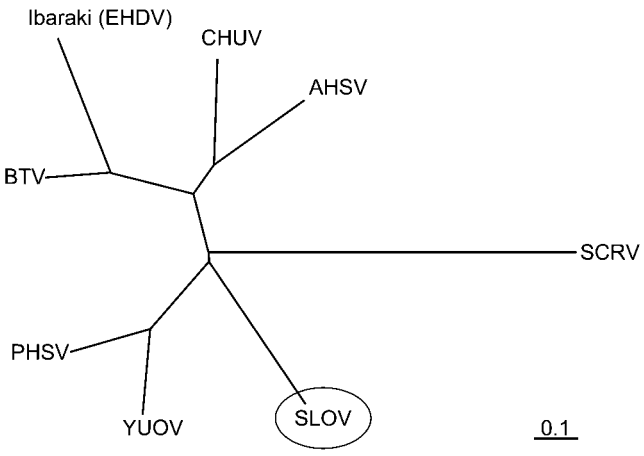


**Fig. 4.** Phylogenetic tree of the genus *Orbivirus* based on full-length VP1 amino acid sequences. The loose ends of the multiple sequence alignment were trimmed prior to generating the distance matrix. The scale bar represents relative genetic distance in nucleotide substitutions per site. GenBank accession nos: SLOV, EU718677; YUOV, NC_007656; PHSV, NC_007748; BTV, NC_006023; Ibaraki virus (EHDV), AB186040; CHUV, NC_005990; AHSV, NC_006021; SCRV, NC_005997.

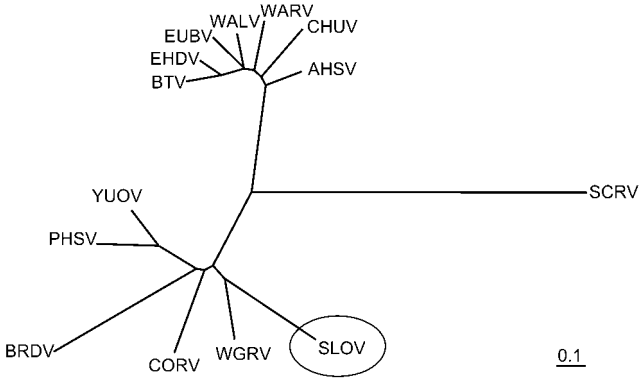


**Fig. 5.** Phylogenetic tree of the genus *Orbivirus* based on partial T2 amino acid sequences. The analysis was performed on a 255 aa region relative to residues 389–643 of SLOV T2. The scale bar represents relative genetic distance in nucleotide substitutions per site. GenBank accession nos: SLOV, EU718677; WGRV (Wongorr virus), U56994; CORV (Corriparta virus), AF530086; BRDV, M87875.1; PHSV, NC_007749; YUOV, NC_007657; BTV, NC_006014; EHDV, AB078629; EUBV (Eubenangee virus), AF530087; WALV (Wallal virus), AF530084; WARV (Warrego virus), EF213555; CHUV, NC_005989; AHSV, NC_006017; SCRV, NC_005998.

**Table 1.** Summary of isolates of SLOV identified by RT-PCR and other methods

| Isolate | Date of collection | Location of collection | Mosquito species | No. in pool |
|---|---|---|---|---|
| CSIRO1747 | 1984 | Peachester, Queensland | *Culex orbostensis* | 66 |
| K23270 | 24 April 1995 | Roebuck Station, Broome, Kimberley, Western Australia | *Culex annulirostris* | 25 |
| P5224 | 7 March 1997 | Fortescue River crossing, Newman, Pilbara, Western Australia | *C. annulirostris* | 25 |
| DPP5368 | 12 July 2001 | Oenpelli, Northern Territory | *C. annulirostris* | 25 |
| K49460* | 6 April 2002 | Stretch Lagoon, Kimberley, Western Australia | *C. annulirostris* | 25 |
| K49472 | 6 April 2002 | Stretch Lagoon, Kimberley, Western Australia | *C. annulirostris* | 25 |
| K50877 | 15 March 2003 | Stretch Lagoon, Kimberley, Western Australia | *Aedes normanensis* | 25 |

*K49460 is the prototype isolate of SLOV.

species. However, pending T2 sequence analysis of all known orbiviruses, we cannot know whether other examples of this species have already been isolated.

YUOV, BRDV and SCRV have a T2=VP2 arrangement, as opposed to BTV, EHDV, African horse sickness virus (AHSV) and Chuzan virus (CHUV), which have a T2=VP3 arrangement. Insufficient information is available to determine the arrangement of the remaining orbiviruses. Based on its position in the phylogenetic tree, SLOV is predicted to follow the T2=VP2 arrangement and we have used the arrangement of the YUOV genome to model the organization of the SLOV genome. This will be resolved once the remaining genome segments of the prototype isolate of SLOV have been sequenced.

Although T2 is considered to be the most conserved gene amongst orbiviruses, the VP1 protein of SLOV was more similar to the other sequenced orbiviruses than its T2 protein. Based on partial sequence data from three genome segments, isolate CSIRO1747 appears to be a distinct second genotype of SLOV. Isolate K50877 is markedly different from other SLOVs in the sequence encoding VP4 (helicase), considered to be one of the most conserved genes amongst orbiviruses (Mertens *et al.*, 2005), which may be evidence of segment reassortment. As we were unable to compare all ten gene segments between the different viruses at this stage, additional differences may yet be identified. In particular, the sequence of the major outer coat protein (VP3) may vary substantially among isolates.

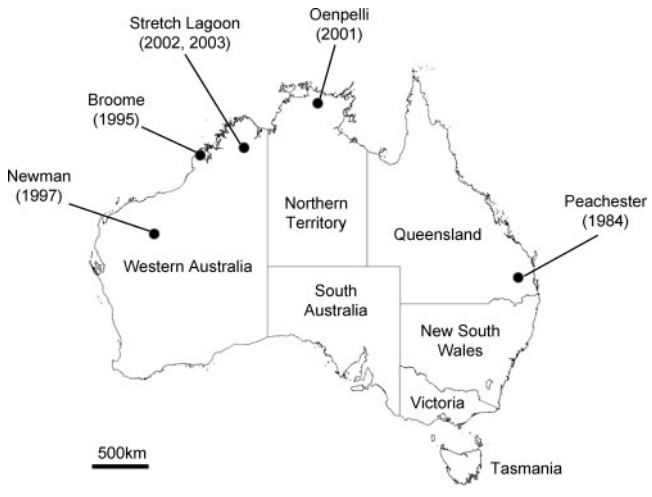


**Fig. 6.** Map of Australia featuring locations referred to in the text. The years when SLOV was isolated at each location are shown in parentheses.

Horses, donkeys and goats were identified as potential vertebrate hosts for SLOV in the serological survey. None of these species is routinely tested for arboviruses in Australia, so it is not surprising that this virus has not been isolated or identified previously in these species, if it is indeed present in them. The observed antibody titres were quite low; however, given the genetic diversity that has been seen in some isolates, it is possible that these animals may have been infected with a strain of SLOV with antigenic differences. SLOV has been isolated from three different species of mosquitoes, but we cannot conclude from this finding that any of these species are true vectors of SLOV. The insect virus collection is subject to severe sampling bias, as it contains a limited range of insect species and covers a limited sampling period and geographical range. Furthermore, vector competency must be determined experimentally. SLOV was isolated on five separate occasions from *C. annulirostris*; however, this is the most common mosquito species in Australia.

In summary, the characterization of SLOV has revealed the existence of a previously undescribed orbivirus species circulating in northern Australia. The development of a rapid diagnostic test permitted identification of additional uncharacterized viral isolates and can now be used for routine identification of future isolates. An isolate from southern Queensland (CSIRO1747) showed that SLOV has been present in Australia since at least 1984, and isolations as recently as 2006 show that it is still in circulation. Serology suggests that SLOV may be infectious in horses, donkeys and goats. In addition, substantial genetic variation was observed in some of the isolates, indicating that SLOV may consist of a group of related viruses, potentially representing a single gene pool.

**Table 2.** List of serum samples that tested positive for antibodies to SLOV by virus neutralization test in a multi-species serological survey

Titres indicate the highest dilution of sera that was able to neutralize 100 $TCID_{50}$ SLOV at 5 days p.i. Sera that caused partial neutralization of SLOV are also indicated.

| Species | Years positive sera collected | No. positive sera (complete neutralization) | Titre range | No. positive sera (partial neutralization) | Total no. positive sera/no. tested |
|---|---|---|---|---|---|
| Horse | 2000, 2001, 2002, 2006 | 10 | 5–10 | 9 | 19/107 (17.8 %) |
| Donkey | 2000, 2003, 2005 | 11 | 5–20 | 4 | 15/32 (46.9 %) |
| Goat | 2000, 2002, 2003, 2004, 2006 | 3 | 5–10 | 3 | 6/109 (5.50 %) |
| Cattle | 1995, 1996, 1997 | 1 | 5 | 2 | 3/670 (0.45 %) |
| Bat | 2001 | 0 | – | 1 | 1/80 (1.25 %) |
| Camel | 2000 | 0 | – | 1 | 1/23 (4.35 %) |
| Antelope | 2002 | 0 | – | 1 | 1/4 (25.0 %) |

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