

Genomic and phylogenetic characterization of Leanyer virus, a novel orthobunyavirus isolated in northern Australia

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Leanyer virus (LEAV), currently classified as a member of the genus *Orthobunyavirus*, in the family *Bunyaviridae*, was originally isolated from a pool of *Anopheles meraukensis* mosquitoes, collected at Leanyer, Northern Territory, Australia in 1974. When it failed to react in serological tests with antisera from other known viruses, full-length genomic sequencing was pursued to determine the relationship of LEAV to other orthobunyavirus species. Genetic and serological characterization confirmed its antigenic distance from other orthobunyaviruses, including to its closest genetic neighbours, the Simbu group viruses, suggesting that it may represent a new antigenic complex.

INTRODUCTION

The family *Bunyaviridae* constitutes one of the largest taxonomic groupings of RNA viruses, containing more than 350 viruses. The family *Bunyaviridae* (commonly known as bunyaviruses) comprises five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus* and *Tospovirus*, based on serological and molecular characteristics (<http://www.ictvonline.org/virusTaxonomy.asp?version=2008>). All members of the family share certain common characteristics including: (i) a tri-segmented genome; (ii) a negative or ambisense coding strategy; (iii) four structural proteins; (iv) cytoplasmic replication; and (v) assembly and maturation at the Golgi apparatus. Viruses within each genus share similar segment and structural protein sizes, and nucleotide sequences at the 3' and 5' termini of each segment.

Viruses in the genera *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* are transmitted by arthropods (mosquitoes, midges, sandflies, ticks or thrips). In contrast, viruses in the genus *Hantavirus* are not transmitted by arthropods, but are acquired by aerosol exposure to virus-contaminated excreta or by a bite from their rodent or shrew hosts (Nichol *et al.*, 2005; Ramsden *et al.*, 2009). Viruses that impinge on human health, either directly by causing illness, or indirectly by causing disease and economic loss in domestic animals or crop plants, are found in each of the five genera (Elliott, 1997; Nichol *et al.*, 2000).

The largest genus in the family is the *Orthobunyavirus*, which currently contains more than 170 viruses, assigned to 48 distinct species based on serological relatedness by complement fixation (CF) test (mediated by the N protein) or haemagglutination inhibition (HI) and neutralization tests (mediated by glycoproteins) (Nichol *et al.*, 2005). Despite the association of many of the orthobunyaviruses with human and animal disease, molecular characterizations of the entire genus has been limited to four serological groups, namely California encephalitis, Bunyamwera, Group C and Simbu (Bowen *et al.*, 1995; Dunn *et al.*, 1994; Nunes *et al.*, 2005b; Saeed *et al.*, 2000). These antigenic complexes bring

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Three supplementary tables and a supplementary figure are available with the online version of this paper.

together several viruses (e.g. Guaroa, Kairi and Bunyamwera species are all grouped in the Bunyamwera complex). Because of the paucity of genetic data for many of the orthobunyaviruses, taxonomic placements must be regarded as fluid, since antigenic relatedness among segmented viruses may vary depending on the particular serological test used or because of natural reassortment among closely related viruses.

Leanyer virus (LEAV) was initially believed to be a member of the family *Togaviridae* based on virion size (Doherty *et al.*, 1977), but was later shown to be morphologically consistent with a bunyavirus (Stuckly & Wright, 1983). Initial analysis of partial sequences showed LEAV to be divergent compared with other orthobunyaviruses, making it an interesting target for further characterization. Here, we describe rapid full-length genome sequencing of LEAV, and provide evidence that it represents a new orthobunyavirus species in a previously unidentified antigenic complex.

RESULTS

Serology

A list of antibodies specific to each viruses' proteins used for serological analysis of LEAV is given in Table 1. In summary, LEAV mouse brain antigen (haemagglutinin titre 1:640 at pH 5.75–6.0) reacted in HI tests with a hyperimmune mouse LEAV antibody (homologous titre 1:5120), but it failed to react with antibodies to any of the other viruses shown in Table 1. Furthermore, LEAV antibodies failed to react with Oropouche virus (OROV), Aino virus (AINOV), Akabane virus (AKAV), Koongol virus (KOOV), Wongal virus (WONV) and Tete virus (TETEV) antigens in CF tests (Supplementary Table S1,

available in JGV Online) and failed to react with OROV and WONV in HI tests (Supplementary Table S2, available in JGV Online). LEAV antibodies also failed to detect proteins from AINOV and OROV in Western blot (data not shown).

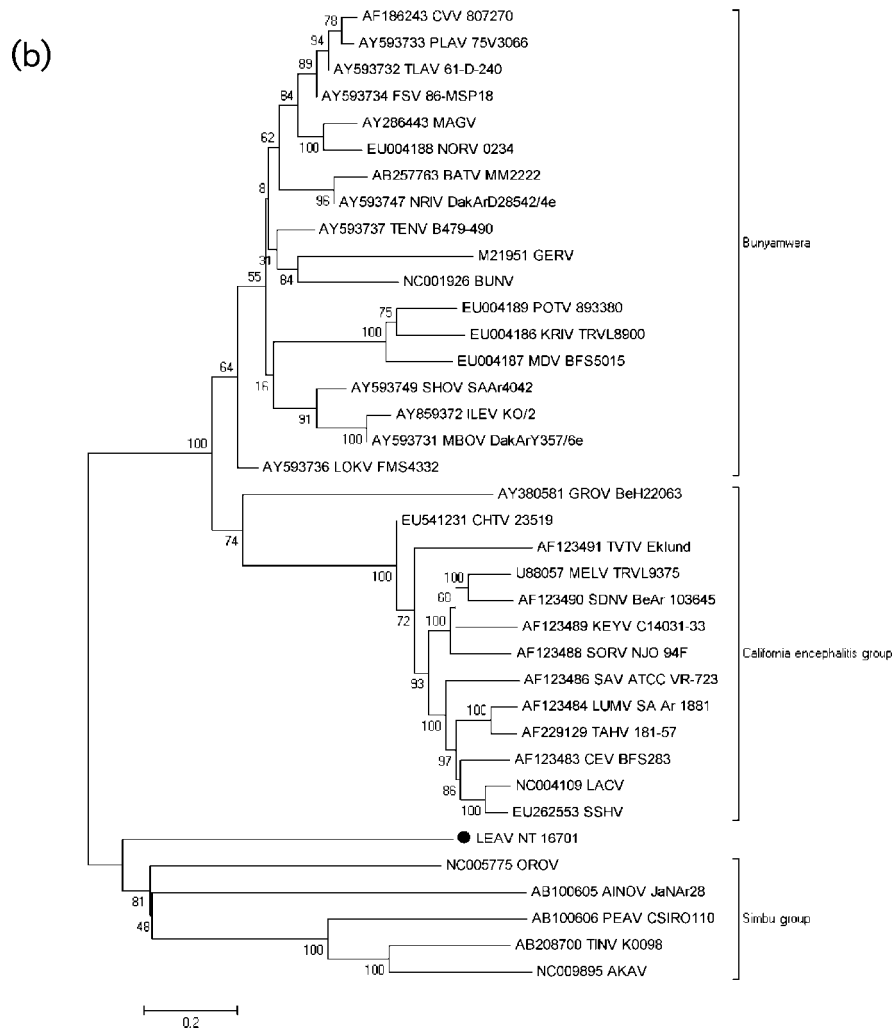
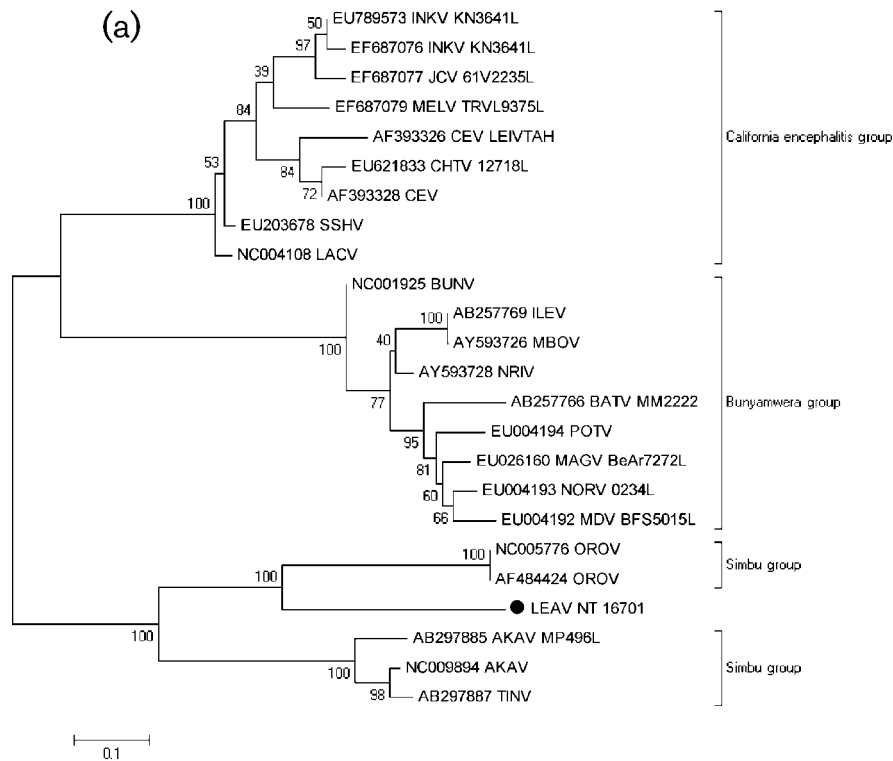
Sequence acquisition and analysis

Consistent with the genomic organization of orthobunyaviruses (Gentsch *et al.*, 1977; Gentsch & Bishop, 1978; Obijeski *et al.*, 1976a, b), the genome of LEAV comprises three RNA segments: a large (L) segment that encodes a large (L-) RNA-dependent RNA polymerase (RdRp)-related ORF in the negative-sense orientation (GenBank accession no. HM627178); a medium (M) segment that encodes the polyprotein (M) in the negative-sense orientation (GenBank accession no. HM627176); and a small (S) segment that encodes a nucleocapsid protein (NP) and a non-structural protein (NSs), both in the negative-sense orientation, but in different ORFs (GenBank accession no. HM627177). The results of the phylogenetic analyses of the L, M and NP ORFs indicate that LEAV is distantly related to members of the Simbu serogroup. It is most closely related to OROV in all three amino acid trees (Fig. 1a–c, respectively). Nucleotide trees show similar topology, though interpretation is limited given sequence divergence (Supplementary Fig. S1). No evidence for reassortment was detected.

LEAV sequences were compared with published orthobunyavirus sequences for members of Bunyamwera, California encephalitis, Simbu and Group C complexes to determine mean intragroup and intergroup distances, using strategies accepted for other virus groups (Collao *et al.*, 2009; Ward *et al.*, 1992). The intragroup and intergroup p-distances were clearly distinguishable for the polymerase and the nucleoprotein (Fig. 2a, b, respectively).

Table 1. Antisera tested by serology (HI and/or neutralization test) against LEAV at the Queensland Institute of Medical Research and University of Texas Medical Branch

<i>Alphavirus</i>	<i>Flavivirus</i>	<i>Bunyaviridae</i>	<i>Orthobunyavirus</i>	<i>Orbivirus</i>	<i>Orthomyxovirus</i>	<i>Rhabdoviridae</i>	<i>Lyssavirus</i>	<i>Nairovirus</i>
Getah	Alfuy	Belmont	AINOV	Corriparta	Dhori	Alpimwar	Bovine ephemeral fever	Kao Shuan
Ross River	Edge Hill	Gan Gan	AKAV	D'Aguillar	Johnston Atoll	Charleville		Taggart
Sindbis	Kokobera	Kowanyama	Facey's Paddock (Ch 16129)	Eubenangee	Upolu	Ngaingan		
Murweh (Ch 16313)	Kunjin	Maprik	KOOV	Mitchell River				
	Murray Valley encephalitis	Mapputta	OROV	Mudjinbarry				
	Saumarez Reef	Trubanaman	PEAV	Nugget				
	Stratford		TETEV	Tilligerry				
			WONV	Wallal				
			Yacaaba	Warrego				



(c)

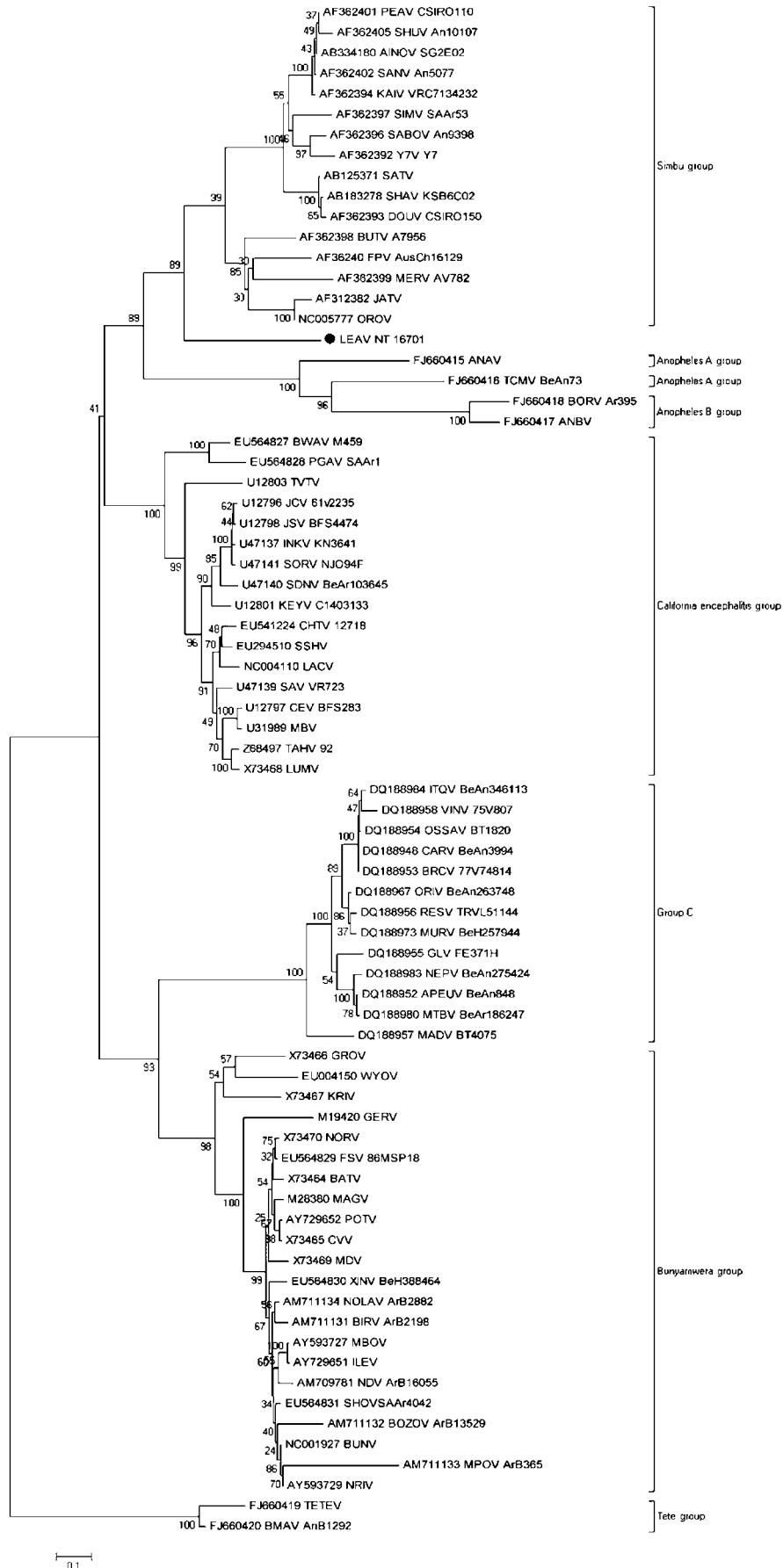


Fig. 1. Phylogenetic analysis of the (a) polymerase, (b) polyprotein and (c) nucleoprotein of orthobunyaviruses. A set of all complete and partial sequences from GenBank were aligned using the CLUSTAL algorithm (as implemented in the MEGA package version 3) at the amino acid level for the L, M and S segments with additional manual editing to ensure the highest possible quality of alignment. A set of these sequences representing different serogroups were used for analysis, with partial sequences removed for correct tree topology. Neighbour-joining (NJ) analysis at the amino acid level was performed given the high observed variability of the nucleotide sequences. Statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1000 times (bootstrap values are shown next to the branches). Phylogenetic analyses were performed using MEGA software (Kumar *et al.*, 2004). LEAV is marked with a black dot. Bars indicate amino acid substitutions per site.

Cut-offs of 59 and 60% similarity were established, respectively, at the amino acid level. However, some overlap was observed in the polyprotein. The mean similarity between LEAV and Simbu viruses was $47.6 \pm 3.5\%$ and $53.7 \pm 3.1\%$ in the polymerase and the nucleoprotein, respectively. Comparison between LEAV and

OROV and AINOV showed 59 and 36.4% similarity in the polymerase and polyprotein, respectively, with OROV and 30.9% with the AINOV polyprotein. These values are in the range of similarity values observed between species belonging to different antigenic groups (Table 2).

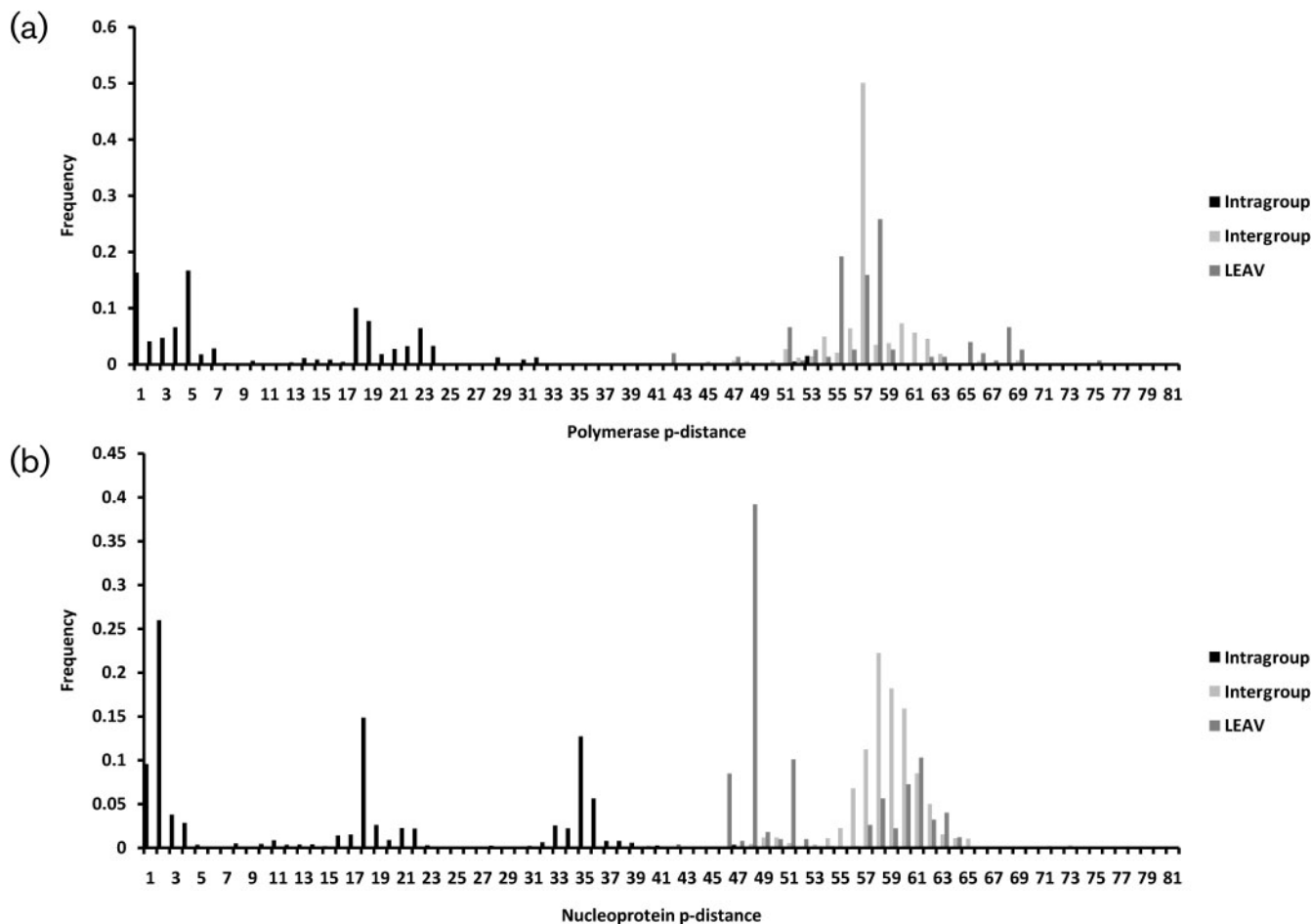


Fig. 2. Pairwise sequence analysis for polymerase (a) and nucleoprotein (b). The sequence of LEAV was compared with published orthobunyavirus sequences to determine intergroup and intragroup mean p-distances and to classify LEAV. Calculations were performed using p-distance at the amino acid level. P-distance values were grouped into three groups: intragroup, distances among different viruses belonging to the same serogroup (black); intergroup, distances between members of different viruses belonging to different serogroups (pale grey); and distance between LEAV and other viruses in the genus (dark grey).

Table 2. Nucleotide (and amino acid) sequence difference values (%) between LEAV and viruses of recognized orthobunyavirus serogroups

NA, Not applicable.

Comparison	L segment	M segment	S segment
Within Simbu	13.0 ± 0.6 (9.3 ± 0.8)	21.0 ± 0.3 (20.7 ± 0.4)	16.9 ± 0.7 (15.0 ± 1.2)
Within Bunyamwera	24.1 ± 1.1 (17.0 ± 1.7)	31.4 ± 0.8 (30.2 ± 1.5)	22.9 ± 0.9 (20.8 ± 1.3)
Within Group C	NA	26.0 ± 1.6 (22.6 ± 2.6)	16.4 ± 0.9 (9.3 ± 1.2)
Within California encephalitis	16.9 ± 1.3 (10.9 ± 1.7)	22.3 ± 0.9 (16.9 ± 1.4)	14.7 ± 0.7 (10.8 ± 1.2)
Between LEAV and Bunyamwera	50.5 ± 1.3 (61.8 ± 3.0)	50.0 ± 1.2 (64.1 ± 2.4)	49.6 ± 1.6 (59.1 ± 2.9)
Between LEAV and California encephalitis	46.6 ± 1.7 (57.2 ± 3.4)	50.5 ± 1.8 (61.7 ± 3.4)	46.0 ± 1.6 (58.2 ± 3.0)
Between LEAV and Group C	NA	47.2 ± 2.4 (60.0 ± 4.1)	50.0 ± 1.7 (61.4 ± 3.0)
Between LEAV and Simbu	45.3 ± 1.8 (52.4 ± 3.5)	54.1 ± 0.6 (67.8 ± 1.2)	40.8 ± 1.5 (46.7 ± 3.1)
Between Bunyamwera and California encephalitis	45.2 ± 1.8 (55.0 ± 3.4)	43.7 ± 1.2 (50.0 ± 2.6)	46.0 ± 1.4 (54.6 ± 2.7)
Between Bunyamwera and Group C	NA	42.7 ± 1.9 (51.7 ± 3.6)	45.5 ± 1.4 (55.1 ± 2.8)
Between Bunyamwera and Simbu	49.0 ± 1.4 (57.3 ± 3.3)	53.9 ± 0.8 (68.4 ± 1.7)	49.9 ± 1.4 (59.3 ± 2.8)
Between California encephalitis and Group C	NA	46.0 ± 2.0 (56.5 ± 3.9)	44.8 ± 1.5 (54.8 ± 2.9)

ORFs

Large RdRp (L). The 2260 aa LEAV viral RdRp (264 kDa, pI=6.4) is similar in size to other orthobunyavirus reference strains falling between the 2238 aa Bunyamwera virus (BUNV) and the 2263 aa La Crosse virus (LACV) L proteins. Certain areas overlap conserved regions among all orthobunyaviruses, suggesting an association with function; region I is located in the amino terminus and is centred on aa P₇₅D; region II, also located in the amino terminus, is centred on aa R₆₅₁Y. Regions I and II have been found to also be conserved among all bunyaviruses (Müller *et al.*, 1994). Region III (948–1239) is located in the centre of the protein and contains the polymerase motifs that comprise the polymerase module [A (1045–1062), B (1129–1151), C (1170–1184), D (1214–1225)] found in all RNA-dependent polymerases ranging from the RdRp encoded by reovirus elements to the RdRp encoded by the positive, negative and dsRNA viruses (Poch *et al.*, 1989; Xiong & Eickbush, 1990). The pre-A (948–977) and E motifs (1228–1239) identified in region III by Müller *et al.* (1994) were also found in LEAV. The fourth conserved region (1240–1343), identified by Aquino *et al.* was also conserved in LEAV (Fig. 3) (Aquino *et al.*, 2003).

Polyprotein (M). The 1419 aa LEAV polyprotein (161.8 kDa, pI=8.4) is cotranslationally cleaved into the 285 aa Gn (32.7 kDa, pI=8.9), 947 aa Gc (107.9 kDa, pI=6.7) and 173 aa NSm (19.7 kDa, pI=9.2). Stuckely and Wright determined the Gn and Gc to be 35 and 115 kDa, respectively, both within 10% of our predictions and similar in size to the BUNV (Stuckely & Wright, 1983). When compared with other orthobunyaviruses reference strains and AINOV, the M proteins of LEAV are comparably sized (Table 3) (Wang *et al.*, 2001; Yanase *et al.*, 2003).

Since the Gn is poorly conserved among bunyaviruses, this is the area that is least conserved of the whole polyprotein.

The N-terminal sequence is consistent with a functional signal peptide for membrane translocation (Blobel & Dobberstein, 1975; Lingappa *et al.*, 1978; von Heijne, 1988) similar to those of other viruses in the genus (Fazakerley *et al.*, 1988). Cleavage of the signal peptide between aa 14 and 15 with respect to the first methionine is compatible with conservation of terminal amino acid tripeptides (Lees *et al.*, 1986). Prediction of signalase cleavage by SignalP 3.0 between aa 14 and 15 supports this view. The Gn contains the conserved arginine in position 299 suggesting cleavage of the mature Gn from the downstream NSm at the carboxy terminus of the Gn is probably mediated by an enzyme that has specificity for basic residues (Fazakerley *et al.*, 1988). There is little conservation near the NSm/Gc junction, so a potential cleavage site, possibly executed by signalase (Fazakerley *et al.*, 1988), is not obvious. Cleavage after a conserved alanine residue (A₄₇₂) analogous to the termination of NSm in the California serogroup viruses (Campbell & Huang, 1999) is possible. SignalP predicts cleavage at VVA₄₇₂-EI, which would result in -3=V and -1=A, one of the most frequent combinations in signalase sites. Although close to a potential glycosylation site, it is greater than the 'minimum glycosylation distance' of 13 aa that has been determined for cleaved internal signals (Nilsson *et al.*, 1994).

Six potential glycosylation sites were identified using NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services>), two in the Gn, one in the NSm and three in the Gc. Two in the Gn and one in each of the NSm and Gc are unique. The glycosylation site in the amino terminus of the Gc is conserved among Peaton virus (PEAV), Tinaroo virus (TINV) and AKAV and one in the Gc is conserved among AKAV and AINOV. Only one glycosylation site in the carboxy terminus of the Gc is conserved with OROV. PEAV, TINV, AKAV, AINOV and OROV are all Simbu serogroup viruses (Fig. 4).

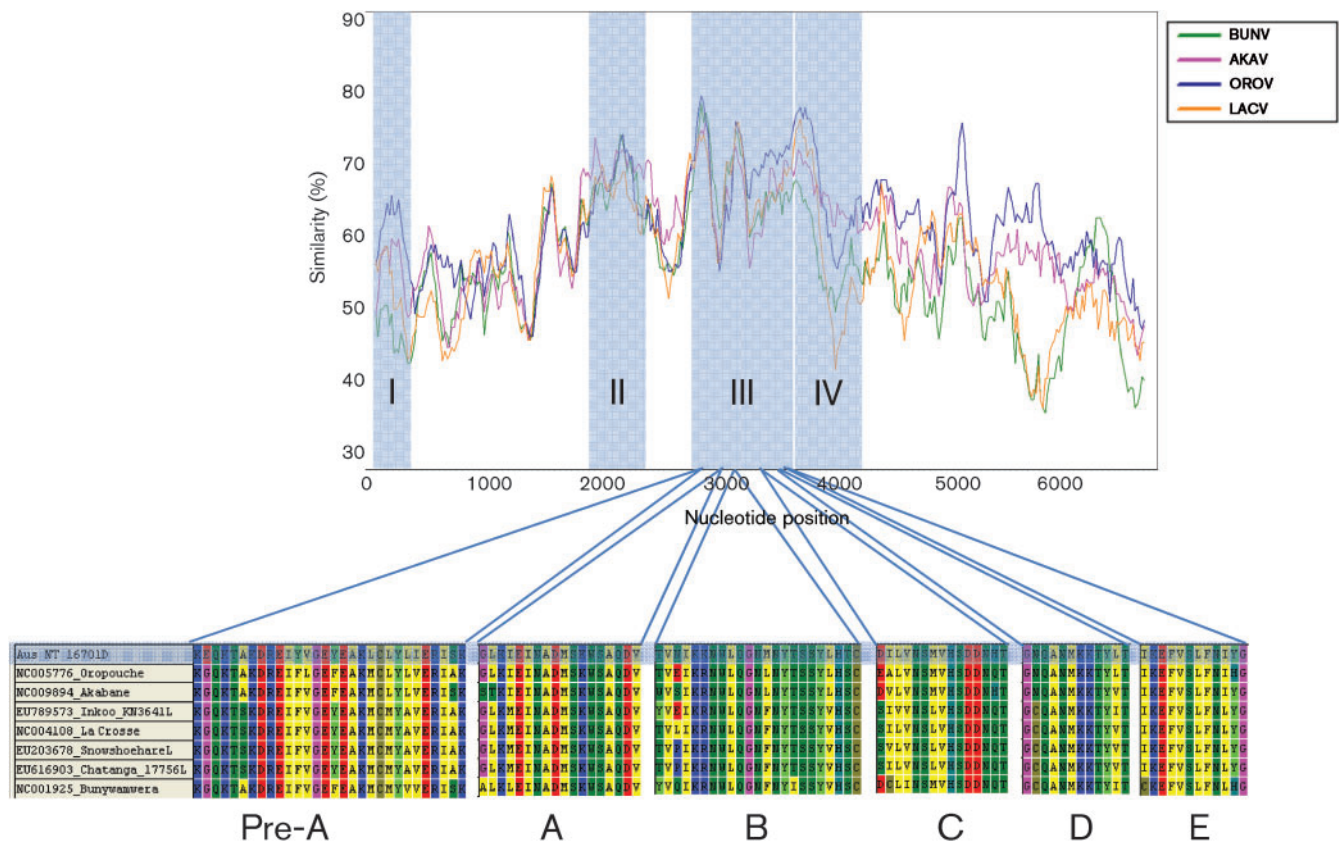


Fig. 3. Large RdRp (L) conserved regions. Simplot analysis identifies regions I–IV with the pre-A, A, B, C, D and E motifs shown from region III.

Prediction of transmembrane regions using the transmembrane hidden Markov model in the program TopPred0.01 (<http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred>), predicts eight transmembrane regions: two in the Gn (200–220 and 223–243), three in the NSm (306–326, 357–377 and 454–474) and three in the Gc (956–976, 1046–1066 and 1375–1395). The two transmembrane regions in the Gn together form a very long hydrophobic sequence (200–243), which are subsequently followed by charged amino acids (244–252) similar to the stop-transfer sequences seen in the transmembrane domains of other viral envelope proteins (Garoff *et al.*, 1980; Jou *et al.*, 1980; Rose *et al.*, 1980). The transmembrane region predicted at position 1375–1395 in

the Gc acts as a potential membrane anchor (Fazakerley *et al.*, 1988; Pekosz *et al.*, 1995) (Fig. 4).

The LEAV Gn protein consists of 299 aa with a predicted cytoplasmic tail (CT) of 69 residues. The Gc consists of 961 aa and has a CT of 24 aa. These values are consistent with predictions of the CT for BUNV, the prototype of the genus *Orthobunyavirus* (Elliott, 1990; Lees *et al.*, 1986).

The fusion peptide identified in LACV from aa 1066–1087 is conserved in LEAV from aa 1043–1064, suggesting that the Gc of LEAV acts as a class II fusion protein, similar to the E1 fusion peptide of the alphaviruses, Sindbis virus and Semliki Forest virus (Plassmeyer *et al.*, 2007). None of the six epitopes identified in California encephalitis group viruses by Cheng *et al.* (2000) are conserved in LEAV. The overall topology of the virus appears to be well conserved, as indicated by the conservation of 71 cysteines with all other orthobunyaviruses (Grady *et al.*, 1987; Lees *et al.*, 1986; Pardigon *et al.*, 1988), 17 in the Gn, 10 in the NSm, and 44 in the Gc (Fig. 4).

Table 3. M segment protein sizes

Virus name	Gn size (aa)	NSm size (aa)	Gc size (aa)
LEAV	285	173	947
LACV	277	173	967
BUNV	292	174	955
OROV	290	175	939
AKAV	292	156	936
AINOV	291	155	941

NP and NSs proteins. The LEAV 235 aa NP (26.3 kDa, pI=8.9) is consistent with Stuckley and Wright’s NP of 29 kDa (Stuckley & Wright, 1983). It shows between 24 (TETEV) and 60% (AINOV) conservation at the amino

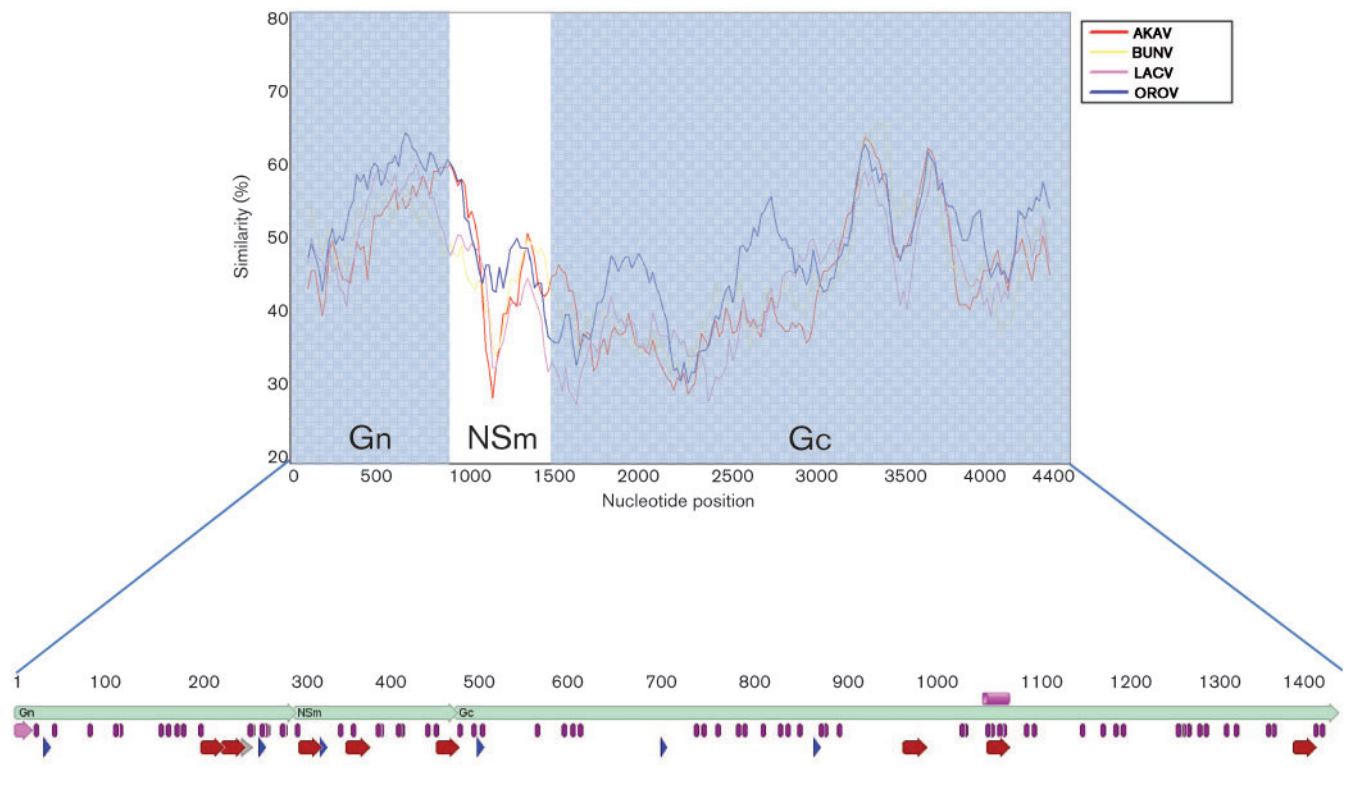


Fig. 4. Polyprotein functional regions. Transmembrane regions (red arrows), glycosylation sites (blue arrows), signal peptide (pink), charged amino acids (grey arrow) and cysteines (purple) are all indicated. The pink cylinder represents the conserved fusion peptide sequence.

acid level and between 40 (TETEV) and 63 % (AINOV) at the nucleotide level to other orthobunyaviruses, thus satisfying the minimum 10 % divergence requirement of the International Committee for Taxonomy of Viruses (ICTV) in the NP for a novel species. Interestingly, of the six conserved regions identified in the Simbu serogroup viruses by Saeed *et al.* (2001), regions two through to six are well conserved with NT 16701 (the prototype strain of LEAV), whereas region one is only somewhat conserved. There are also several individual amino acids that have been identified as being globally conserved in the NP among the four major serogroups: Buyamwera, California, Group C and Simbu. There are 46 positions that are strictly conserved in all 51 viruses in these four groups, while a further 14 are conserved in at least 45 (90 %) of the N protein sequences (Eifan & Elliott, 2009). These residues are presumably critical for the N protein function. Of the 60 conserved amino acids, 50 are conserved in LEAV. The four residues (P₁₂₅, G₁₃₁, Y₁₅₈ and I₂₃₁) involved in formation of ribonucleoprotein complexes (Eifan & Elliott, 2009) are all conserved in LEAV (Fig. 5). Of the 10 residues identified as involved in RNA synthesis, eight are conserved in NT 16701. LEAV encodes a NSs, as do most other orthobunyaviruses, in a second ORF of the S segment. The 93 aa (10.8 kDa, pI=10.8) falls within the range of NSs sizes of 83–109 residues (Dunn *et al.*, 1994) and may

correspond to the p8 protein identified by Stuckly & Wright (1983). The NSs is poorly conserved when compared to Simbu group viruses, showing between 28 and 37 % similarity at the amino acid level (55–60 % at the nucleotide level).

DISCUSSION

According to the ICTV, a virus belongs to a serogroup if it cross-reacts with members of that group by one or more serological tests (Nichol *et al.*, 2005). Previous studies of Simbu group viruses have demonstrated extensive cross-reactivity through CF tests (Kinney & Calisher, 1981). LEAV does not show cross-reactivity with other orthobunyaviruses and phylogenetic analyses of the M and S segments of LEAV show it to be only distantly related to Simbu serogroup viruses. Furthermore, p-distance frequency calculations demonstrate that differences in amino acids specified by the L and S segments of LEAV and other orthobunyaviruses are consistent with intergroup distances. Although this investigation is based on the limited number of viruses tested, genetic and serological evidence indicate that LEAV represents a new species in the genus *Orthobunyavirus* and may represent a new antigenic complex.

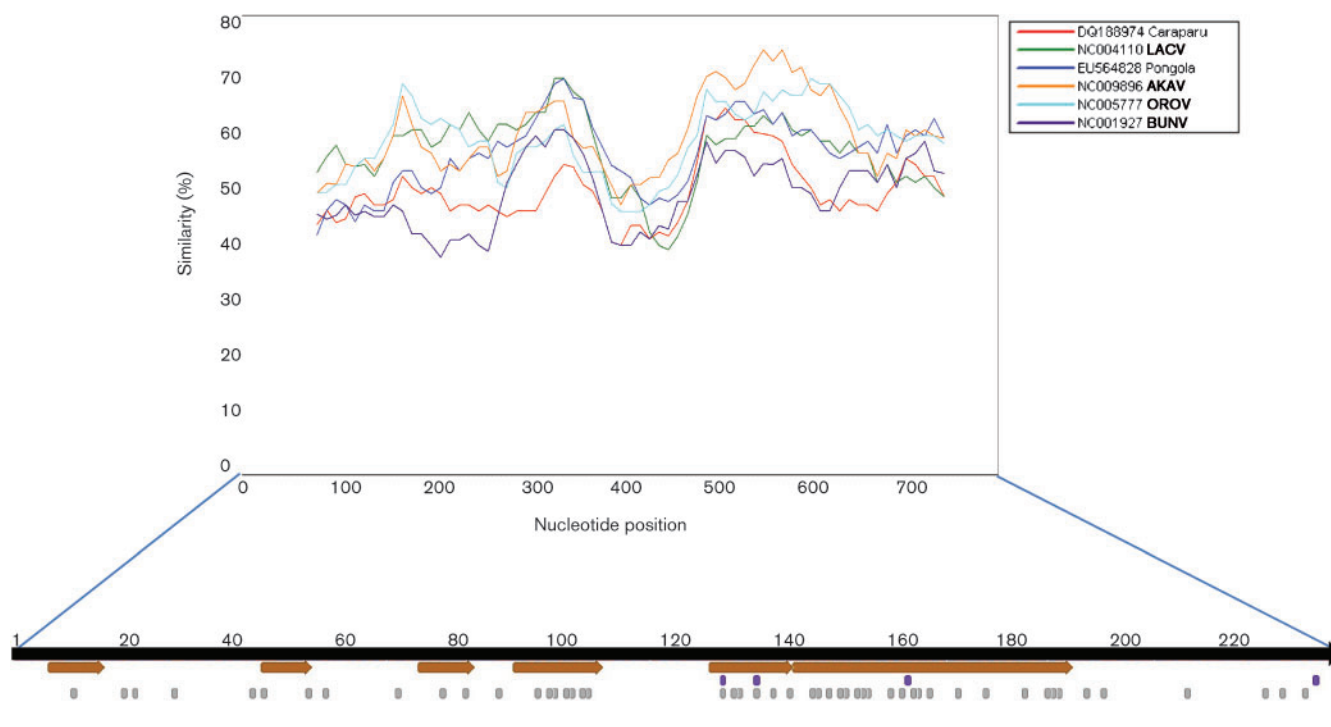


Fig. 5. Nucleocapsid conserved regions. Conserved amino acids among all orthobunyaviruses, which are also conserved in LEAV, are indicated by grey blocks. The orange arrows indicate regions identified by Saeed *et al.* (2001) as being conserved among all Simbu group viruses. The purple blocks represent the four residues involved in the formation of ribonucleoprotein complexes.

Many other Simbu group viruses have been isolated from sentinel cattle and from insects in northern Australia, including AINOV, AKAV, Douglas virus, PEAV and TINV (Gard *et al.*, 1988). AKAV and AINOV have economic and veterinary importance. AKAV causes periodic outbreaks of abortions, stillbirths and congenital malformations in cattle, sheep and goats in Australia, the Middle East and in sub-Saharan Africa (Schmaljohn & Nichol, 2007). AINOV has been associated with abortions, stillbirths and congenital defects in cattle, sheep and goats in Australia and Japan. As the second closest phylogenetic relative in the M, high relative sequence similarity in the N, and geographical overlap, AINOV and LEAV may have evolved from a similar, but distant, ancestor.

Although distantly related, LEAV appears consistently paired with OROV, which has only been isolated in Central and South America (Pinheiro *et al.*, 2004). OROV is recognized as an important cause of acute febrile illness, known as Oropouche fever, among people living in rural and urban communities in tropical South America (Nunes *et al.*, 2005a).

Antibodies to LEAV were detected in nine out of 30 (30%) cattle initially tested by Doherty *et al.* (1977) in the Northern Territory of Australia. Neutralizing antibodies were not detected in a limited survey of humans in northern Australia, but were detected in cattle in Queensland, suggesting a geographical distribution beyond

the Northern Territory (Doherty *et al.*, 1977). Thus, a more extensive survey, with regard to sample size and geography is necessary to better understand the distribution of this virus and its role in human, livestock and wildlife diseases.

METHODS

Virus isolation and antigenic characterization. The prototype strain of LEAV (NT 16701) was originally isolated in newborn mice inoculated intracranially with a clarified homogenate of 100 *Anopheles meraukensis* mosquitoes collected at Leanyer, Northern Territory, Australia in April 1974 (Doherty *et al.*, 1977). It was subsequently reisolated from *Culicoides marksii* at Beatrice Hill, also in northern Australia (Standfast *et al.*, 1984). Initial characterization of NT 16701 was done at the Queensland Institute of Medical Research, Brisbane, Queensland, Australia at the time of isolation. Methods used to prepare antigens for the complement fixation (CF) tests and for making immune ascitic fluids have been described previously (Beatty *et al.*, 1989; Travassos da Rosa *et al.*, 1983; Xu *et al.*, 2007). Antigens and antibodies were both prepared in mice. CF tests were performed by the microtitre technique (Beatty *et al.*, 1989; Xu *et al.*, 2007), using two units of guinea pig complement and overnight incubation of the antigen and antibody at 4 °C. CF titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement. Titres of 1:8 were considered positive. Haemagglutination inhibition (HI) testing was done in microtitre plates as described previously (Travassos da Rosa *et al.*, 1983). HI tests were performed with 4 haemagglutination units of virus at the optimal pH (5.75) against serial twofold antiserum dilutions starting at 1:20. HI titres of 1:20 were considered positive.

By CF, HI and mouse neutralization tests, NT 16701 was found to be antigenically distinct from 40 suspected arboviruses known from Australia and New Guinea at that time (Doherty, 1977; Doherty *et al.*, 1977). Based on these initial studies, NT 16701 was designated a new virus, and named 'Leanyer virus' (LEAV). Negative contrast electron microscopy of LEAV-infected mouse brain showed poorly defined spherical 50 nm diameter particles with dense cores (Doherty *et al.*, 1977). It was later discovered that LEAV virions were approximately 110 nm in diameter, a size consistent with other bunyaviruses (Stuckly & Wright, 1983). A limited survey done by neutralization test with sera from humans and other vertebrates from Australia revealed neutralizing antibodies to LEAV in cattle, wallabies and dogs but not in humans (Doherty *et al.*, 1977). Additional HI tests were done at the University of Texas Medical Branch, Galveston with other orthobunyaviruses and ungrouped bunyaviruses, including OROV and AKAV (Table 1).

Genome sequencing. LEAV was extracted using TRIzol LS (Invitrogen). Total RNA extracts were treated with DNase I (DNA-Free; Ambion) and cDNA was generated using the Superscript II system (Invitrogen) using random hexamers that were linked to an arbitrary 17-mer primer sequence (Palacios *et al.*, 2007). Resulting cDNA was treated with RNase H and then amplified by random PCR (Palacios *et al.*, 2007). Products greater than 70 bp were selected by column purification (MinElute; Qiagen) and ligated to specific adapters for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences) without fragmentation of the cDNA (Cox-Foster *et al.*, 2007; Margulies *et al.*, 2005; Palacios *et al.*, 2008). Software programs accessible through the analysis applications at the GreenePortal website (<http://tako.cpmc.columbia.edu/Tools/>) were used for the removal of primer sequences, redundancy filtering and sequence assembly. Primers were designed using pyrosequencing data to fill gaps in the sequence (Supplementary Table S3, available in JGV Online). Conventional PCRs were performed with BIO-X-ACT polymerase (Bioline) on PTC-200 thermocyclers (Bio-Rad): an enzyme activation step of 5 min at 95 °C was followed by 45 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 68 °C for 1–3 min depending on the expected amplicon size. PCR products were run on 1% agarose gels, gel extracted and purified (MiniElute; Qiagen), and directly sequenced in both directions with ABI Prism Big Dye Terminator 1.1 Cycle Sequencing kits on ABI Prism 3700 DNA Analysers (Perkin-Elmer Applied Biosystems). Terminal sequences were generated using a universal orthobunyavirus primer, targeting the conserved viral termini (5'-AGTAGTGTRCTCCAC-3'). Sequences of the genomes were verified by classical Sanger sequencing using primers designed to create amplicons of ~1000 bp with 500 bp overlap. The assembled data revealed a classical orthobunyavirus genome (GenBank accession nos HM627176, HM627177 and HM627178).

Phylogenetic analysis. A set of orthobunyavirus sequences (151 for the L segment; 243 for the polyprotein M segment and 502 for the nucleocapsid gene) comprising all sequences from GenBank were used to determine the phylogenetic history of LEAV strain NT 16701. All orthobunyavirus sequences were aligned using the CLUSTAL algorithm (as implemented in the MEGA package version 4) at the amino acid level, with additional manual editing to ensure the highest possible quality of the alignment. UPGMA (Unweighted Pair Group Method with Arithmetic Mean) analysis at the amino acid level was performed due to the observed high variability of the underlying nucleotide sequences. Nucleotide phylogenetic trees were also investigated using the neighbour-joining algorithm and the Kimura two-parameter model. The statistical significance of the tree topology was evaluated by bootstrap resampling of the sequences 1000 times. Phylogenetic analyses were performed by using MEGA software (Kumar *et al.*, 2004).

Sequence analysis. Geneious 4.7.5 (Biomatters) was used for sequence assembly and analysis. Topology and targeting predictions were generated by employing SignalP, NetNGlyc, TMHMM (<http://www.cbs.dtu.dk/services>), the web-based version of TopPred0.01 (<http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred>), and integrated predictions in Geneious (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Kahsay *et al.*, 2005; Käll *et al.*, 2004; Krogh *et al.*, 2001).

Pairwise sequence analysis. To establish a potential cut-off for classification of LEAV, we used pairwise sequence comparison to compare its sequences with all published orthobunyavirus sequences. Calculations were performed using MEGA software (Kumar *et al.*, 2004) to calculate the p-distance of the S segment, which is used by the ICTV for demarcation of species, at both the nucleotide and amino acid level using pairwise deletion. Calculations were performed using MEGA software (Kumar *et al.*, 2004) to calculate the p-distance of each segment at the nucleotide level.

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