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

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Summary

Leanyer virus (LEAV), currently classified as a member of the genus *Orthobunyavirus*, 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 neighbors, 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: (1) a tri-segmented genome; (2) a negative or ambisense coding strategy; (3) four structural proteins; (4) cytoplasmic replication; and (5) assembly and maturation at the Golgi apparatus. Viruses within each genus share similar segment and structural protein sizes, and nucleotide (nt) 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 arthropod transmitted, but are acquired by aerosol exposure to virus-contaminated excreta or by bite of 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 test (mediated by the N protein) or hemagglutination-inhibition 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 serologic 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 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 serologic 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 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 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 (HA titer 1:640 at pH 5.75-6.0) reacted in HI tests with a hyperimmune mouse LEAV antibody (homologous titer 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 (OROV), Aino (AINOV), Akabane (AKAV), Koongol (KOOV), Wongal (WONV), and Tete (TETEV) virus antigens in CF (**Supplemental Table 1**) and failed to react with OROV and WONV in HI tests (**Supplemental Table 2**). 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; Obijeski *et al.*, 1976b), the genome of LEAV comprises three RNA segments: a large (L) segment that encodes a large (L-) polymerase-related open reading frame (ORF) in the negative sense orientation (GenBank Accession number HM627178); a medium (M) segment that encodes the polyprotein (M) in the negative sense orientation (GenBank Accession number 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 Number HM627177). The results of the phylogenetic analyses of the L, M, and NP ORFs indicate that LEAV virus is distantly

related to members of the Simbu serogroup. It is most closely related with OROV in all three amino acid trees (**Figure 1A, B, C, respectively**). Nucleotide trees show similar topology, though interpretation is limited given sequence divergence. **(Supplementary Figure 1)**. 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 intragroup and intergroup average 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 (**Figure 2A, B, respectively**). Cut offs of 59% and 60% similarity were established, respectively, at the aa level. However, some overlap was observed in the polyprotein. The average similarity between LEAV and Simbu viruses was 47.6% ± 3.5% and 53.7% ± 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**).

Open Reading Frames (ORFs)

Large RNA-dependent RNA polymerase (L). The 2260 aa LEAV viral RNA-dependent RNA polymerase (RdRp; 264 kDa, pI=6.4) is similar in size to other orthobunyavirus reference strains falling between the 2238 aa Bunyamwera and the 2263 aa LaCrosse virus 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 centered on aa $P_{75}D$; region II, also located in the amino terminus, is centered on aa $R_{651}Y$. Regions I and II have been found to also be conserved among all bunyaviruses (Muller *et al.*, 1994). Region III (948-1239) is located in the center 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 RNA-dependent DNA polymerase encoded by retroid elements to the RNA-dependent RNA polymerases encoded by the positive, negative, and double stranded RNA viruses (Poch *et al.*, 1989; Xiong & Eickbush, 1990). The pre-A (948-977) and E motifs (1228-1239) identified in region 3 by Muller et al. were also found in LEAV. The fourth conserved region (1240-1343), identified by Aquino et al., was also conserved in LEAV **(Figure 3)** (Aquino *et al.*, 2003)**.**

Polyprotein (M): The 1419 aa LEAV polyprotein (161.8 kDa, pI=8.4) is cotranslationally cleaved into the 285aa Gn (32.7 kDa, pI=8.9), 947aa Gc (107.9 kDa, pI=6.7), and 173aa NSm (19.7 kDa, pI=9.2). Stuckely and Wright determined the Gn and Gc to be 35 and115 kDa, respectively, both within 10% of our predictions and similar in size to the Bunyamwera virus (Stuckly & Wright, 1983). When compared with other orthobunyaviruses reference strains and Aino virus, 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 of less conservations 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 aa 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 likely 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 (A_{472}) analogous to the termination of NSm in California serogroup viruses (Campbell & Huang, 1999) is possible. SignalP predicts cleavage at VVA_{472}-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 glycosylations sites were identified using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services), two in the Gn, one in the NSm and four in the Gc. Two in the Gn and one in each the NSm, and Gc are unique. The glycosylation site in the amino terminus of the Gc is conserved among Peaton, Tinaroo, and Akabane viruses and one in the Gc is conserved among Akabane and Aino viruses. Only one glycosylation site in the C-terminus of the Gc is conserved with Oropouche. Peaton, Tinaroo, Akabane, Aino and Oropouche are all Simbu serogroup viruses (**Figure 4**).

Prediction of transmembrane regions using the transmembrane hidden Markov model in the program TopPred2

(http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), predicts 8 transmembrane regions: two in the Gn (200-220, 223-243), three in the NSm (306-326, 357-377, 454-474), and three in the Gc (956-976, 1046-1066, 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) (**Figure 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 Bunyamwera virus, the prototype of the genus *Orthobunyavirus* (Elliott, 1990; Lees *et al.*, 1986).

The fusion peptide identified in La Crosse virus (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. (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 51 cysteines with all other orthobunyaviruses (Grady *et al.*, 1987; Lees *et al.*, 1986; Pardigon *et al.*, 1988), 14 in the Gn, 8 in the NsM, and 29 in the Gc (**Figure 4**).

Nucleocapsid (NP) and Non-Structural (NSs) proteins: The LEAV 235 aa NP (26.3kDa, pI=8.9) is consistent with Stuckley and Wright's NP of 29 kDa (Stuckly & Wright, 1983). It shows between 24% (Tete virus) and 60% (Aino virus) conservation at the aa level and between 40% (Tete virus) and 63% (Aino virus) at the nt level to other orthobunyaviruses, thus satisfying the minimum 10% divergence requirement of the ICTV in the NP for a novel species. Interestingly, of the six conserved regions identified in the Simbu serogroup viruses by Saeed et al.(Saeed *et al.*, 2001), regions two through six are well conserved with NT 16701, whereas region one is only somewhat conserved. There are also several individual aa that have been identified as being globally conserved in the NP among the 4 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 (P_{125}, G_{131}, Y_{158}, I_{231}) involved in formation of ribonucleoprotein complexes (Eifan & Elliott, 2009) are all conserved in LEAV (**Figure 5**). Of the 10 residues identified as involved in RNA synthesis, 8 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 (10.8kDa, pI=10.8) falls within the range of NSs sizes of 83 to 109 residues (Dunn *et al.*, 1994) and may correspond to the p8 protein identified by Stuckley and Wright (Stuckly & Wright, 1983). The NSs is poorly conserved when compared to Simbu group viruses, showing between 28% and 37% similarity at the aa level (55% to 60% at the nt 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 complement fixation 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 aa 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 serologic evidence indicate that LEAV represents a new species in the genus *Orthobunyavirus* and may represent a new antigenic complex.

Many other Simbu group viruses have been isolated from sentinel cattle and from insects in northern Australia, including Aino, Akabane, Douglas, Peaton, and Tinaroo viruses (Gard *et al.*, 1988). Akabane and Aino viruses have economic and veterinary importance. Akabane virus 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). Aino virus 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 geographic overlap, Aino virus 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 9/30 (30%) of 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 regards to sample size and geography is necessary to better understand the distribution of this virus and its role in human, livestock, and wildlife diseases.

Materials and 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 marksi* 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, Qlds., 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 (Beaty *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 microtiter technique (Beaty *et al.*, 1989; Xu *et al.*, 2007), using two units of guinea pig complement and overnight incubation of the antigen and antibody at 4^{o}C. CF titers were recorded as the highest dilutions giving 3+ or 4+ fixation of complement. Titers of 1:8 were considered positive. Hemagglutination inhibition (HI) testing was done in microtiter plates as described previously (Travassos da Rosa *et al.*, 1983). HI tests were performed with 4 hemagglutination units of virus at the optimal pH (5.75) against serial two-fold antiserum dilutions starting at 1:20. HI titers of 1:20 were considered positive.

By complement fixation (CF), hemagglutination-inhibition (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 as 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 110nm 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 Oropouche and Akabane (**Table 1**).

Genome sequencing

LEAV was extracted using TRIzol LS (Invitrogen, Carlsbad, CA, USA). Total RNA extracts were treated with DNase I (DNA-Free, Ambion, Austin, TX, USA) 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 base pairs (bp) were selected by column purification (MinElute, Qiagen, Hilden, Germany) and ligated to specific adapters for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA) 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 removal of primer sequences, redundancy filtering, and sequence assembly. Primers were designed using pyrosequencing data to fill gaps in the sequence (**Supplemental Table 3**). Conventional PCRs were performed with BIO-X-ACT polymerase (Bioline, Taunton, MA, USA) on PTC-200 thermocyclers (Bio-Rad, Hercules, CA, USA): 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 to 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 Analyzers (Perkin-Elmer Applied Biosystems, Foster City, CA). Terminal sequences were generated using a universal orthobunyavirus primer, targeting the conserved viral termini (5'- AGT AGT GTR CTC CAC-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 numbers 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 phylogenic 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 (aa) level, with

additional manual editing to ensure the highest possible quality of the alignment. UPGMA analysis at the amino acid level was performed due to the observed high variability of the underlying nt sequences. Nucleotide phylogenic trees were also investigated using the Neighbor-joining algorithm and the kimura 2-parameter model. The statistical significance of the tree topology was evaluated by bootstrap re-sampling of the sequences 1000 times. Phylogenetic analyses were performed by using MEGA software (Kumar *et al.*, 2004).

Sequence Analysis

Geneious 4.7.5 (Biomatters Inc., Auckland, New Zealand) 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 TopPred2 (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html), and integrated predictions in Geneious (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Kahsay *et al.*, 2005; Kall *et al.*, 2004; Krogh *et al.*, 2001).

Pairwise Sequence Analysis

To establish a potential cutoff 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 International Committee for Taxonomy of Viruses (ICTV) for demarcation of species, at both the nt and aa level

using pairwise deletion. Calculations were performed using MEGA software (Kumar *et al.*, 2004) to calculate the p-distance of each segment at the nt level.

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538 **Table 1.** Antisera tested by serology (HI and/or NT) against Leanyer virus at the Queensland Institute of Medical

539 Research and University of Texas Medical Branch.

540

Alphavirus	***Flavivirus***	***Bunyaviridae***	***Orthobunyavirus***	***Orbivirus***
Getah	Alfuy	Belmont	Aino	Corriparta
Ross River	Edge Hill	Gan Gan	Akabane	D'Aguillar
Sindbis	Kokobera	Kowanyama	Facey's Paddock (Ch 16129)	Eubenangee
Murweh (Ch 16313)	Kunjin	Maprik	Koongol	Mitchell River
	Murray Valley encephalitis	Mapputta	Oropouche	Mudjinbarry
	Saumarez Reef	Trubanaman	Peaton	Nugget
	Stratford		Tete	Tilligerry
			Wongal	Wallal
			Yacaaba	Warrego
Orthomyxovirus	***Rhabdoviridae***	***Lyssavirus***	***Nairovirus***	Wongorr
Dhori	Alpimwar	Bovine Ephemeral fever	Kao Shuan	
Johnston Atoll	Charleville		Taggert	
Upolu	Ngaingan			

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

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	N/A	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	N/A	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	N/A	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	N/A	46.0 ± 2.0 (56.5 ± 3.9)	44.8 ± 1.5 (54.8 ± 2.9)
Between California encephalitis and Simbu	48.9 ± 2.0 (55.9 ± 3.8)	55.5 ± 1.2 (69.7 ±2.3)	47.2 ± 1.4 (57.1 ± 2.8)
Between Group C and Simbu	N/A	46.2 ± 2.0 (54.7 ± 3.7)	45.8 ± 1.4 (56.2 ± 2.9)

Table 3. M Segment Protein Sizes.

Virus Name	Gn Size (aa)	NSm Size (aa)	Gc Size (aa)
Leanyer virus	285	173	947
LaCrosse virus	277	173	967
Bunyamwera virus	292	174	955
Oropouche virus	290	175	939
Akabane virus	292	156	936
Aino virus	291	155	941

Figure Legends

Figure 1. Phylogenetic analysis of the A) polymerase, B) polyprotein, C) and nucleoprotein. 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 aa 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. Neighbor-joining (NJ) analysis at the aa level was performed given the high observed variability of the nt sequences. Statistical significance of the tree topology was evaluated by bootstrap re-sampling of the sequences 1000 times. Phylogenetic analyses were performed using MEGA software (Kumar *et al.*, 2004). Sequence marked with a black dot represents LEAV.

Figure 2. Pairwise sequence analysis. The sequence of LEAV was compared with published orthobunyavirus sequences to determine intergroup and intragroup averages and to classify LEAV. Calculations were performed using p-distance at the aa level. P-distance values were grouped into three groups: red, distances among different viruses belonging to the same serogroup (intragroup); green, distances between members of different viruses belonging to different serogroups; and purple, distance between LEAV and other viruses in the genus.

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

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

Figure 5. Nucleocapsid. 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. as being conserved among all Simbu Group viruses.

Supplementary Figure 1. Phylogenetic analysis of the A) polymerase, B) polyprotein, C) and nucleoprotein. 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 nt 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. Neighbor-joining (NJ) analysis at the nt level was performed given the high observed variability of the nt sequences. Statistical significance of the tree topology was evaluated by bootstrap re-sampling of the sequences 1000 times. Phylogenetic analyses were performed using MEGA software (Kumar *et al.*, 2004). Sequence marked with a black dot represents LEAV.

Supplemental Table 1

Antigens	Complement Fixation test Antibodies						
	Leanyer 5.10.76	**Oropouche** T-34161	**Aino** 6.10.69	**Akabane** T-33756	**Koongol** 1.26.66	**Wongal** 4.27.67	**Tete** 8.18.84
Leanyer T-36190	**128 ≥64**	0	0*	0	0	0	0
Oropouche 3.19.85	0	**≥64 ≥Φ**	0	**8 ≥Φ**	0	0	0
Aino 5.8.69	0	0	**≥64 ≥Φ**	**64 ≥Φ**	0	0	0
Akabane 5.6.69	0	0	**32 ≥Φ**	**≥64 ≥Φ**	0	0	0
Koongol 1.8.64	0	0	0	0	**16 ≥Φ**	**8 ≥Φ**	0
Wongal 5.26.67	0	0	0	0	**32 ≥Φ**	**16 ≥Φ**	0
Tete TVP-11869	0	0	0	0	0	0	**64 ≥Φ**

*<8/<8 Numerator means serum titer and denominator antigen titer

Supplemental Table 2

Antibodies	Hemagglutination Inhibition test Antigens 4u.		
	Leanyer	**Oropouche**	**Wongal**
Leanyer	**1:5120**	0	0
Aino	0*	0	0
Akabane	0	0	0
Koongol	0	0	**1:160**
Oropouche	0	**≥1:640**	0
Tete	0	1:20?	0
Wongal	0	0	**1:160**

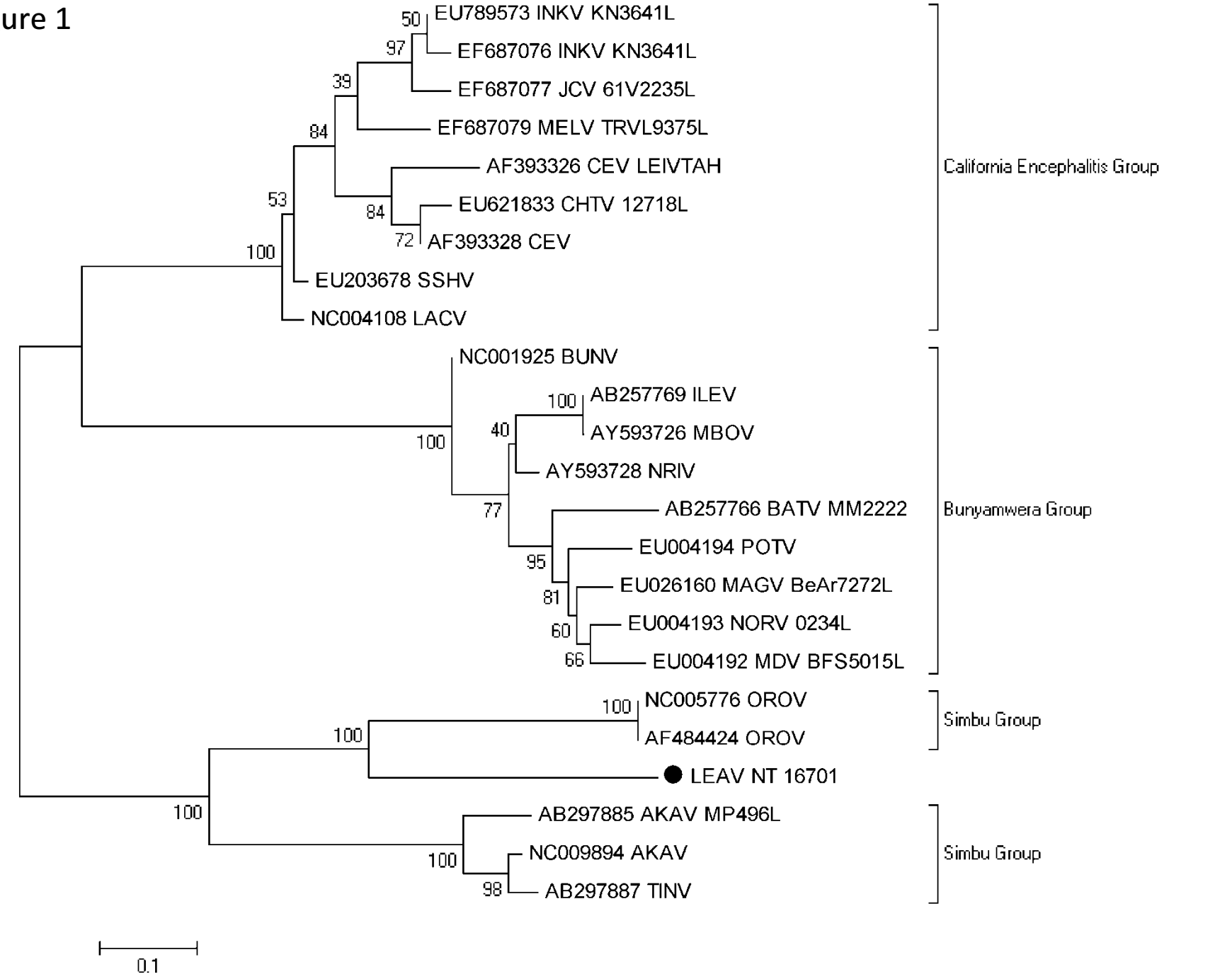
*<1:20

Supplemental Table 3. Primers designed from pyrosequencing data.

Name	Sequence
LEAV_L_3776R	TGCAGTCAAAAGGAATCTACCAAA
LEAV_L_3823R	TCGCTGTTTGAGTAGCTGAAAGTC
LEAV_L_3862R	TAGACAACCAGGCTAAAGATGCAG
LEAV_L_2941R	GATCTGCCCAGGCATCATGT
LEAV_L_4934F	AAATTTGATTTCCCAAAAGTGGAA
LEAV_L_4961F	GAAGAGGAGCTCAGAAGGGATGTA
LEAV_L_5028F	AAAAATGTTAGAACGAATTGCAGAAA
LEAV_L_5091F	TTGTTCCAGTTGAAAGAATTAACAAA
LEAV_L_5215F	TGCTGTGCTGTAACAGGGAACTTA
LEAV_M_284F	ACACAGGAACCATCCAAATCACAT
LEAV_M_328R	TGTCCCCATTCCTTGAAACTGATG
LEAV_M_854F	CATCTACAGAAGCCTTGAAGACCC
LEAV_M_1022R	CTGTAATTCCCTTTCACTGGAGCA
LEAV_M_2587F	CCAGAACATTTTGGCGATG
LEAV_M_2687R	TTGCATTCTAGAAGCCATTTTG
LEAV_M_2936F	AAGGGCTGACACTAACCTTACCTA
LEAV_M_3079R	AGACTTCTGAATGCACCATGTCTA
LEAV_M_3297F	TAGCTTAAGCAGCTATTGCCACAA
LEAV_M_3328R	AGGTCAGGTAGATTGGAGACAGTT
LEAV_M_3794F	GCACAGAAGCAGTGATCTGTACTC
LEAV_M_3903R	CCCTTCAGGGATTTTCATCTGTGT
LEAV_S_452R	GCCATCTCCCCATTTGCAGCCCTT
LEAV_S_482R	ACCTGGTGCAAATGCCAAATAGAGAGCA
LEAV_S_500F	AGACTTTGAATTCTATCCTCTTGC
LEAV_S_532F	CAAAACATAGTGGAAATGGGCGAC

Figure 1

A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EU621833 CHTV 12718L
AF393328 CEV
EU203678 SSHV
NC004108 LACV
California Encephalitis Group
NC001925 BUNV
AB257769 ILEV
AY593726 MBOV
AY593728 NRIV
AB257766 BATV MM2222
EU004194 POTV
EU026160 MAGV BeAr7272L
EU004193 NORV 0234L
EU004192 MDV BFS5015L
Bunyamwera Group
NC005776 OROV
AF484424 OROV
LEAV NT 16701
Simbu Group
AB297885 AKAV MP496L
NC009894 AKAV
AB297887 TINV
Simbu Group
50
97
39
84
53
84
72
100
100
40
100
77
95
81
60
66
100
100
100
100
98
0.1

Figure 1

B


AF186243 CVV 807270
AY593733 PLAV 75V3066
AY593732 TLAV 61-D-240
AY593734 FSV 86-MSP18
AY286443 MAGV
EU004188 NORV 0234
AB257763 BATV MM2222
AY593747 NRIV DakArD28542/4e
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M21951 GERV
NC001926 BUNV
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EU004186 KRIV TRVL8900
EU004187 MDV BFS5015
AY593749 SHOV SAAr4042
AY859372 ILEV KO/2
AY593731 MBOV DakArY357/6e
AY593736 LOKV FMS4332
AY380581 GROV BeH22063
EU541231 CHTV 23519
AF123491 TVTV Eklund
U88057 MELV TRVL9375
AF123490 SDNV BeAr 103645
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AF123484 LUMV SA Ar 1881
AF229129 TAHV 181-57
AF123483 CEV BFS283
NC004109 LACV
EU262553 SSHV
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NC005775 OROV
AB100605 AINOV JaNAr28
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Bunyamwera
California Encephalitis Group
Simbu Group
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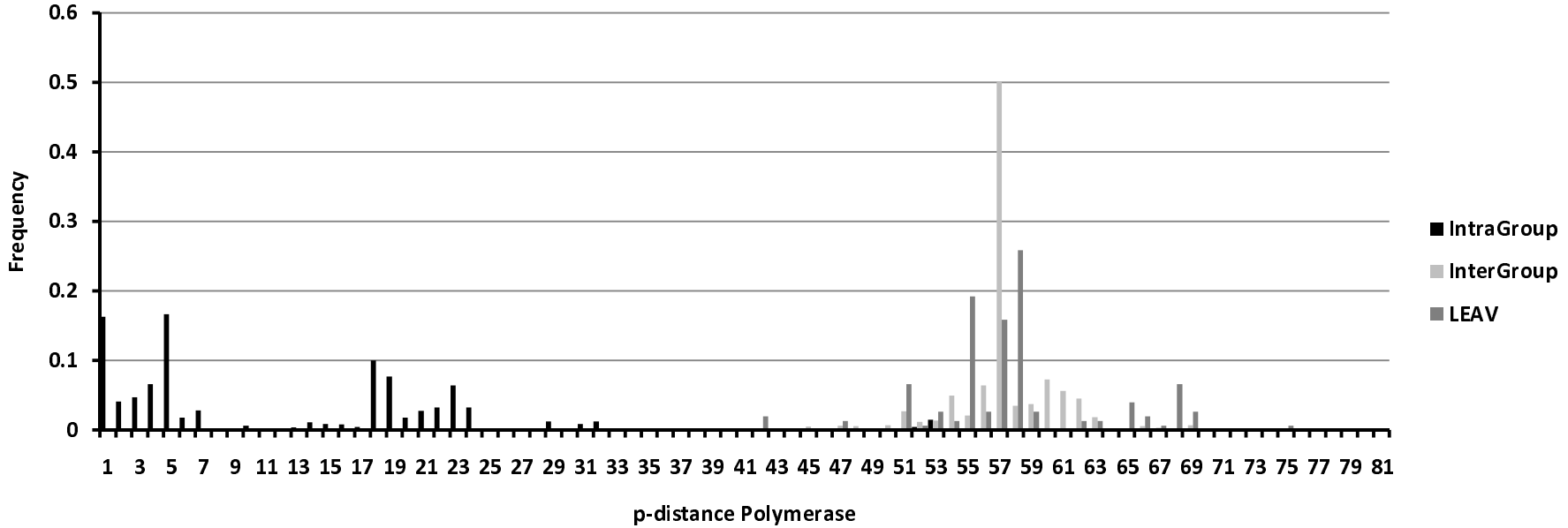
Figure 2

A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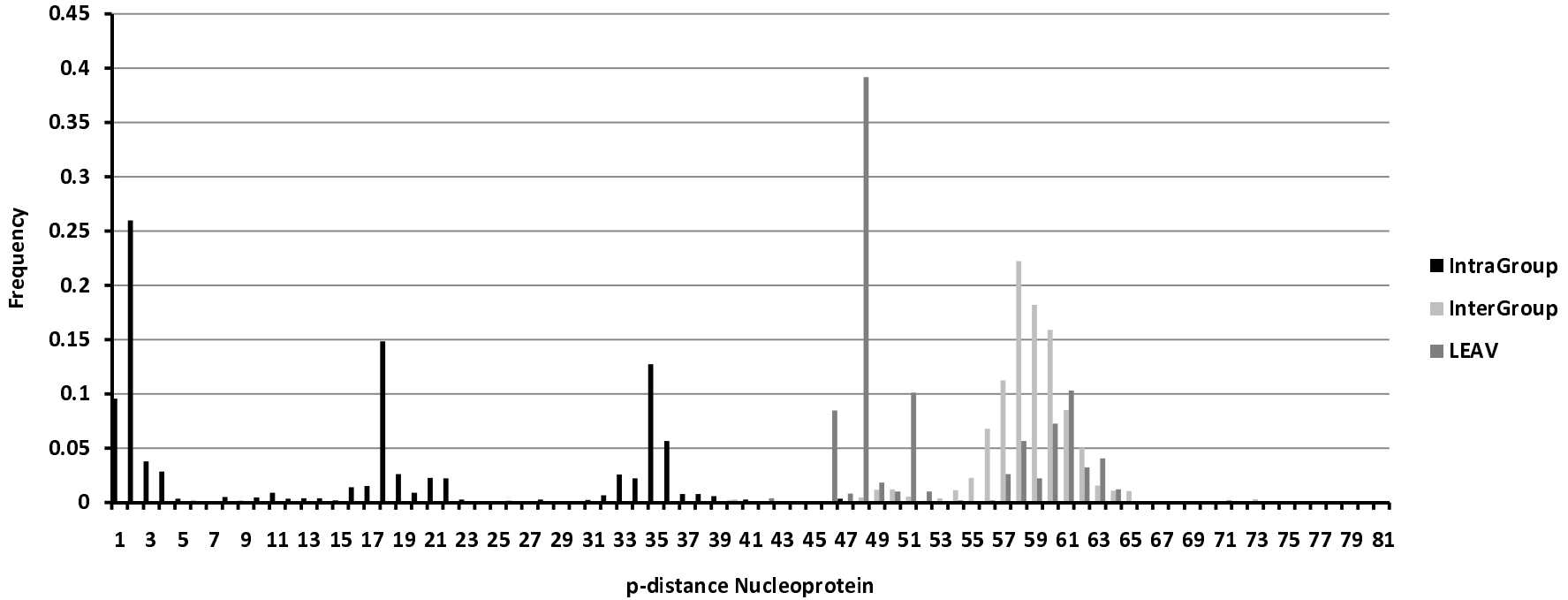
IntraGroup
InterGroup
LEAV

B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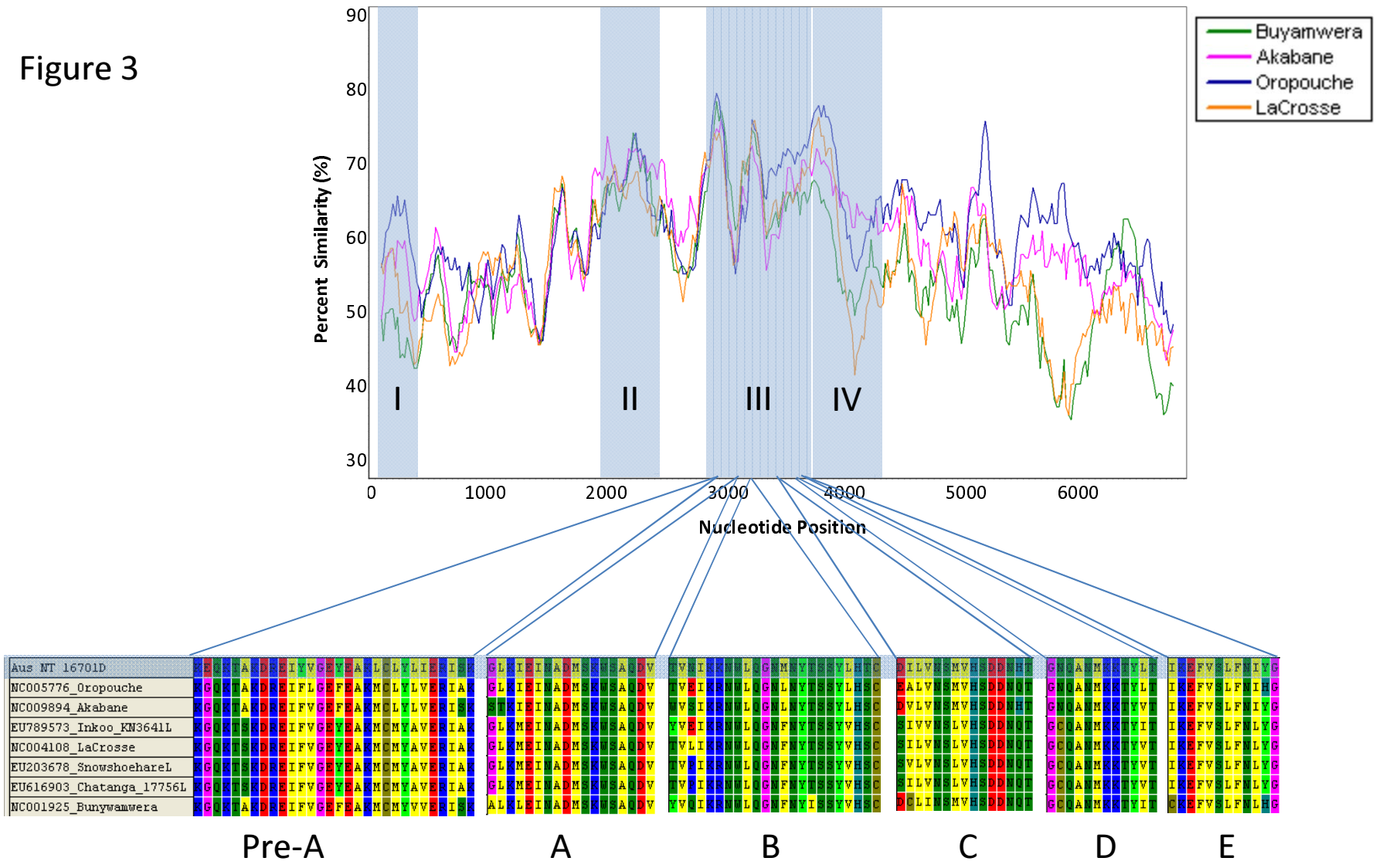
p-distance Nucleoprotein
IntraGroup
InterGroup
LEAV

Figure 3


Buyamwera
Akabane
Oropouche
LaCrosse
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40
30
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III
IV
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1000
2000
3000
4000
5000
6000
Nucleotide Position
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NC009894_Akabane
EU789573_Inkoo_KN3641L
NC004108_LaCrosse
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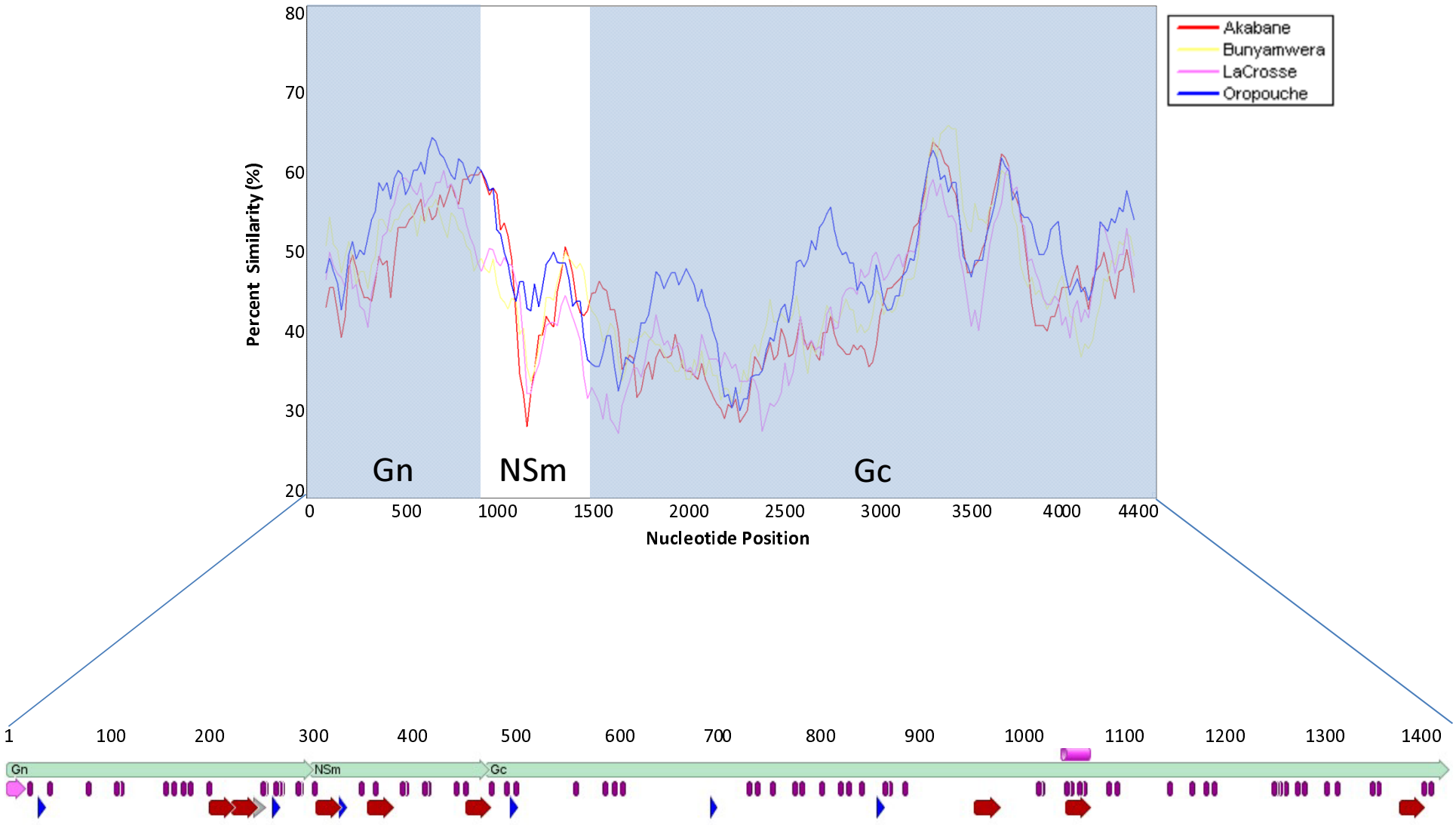
Pre-A
A
B
C
D
E

Figure 4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Nucleotide Position
Gn
NSm
Gc
Akabane
Bunyamwera
LaCrosse
Oropouche
1
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
Gn
NSm
Gc

Figure 5

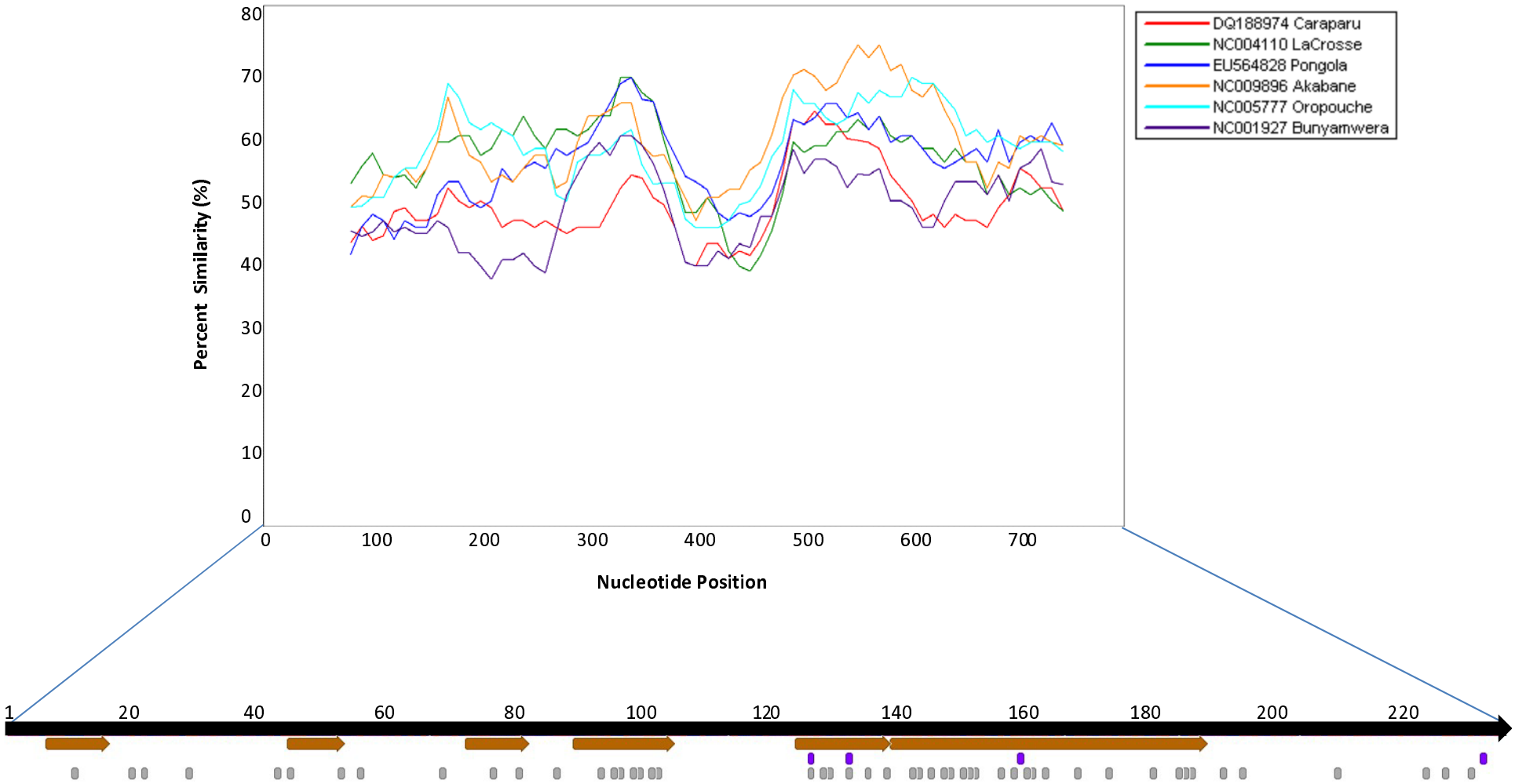
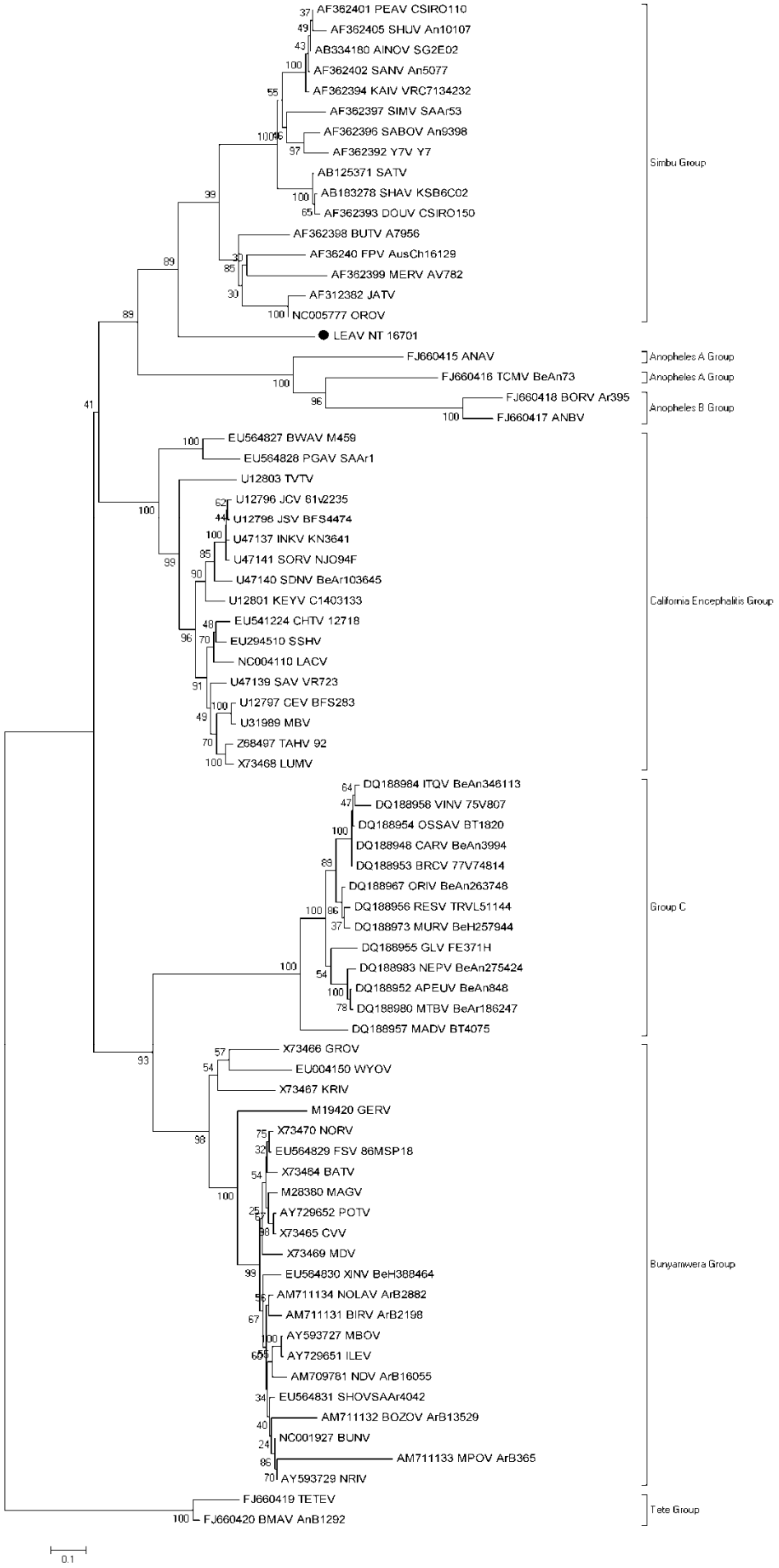

Percent Similarity (%)
80
70
60
50
40
30
20
10
0
0
100
200
300
400
500
600
700
Nucleotide Position
DQ188974 Caraparu
NC004110 LaCrosse
EU564828 Pongola
NC009896 Akabane
NC005777 Oropouche
NC001927 Bunyamwera
1
20
40
60
80
100
120
140
160
180
200
220

Figure 1

C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AF362405 SHUV An10107
AB334180 AINOV SG2E02
AF362402 SANV An5077
AF362394 KAIV VRC7134232
AF362397 SIMV SAAr53
AF362396 SABOV An9398
AF362392 Y7V Y7
AB125371 SATV
AB183278 SHAV KSB6C02
AF362393 DOUV CSIRO150
AF362398 BUTV A7956
AF36240 FPV AusCh16129
AF362399 MERV AV782
AF312382 JATV
NC005777 OROV
LEAV NT 16701
Simbu Group
FJ660415 ANAV
FJ660416 TCMV BeAn73
FJ660418 BORV Ar395
FJ660417 ANBV
Anopheles A Group
Anopheles A Group
Anopheles B Group
EU564827 BWAV M459
EU564828 PGAV SAAr1
U12803 TVTV
U12796 JCV 61v2235
U12798 JSV BFS4474
U47137 INKV KN3641
U47141 SORV NJO94F
U47140 SDNV BeAr103645
U12801 KEYV C1403133
EU541224 CHTV 12718
EU294510 SSHV
NC004110 LACV
U47139 SAV VR723
U12797 CEV BFS283
U31989 MBV
Z68497 TAHV 92
X73468 LUMV
California Encephalitis Group
DQ188984 ITQV BeAn346113
DQ188958 VINV 75V807
DQ188954 OSSAV BT1820
DQ188948 CARV BeAn3994
DQ188953 BRCV 77V74814
DQ188967 ORIV BeAn263748
DQ188956 RESV TRVL51144
DQ188973 MURV BeH257944
DQ188955 GLV FE371H
DQ188983 NEPV BeAn275424
DQ188952 APEUV BeAn848
DQ188980 MTBV BeAr186247
DQ188957 MADV BT4075
Group C
X73466 GROV
EU004150 WYOV
X73467 KRIV
M19420 GERV
X73470 NORV
EU564829 FSV 86MSP18
X73464 BATV
M28380 MAGV
AY729652 POTV
X73465 CVV
X73469 MDV
EU564830 XINV BeH388464
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AM711131 BIRV ArB2198
AY593727 MBOV
AY729651 ILEV
AM709781 NDV ArB16055
EU564831 SHOVSAAr4042
AM711132 BOZOV ArB13529
NC001927 BUNV
AM711133 MPOV ArB365
AY593729 NRIV
Bunyamwera Group
FJ660419 TETEV
FJ660420 BMAV AnB1292
Tete Group
0.1