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Genomic and phylogenetic characterization of Merino Walk virus, a novel arenavirus isolated in South Africa

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Merino Walk virus (MWV), a proposed novel tentative species of the family *Arenaviridae*, was isolated from a rodent, *Myotomys unisulcatus*, collected at Merino Walk, Eastern Cape, South Africa, in 1985. Full-length genomic sequence confirmed MWV as an arenavirus related distantly to Mobala, Mopeia and Ippy viruses, all members of the Old World arenavirus complex. We propose MWV as a tentative novel species in the Lassa–lymphocytic choriomeningitis virus complex, based on its isolation from a novel rodent species and its genetic and serological characteristics.

INTRODUCTION

The family *Arenaviridae* currently includes 23 antigenically related viruses classified into two groups: Old World (OW; Lassa–lymphocytic choriomeningitis complex) and New World (NW; Tacaribe complex) (http://www.ictvonline.org/virusTaxonomy.asp?), based on geographical, genetic, antigenic and host relationships (Bowen *et al.*, 1997; Moncayo *et al.*, 2001). The arenavirus genome is bisegmented, comprising a larger (L) and a smaller (S) segment, each encoding two open reading frames (ORFs) using an ambisense coding strategy (Auperin *et al.*, 1984; Salvato & Shimomaye, 1989). Nonetheless, members of the *Arenaviridae*, together with the families *Orthomyxoviridae* and *Bunyaviridae*, are classified as segmented, single-stranded, negative-sense RNA viruses. Some members of the family *Arenaviridae*, such as the South American haemorrhagic fever viruses [Junín (JUNV; Parodi *et al.*, 1958; Pirosky *et al.*, 1959), Machupo (MACV; Johnson *et al.*, 1965), Guanarito (GTOV; Salas *et al.*, 1991) and Sabiá (SABV; Lisieux *et al.*, 1994) viruses] and the African Lassa virus (LASV) are listed as potential biowarfare or bioterrorism agents (http://www.cdc.gov/od/sap) because of their human pathogenicity and aerosol-transmission potential. These latter five viruses are classified as biosafety level 4 agents.

Arenavirus species are usually maintained in and transmitted by a single rodent species (Bowen *et al.*, 1997), the only exceptions thus far being Tacaribe virus (TCRV; Downs *et al.*, 1963), which was isolated from fruit bats (*Artibeus* sp.), and Amapari virus (AMAV), which has been isolated from two rodent species, *Oryzomys gaeldi* and *Neacomys guianae* (Pinheiro & Woodall, 1969). The NW arenaviruses are associated with rodents in the subfamily Sigmodontinae of the family Cricetidae, whereas the OW arenaviruses are associated with rodents in the subfamily Murinae of the family Muridae. LASV and Mopeia virus (MOPV; Wulff *et al.*, 1977) are hosted by members of the genus *Mastomys*, Mobala virus (MOBV; Gonzalez *et al.*, 1983) by *Praomys* spp., Ippy virus (IPPYV; Meunier *et al.*, 1985; Swanepoel *et al.*, 1985) by *Arvicanthis* spp., and lymphocytic choriomeningitis virus (LCMV; Peters, 2006) by the ubiquitous *Mus musculus*.

Of the OW arenavirus species, only LCMV and LASV were known to cause severe disease in humans (Buckley & Casals, 1970) until September 2008, when a cluster of undiagnosed fatal haemorrhagic fever cases in South Africa and Zambia led to the identification of a novel OW arenavirus, designated Lujo virus (LUJV; Briese *et al.*, 2009; Paweska *et al.*, 2009). The rodent host of this species remains unknown, but efforts to survey wildlife are under way to identify its origin. This sudden outbreak of viral haemorrhagic fever highlights the importance of surveying wildlife, in this case the rodent population, to describe the virome diversity and its ecology. As an example of such surveys, here we describe rapid full-genome sequencing for genetic characterization of another novel OW arenavirus, tentatively termed Merino Walk virus (MWV).

The GenBank/EMBL/DDBJ accession numbers for the sequences of the MWV small and large genome segments reported in this paper are GU078660 and GU078661, respectively.

Five supplementary figures are available with the online version of this paper.

RESULTS AND DISCUSSION

Pathogenicity

Newborn mice inoculated intracerebrally with MWV were moribund and died 10–11 days after infection. Adult ICR mice inoculated intraperitoneally with a 10 % suspension of the infected newborn mouse brains did not become ill, but subsequently developed antibodies to the virus.

In comparison, IPPYV kills intracerebrally inoculated suckling mice between 5 and 8 days after inoculation, with a mortality of 60 % (MOBV and MOPV show also a similar mortality) (Swanepoel *et al.*, 1985). In general, LCMV and LASV are pathogenic for weaned mice, but not suckling mice (Salvato *et al.*, 2005). We do not have any data on MWV serology in mice or humans. Given that MWV was isolated in 1985 and that other OW arenaviruses are associated with outbreaks of viral haemorrhagic fever in the same geographical region, serology should be pursued to determine whether MWV infects humans.

Serology

Results of complement fixation (CF) tests comparing MWV with four other African arenaviruses (MOBV, MOPV, IPPYV and LCMV) are shown in Table 1. By this method, MWV was shown to be related antigenically to MOBV, MOPV and IPPYV, but unrelated to LCMV. However, the cross-CF tests indicate that MWV is antigenically distinct from these other arenaviruses.

Transmission electron microscopy

In ultrathin sections of infected Vero cells, virions with typical arenavirus morphology were observed at the cell surface and in the process of budding from it (Fig. 1). They had diameters of 80–190 nm.

Table 1. Results of CF tests comparing MWV with other African arenaviruses

Values are shown as reciprocal of highest antiserum dilution/reciprocal of highest antigen dilution. Homologous titres are shown in bold.

Antigen	Antibody				
	MWV	MOBV	MOPV	IPPYV	LCMV*
MWV	**256/32**	16/8	64/32	8/8	0†
MOBV	16/32	**512/128**	⩾1024/128	32/32	–
MOPV	16/32	128/128	⩾**1024/128**	32/32	–
IPPYV	16/32	128/128	512/128	**512/512**	–
Control	0	0	0	0	0

*LCMV homologous titre=128/256.

†0=<8/<8.

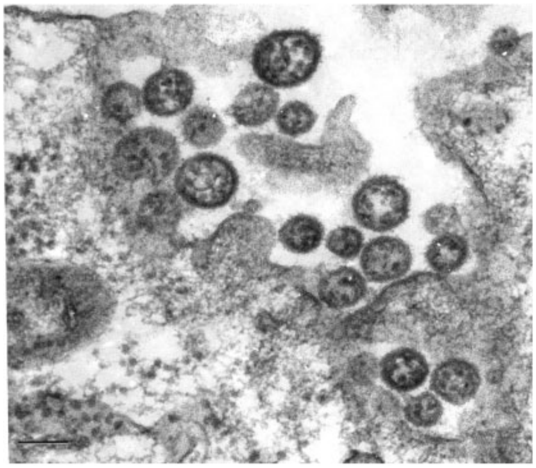

Fig. 1. MWV as observed at the plasma membrane of an infected Vero cell in an ultrathin section. Bar, 100 nm.

Sequence acquisition and analysis

Consistent with the ambisense genome organization characteristic of members of the family *Arenaviridae* (Auperin *et al.*, 1984; Charrel *et al.*, 2008; Salvato & Shimomaye, 1989), the genome of MWV comprises two RNA segments: an L segment that encodes a large (L) polymerase-related ORF in the negative-sense orientation and a small RING-finger protein Z in the positive-sense orientation (GenBank accession no. GU078661), and an S segment that encodes a nucleocapsid protein (NP) in the negative-sense orientation and a glycoprotein precursor protein (GPC) in the positive-sense orientation (GenBank accession no. GU078660).

The result of the phylogenetic analysis of the GPC and L (Fig. 2a, b, respectively) and NP (Supplementary Fig. S1, available in JGV Online) ORFs indicated that, although distant from any other known OW arenavirus, MWV is consistently associated with MOBV, MOPV and IPPYV. No evidence of RNA segment reassortment or intersegmental recombination was discovered. The NP gene sequence of MWV differs from those of other arenaviruses by between 34.5 % (MOPV) and 49.5 % (Tamiami virus, TAMV) on the nucleotide level, and between 31.4 % (MOPV) and 55.5 % (TAMV) on the amino acid level (Table 2). Historically, phylogenetic classification of arenaviruses was based on analysis of partial areas of the NP gene. The degree of divergence is significantly higher than proposed cut-off values within (<10–12 %) or between (>21.5 %) OW arenavirus species (Bowen *et al.*, 2000; Emonet *et al.*, 2006).

ORFs

Z protein. A conserved RING motif and shorter C-terminal domain resembling those present in NW arenaviruses is found in the 89 aa Z protein of MWV (10 kDa, pI=7.6).

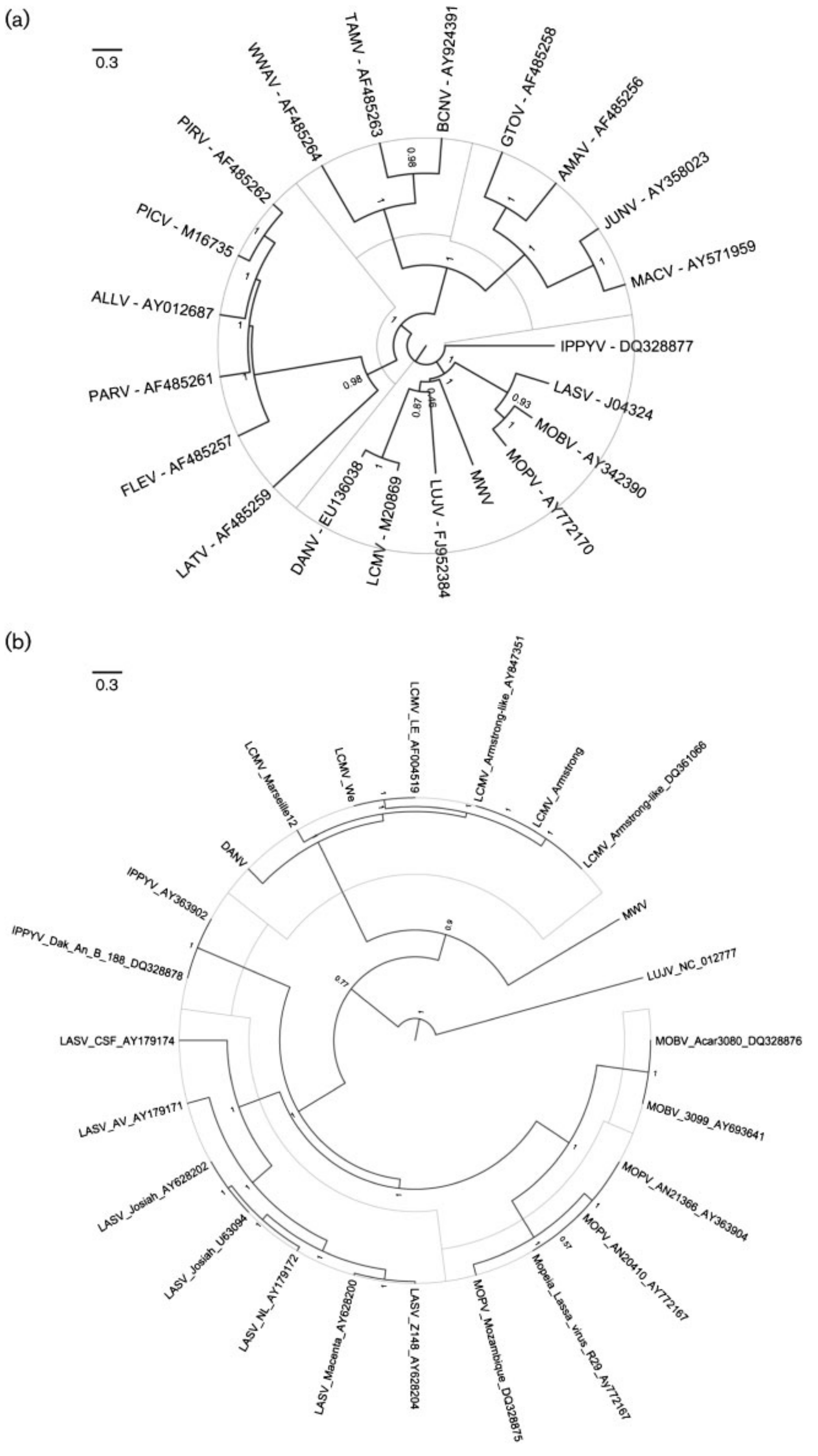

Fig. 2. Phylogenetic analyses of MWV were inferred based on primary nucleotide alignments of (a) GPC and (b) L sequences. Maximum clade credibility trees generated from analysis of arenavirus GPC or L sequences are shown. Posterior probabilities are listed below the branches for supported nodes.

MWV includes the conserved N-terminal myristoylation site G_2, implicated in membrane association during budding and interaction of Z with the viral glycoprotein complex (Capul *et al.*, 2007; Perez *et al.*, 2004; Strecker *et al.*, 2006). The tryptophan residue conserved in the RING motif of other arenavirus Z proteins and in cellular ubiquitin ligases is found in the RING motif of MWV (Joazeiro *et al.*, 1999) (W_{44}; Supplementary Fig. S2, available in JGV Online).

The Z protein of arenaviruses is critical in the budding of viral progeny, similar to matrix proteins of other negative-sense RNA viruses; mutation analysis of the late (L) domains of the LASV and LCMV Z proteins demonstrated

Table 2. Percentage differences in NP sequence between arenavirus isolates

Nucleotide sequence differences (%) are shown above the diagonal; amino acid sequence differences (%) are shown below the diagonal.

Virus	LUJV	DANV	LCMV	MOPV	MOBV	IPPYV	LASV	MWV	GTOV	AMAV	MACV	CPXV	SABV	TCRV	JUNV	CHPV	OLVV	LATV	FLEV	CATV	PIRV	ALLV	BCNV	SKTV	NAAV	TAMV	PARV	WWAV	PICV
LUJV		38.7	38.9	39.7	42.0	40.4	41.6	40.7	46.9	47.6	45.0	46.6	46.5	46.0	46.7	46.7	46.9	48.0	45.5	45.6	44.0	45.1	46.2	47.1	46.4	49.4	46.2	47.2	45.8
DANV	41.3		19.5	39.0	38.4	38.4	39.7	37.3	45.0	46.7	45.2	45.3	45.3	46.4	44.8	45.3	46.0	44.4	44.0	45.2	43.2	45.1	44.8	45.3	46.2	49.2	45.3	45.2	45.3
LCMV	40.8	5.8		38.1	38.3	38.4	39.6	37.9	45.8	46.6	44.3	45.4	44.4	45.7	44.8	45.8	46.2	44.1	44.0	45.3	43.9	44.9	44.2	46.1	46.1	48.6	44.2	45.7	47.3
MOPV	43.7	34.9	36.3		29.8	33.7	31.9	34.5	46.9	46.3	44.7	43.6	45.8	46.2	46.0	46.3	45.3	44.8	45.3	44.7	44.6	44.5	45.1	44.8	46.0	49.3	44.6	44.6	44.2
MOBV	42.4	37.0	37.5	21.6		36.1	32.0	35.3	46.3	46.7	45.2	43.4	44.6	45.7	47.3	45.5	45.7	46.1	45.5	45.1	45.6	44.6	46.3	46.6	46.9	48.3	44.3	45.8	46.1
IPPYV	44.4	37.1	37.2	29.7	29.3		35.0	35.2	46.6	46.9	45.2	45.3	46.9	46.9	45.4	46.3	46.7	47.9	47.8	45.5	44.7	46.7	46.3	45.5	47.1	49.0	45.7	45.8	44.8
LASV	42.3	37.4	38.4	26.2	27.2	31.0		35.2	45.1	47.2	46.6	45.2	45.6	45.1	45.9	47.1	46.0	45.4	45.7	45.8	47.2	44.7	45.3	46.2	46.1	47.9	45.6	46.7	44.4
MWV	43.2	34.2	34.5	31.4	33.3	31.6	32.3		45.8	46.1	44.7	46.6	45.2	46.5	45.2	45.7	47.3	46.7	45.6	44.8	44.5	44.9	45.0	44.9	46.6	49.5	44.7	46.2	44.0
GTOV	52.6	49.6	48.5	48.7	49.9	49.6	49.4	47.7		24.5	30.7	26.6	34.0	32.3	30.4	32.4	39.2	37.6	41.2	42.2	41.3	42.8	42.8	42.3	41.8	45.2	41.4	42.4	41.3
AMAV	52.6	49.3	48.3	49.6	49.7	49.3	50.1	49.0	14.5		32.7	26.6	33.5	32.4	31.9	31.9	36.3	37.8	42.2	43.2	42.4	42.1	43.2	43.0	43.5	47.0	41.3	43.7	41.3
MACV	52.1	49.8	49.0	50.0	49.9	49.8	49.6	49.5	23.7	26.3		31.3	32.9	28.7	23.7	34.3	36.9	37.8	40.7	42.0	40.0	41.6	41.0	42.4	42.8	44.8	41.2	42.3	42.0
CPXV	51.6	47.7	46.7	47.9	48.2	49.4	48.4	50.0	17.9	17.4	25.9		34.2	32.6	32.0	33.0	37.3	37.2	42.4	42.5	42.5	41.4	43.9	43.9	45.1	47.0	41.9	43.2	42.7
SABV	51.7	48.5	47.6	49.4	48.7	49.9	49.3	48.6	29.6	28.2	28.3	29.1		35.3	33.3	25.2	40.1	38.8	43.1	42.7	43.0	41.3	41.7	43.9	44.5	44.9	42.2	42.4	42.3
TCRV	52.2	48.8	49.1	49.6	50.4	49.4	49.5	49.8	28.6	30.0	20.7	30.2	33.5		27.8	33.6	39.0	37.9	42.2	43.5	41.6	41.0	43.0	43.5	44.0	44.7	41.4	43.7	41.9
JUNV	53.0	49.5	49.2	50.2	51.4	50.9	50.4	50.1	25.4	26.6	12.2	27.3	29.8	21.5		32.8	37.4	37.7	41.3	42.7	40.9	41.3	43.7	42.7	44.5	45.4	42.3	43.7	42.3
CHPV	52.6	47.5	47.6	49.0	49.3	49.7	49.5	48.0	28.2	27.7	29.1	28.2	16.2	31.7	30.1		39.2	38.6	42.0	41.7	41.5	42.0	42.0	42.8	43.0	46.1	41.4	43.5	42.6
OLVV	53.5	49.6	49.9	50.4	50.5	51.4	49.7	50.6	38.0	37.6	37.8	36.1	38.2	40.0	39.1	38.0		27.7	39.7	41.8	39.7	39.1	41.6	42.8	42.6	44.5	40.3	42.5	39.7
LATV	53.3	47.7	48.1	49.0	50.4	51.3	50.7	48.6	35.9	37.3	37.2	35.6	37.9	39.7	38.8	38.1	19.2		41.0	41.2	38.7	39.6	41.8	42.4	40.9	43.0	38.4	42.0	41.5
FLEV	53.8	48.9	48.8	50.4	51.5	51.3	50.3	49.4	41.7	42.9	43.1	44.4	43.7	44.2	42.0	41.2	41.6	42.8		36.8	36.0	31.8	37.5	37.3	38.7	41.1	29.8	38.0	35.3
CATV	53.7	50.1	49.3	48.6	50.5	52.5	50.1	50.6	45.0	46.4	45.5	46.9	45.4	46.8	45.9	43.8	44.2	43.1	35.8		36.7	36.5	28.4	22.7	23.0	29.8	37.0	23.0	37.1
PIRV	49.8	45.1	44.8	48.0	50.6	48.0	50.6	47.5	41.7	43.3	43.6	44.1	43.5	43.1	43.3	41.6	40.4	40.3	32.4	37.5		34.8	36.4	36.8	38.4	39.8	35.8	38.0	34.6
ALLV	51.9	47.9	48.5	48.6	49.5	50.0	47.2	47.9	43.5	43.0	42.3	42.9	43.6	41.5	42.4	41.8	41.0	40.5	25.7	38.5	29.6		37.0	36.2	37.6	39.1	32.6	36.6	30.0
BCNV	52.5	48.8	47.8	48.9	51.4	50.7	50.6	49.4	45.2	45.9	45.9	47.0	45.1	46.1	45.7	43.6	43.2	41.8	34.9	17.8	36.4	35.4		26.6	28.6	30.8	36.3	27.9	37.3
SKTV	53.6	49.7	49.5	48.7	50.5	51.6	50.1	49.9	45.3	47.1	47.0	47.4	45.6	46.8	46.8	44.7	44.4	42.0	37.1	11.4	37.6	37.2	16.9		24.2	29.6	38.0	22.9	38.3
NAAV	52.2	49.1	48.6	48.5	51.1	52.4	50.4	50.4	44.2	45.1	46.1	46.2	45.3	45.8	44.5	43.2	44.7	41.7	36.3	14.6	38.1	36.7	20.5	15.4		30.2	37.4	16.0	38.1
TAMV	57.2	54.5	53.6	53.4	55.1	56.8	55.3	55.5	49.1	50.4	49.1	49.7	47.8	49.6	48.0	47.6	45.7	46.1	41.8	22.5	40.9	42.9	25.0	22.3	23.9		39.2	31.3	40.6
PARV	51.9	48.4	47.5	46.6	50.3	50.0	49.4	47.5	42.1	43.2	43.5	44.2	43.7	43.4	42.5	41.6	41.9	41.4	21.9	38.5	32.3	28.1	34.6	38.7	38.2	41.8		37.1	34.8
WWAV	52.8	49.9	49.3	47.8	50.5	50.9	50.3	50.3	44.8	45.3	44.4	46.3	44.9	45.5	45.0	43.6	44.1	42.2	37.8	13.5	37.1	36.9	18.4	14.3	9.3	23.7	39.2		36.7
PICV	52.2	50.2	50.5	48.0	51.0	49.8	50.1	47.7	43.1	43.3	44.2	45.7	43.0	44.0	44.0	42.8	42.9	42.6	30.8	37.0	29.6	23.8	36.1	37.0	36.9	40.4	32.9	35.8	

that their abrogation impairs budding completely (Perez *et al.*, 2003; Strecker *et al.*, 2003). Two L-domain motifs have been identified in the C terminus of the LASV Z protein. The first motif (PTAP) mediates recognition of the tumour-susceptibility gene 101 (Tsg101; Perez *et al.*, 2003), whilst the second motif (PPPY) acts as a Nedd4-like ubiquitin ligase-recognition motif (Staub *et al.*, 1996). Those motifs are also conserved in the OW arenaviruses MOBV, MOPV and IPPYV. In contrast, only one motif has been recognized in the LCMV Z protein (P_{85}PPY) (Perez *et al.*, 2003). It is not possible to recognize any known L domains in the MWV Z ORF. MWV is not unique in this feature; TCRV also does not show canonical L domains. Instead, TCRV Z contains an ASAP sequence that partially mimics the L domain, PTAP. Similarly, MWV contains a PTCP domain that might also mimic the PTAP domain, although more distantly due to its polarity properties. Recently, similar L-domain motifs have been described in other viruses, including YP$(x)_n$L in the p9 region of equine infectious anemia virus Gag protein (Puffer *et al.*, 1997) and the YRKL sequence in the matrix protein of influenza virus (Hui *et al.*, 2003).

Large RNA-dependent RNA polymerase protein (RdRp; L). The less divergent areas of the 2001 aa MWV RdRp (230 kDa, pI=6.8) overlap conserved areas among all other arenaviruses, confirming their association with function: region I (position 1–250), region II (position 500–900) (Supplementary Fig. S3a, available in JGV Online), region III (position 1000–1650; RNA replicase domain) and region IV (position 1750–1900). Region III is characterized by the motifs in the catalytic domain termed pre-A, A, B, C, D and E (Delarue *et al.*, 1990; Müller *et al.*, 1994; Poch *et al.*, 1989; Vieth *et al.*, 2004) (Supplementary Fig. S3b).

NP. The 561 aa NP of MWV (62.2 kDa, pI=9.0) shows between 45 and 53 % conservation at the amino acid level (51–56 % at the nucleotide level) to those of NW arenaviruses, and between 57 and 72 % (59–66 % at the nucleotide level) to those of OW arenaviruses (Table 2; Supplementary Fig. S4, available in JGV Online). The following amino acid motifs previously described in the family are all well-conserved (Gonzalez *et al.*, 1996): (a) mixed-charge segment at the N-terminal region (Parisi *et al.*, 1996); (b) the atypical ribonucleoprotein consensus sequence motif (RNP-1) (Parisi *et al.*, 1996); (c) zinc-finger motif in the C-terminal region (Parisi *et al.*, 1996); and (d) the cytotoxic T-lymphocyte (CTL) epitope G_{123}VYMGNL described in LCMV (Whitton *et al.*, 1989). Another

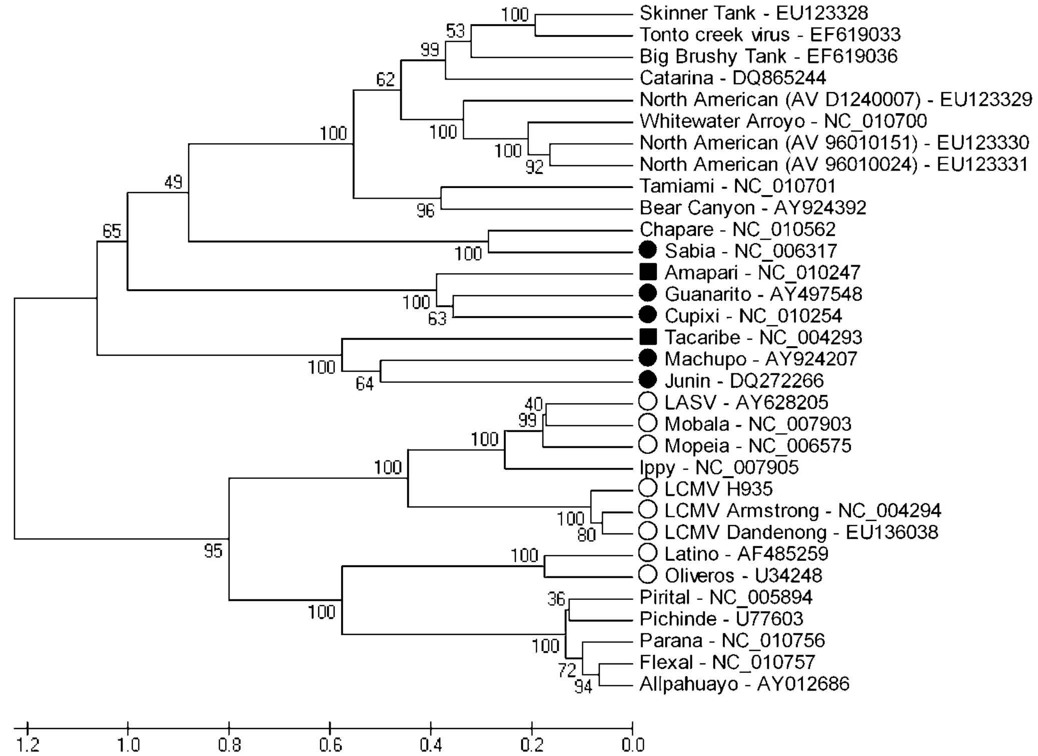

Fig. 3. Phylogenetic analysis of MWV based on secondary structure alignments of G1 sequences. Receptors reported to interact with the G1 glycoprotein moiety are shown, where ● represents NW arenaviruses using TfR1, ■ represents viruses using host TfR1, but not human receptors, and ○ indicates OW and clade C NW arenaviruses that use α-DG.

potential CTL epitope, Y_{315}IACRTSIV as found in LCMV, is not well-conserved, similar to LUJV. A potential antigenic site in the mixed-charge area of the LASV NP (RKSKRND; Gonzalez *et al.*, 1995) has the sequence R_{55}KEKRDD in MWV.

GPC. The 497 aa MWV GPC preprotein precursor (56.3 kDa, pI=8.5) is cleaved cotranslationally into a stable signal peptide and an immature precursor, which is subsequently processed by the host signal signalase (S1P) into the G1 and G2 fragments (Beyer *et al.*, 2003; Burns & Buchmeier, 1991, 1993; Lenz *et al.*, 2001; Neuman *et al.*, 2005; Rojek *et al.*, 2008b). The G1 segment is the area of lowest conservation of the whole GPC (Supplementary Fig. S5a, available in JGV Online).

Using the SignalP algorithm, S1P is predicted to cleave the signal peptide of MWV between A and T_{60}. The two hydrophobic domains found in arenaviruses are in the 59 aa signal peptide of MWV (6.4 kDa, pI=4.9) (Eichler *et al.*, 2004). The sequence also displays a conserved G_2 that has been implicated in myristoylation of JUNV (York *et al.*, 2004).

By comparison with other arenaviruses, S1P cleavage of RRLK_{265} may lead to separation of G1 (261 aa, 29.2 kDa, pI=6.8) from G2 (232 aa, 26.6 kDa, pI=8.5) (Beyer *et al.*, 2003; Lenz *et al.*, 2000; Rojek *et al.*, 2008a). Although the G2 fragment contains unique amino acid residues in several positions, it is generally well-conserved with respect to other arenaviruses (Supplementary Fig. S5b).

Two receptors are reported to interact with the G1 glycoprotein moiety of arenaviruses. Alpha-dystroglycan (α-DG) (Cao *et al.*, 1998) binds OW arenaviruses LASV and LCMV and the non-pathogenic clade C NW arenaviruses Oliveros virus (OLVV) and Latino virus (LATV) (Spiropoulou *et al.*, 2002). Transferring receptor 1 (TfR1) binds the pathogenic NW arenaviruses JUNV, MACV, GTOV and SABV (Radoshitzky *et al.*, 2007) (Fig. 3). Structural alignment of the MWV G1 with those of representative members of the OW and NW clade C arenaviruses might correlate with its receptor usage.

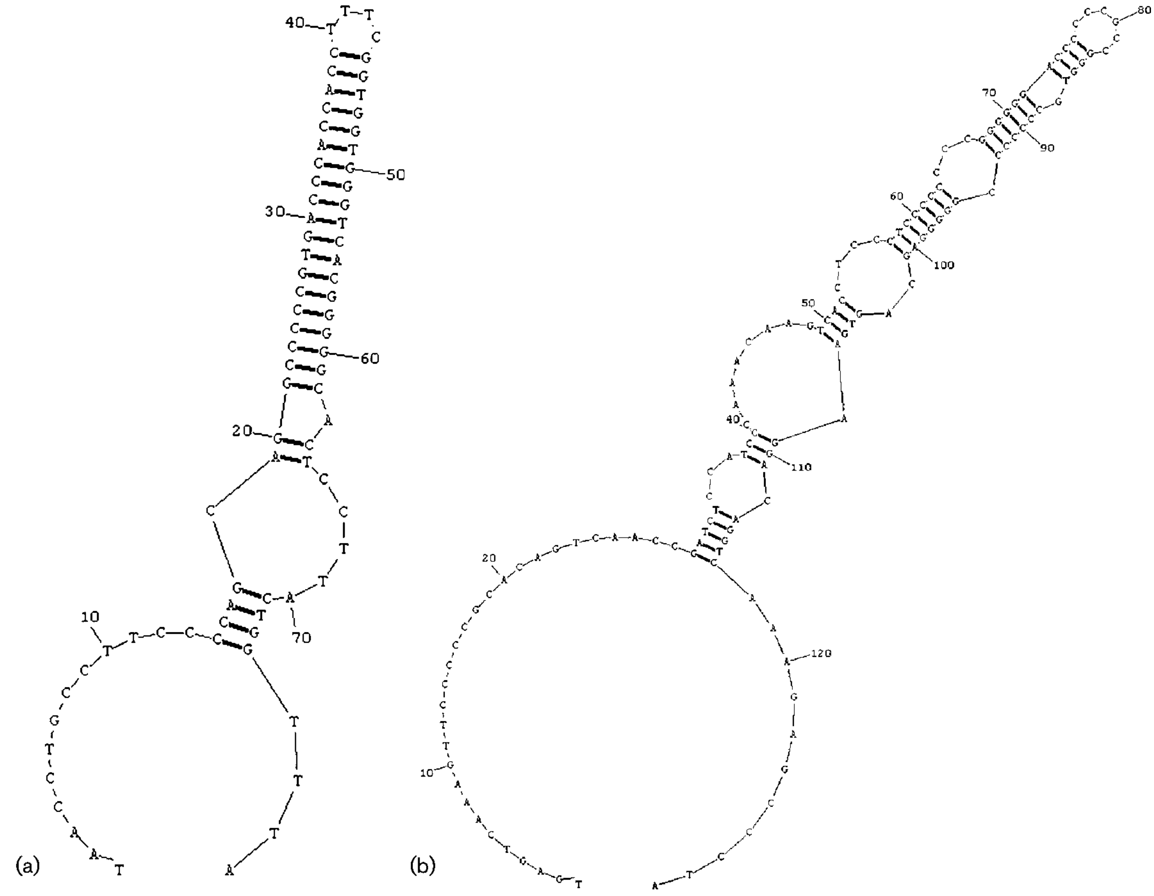

Fig. 4. Predicted secondary structures of the (a) S and (b) L segments. The S segment (a) is predicted to form a single, highly stable stem–loop structure, whereas the L segment (b) is assumed to form a complex folding structure.

Non-translated sequence analysis

The 5′ and 3′ non-coding regions (NCRs) of the S segment are 52 and 60 nt long, respectively. The S segment intergenic region (IR) of MWV comprises 72 nt and is predicted to form a single, highly stable stem–loop structure (Fig. 4a). The S segment IR of SABV has been reported to form three such structures (Gonzalez *et al.*, 1996), whereas those of JUNV, TCRV, OLVV, Flexal virus (FLEV), GTOV, LATV, MACV, AMAV, Cupixi virus (CPXV) (all NW) and of the OW virus MOPV can fold into two (Archer & Rico-Hesse, 2002; Bowen *et al.*, 1996; Ghiringhelli *et al.*, 1991; Iapalucci *et al.*, 1991; Wilson & Clegg, 1991). Those of the NW viruses Pichinde virus (PICV), Paraná virus (PARV), Pirital virus (PIRV), Allpahuayo virus (ALLV; Moncayo *et al.*, 2001) and Whitewater Arroyo virus (WWAV; Fulhorst *et al.*, 1996), and of the OW viruses LASV and LCMV, appear to form only a single stem–loop structure (Archer & Rico-Hesse, 2002; Auperin *et al.*, 1984, 1986; Charrel *et al.*, 2001; Moncayo *et al.*, 2001; Romanowski & Bishop, 1985). The L segment has a 5′ NCR of 99 nt, a 3′ NCR of 23 nt and an IR of 122 nt that can be assumed to form a complex folding structure, as observed in other arenavirus L segment IRs (Fig. 4b) (Archer & Rico-Hesse, 2002).

Conclusion

Our analysis of the genomic and biological characteristics of MWV indicates that it is a novel virus related only distantly to other known arenaviruses. MWV fulfils the majority of the parameters established by the International Committee on Taxonomy of Viruses for species demarcation within the genus *Arenavirus*: (i) association with a specific host species. *Myotomys unisulcatus* is a member of the rodent subfamily Muridae; this is the first arenavirus detected in rodents of the genus *Myotomys*. (ii) Substantive differences in antigenic cross-reactivity. MWV has antigenic cross-reactivity with MOBV, MOPV and IPPYV, but no reactivity with LCMV. (iii) Substantive sequence difference from other known species in the genus. The observed lowest nucleotide sequence divergence with the closest arenavirus species, MOPV, was 34.5 %. The lowest amino acid sequence divergence with the same species was 31.4 %. Two parameters remain undefined: (iv) the distribution of this virus within a defined geographical area (southern Africa) and (v) pathogenicity (or not) for humans. We have tentatively named this species Merino Walk virus (MWV), following the standard nomenclature of naming arenaviruses by their geographical origin, and we propose SPU 353-85 as its reference genome (prototype).

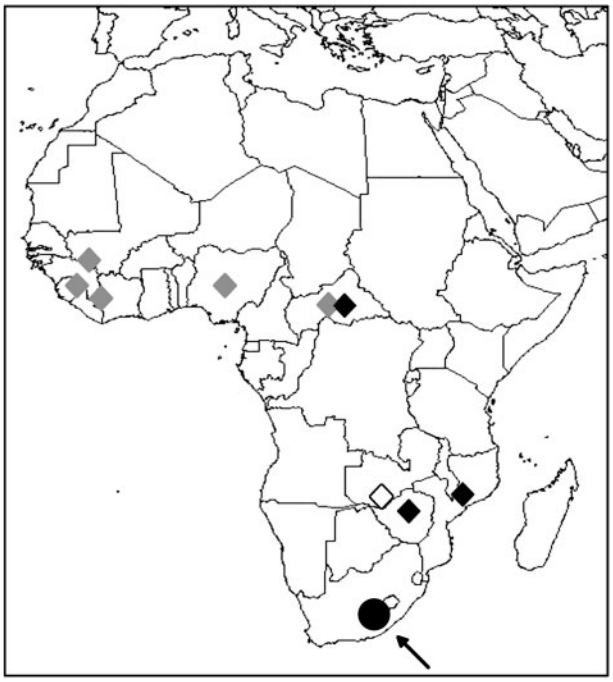

Fig. 5. Known geographical distribution of arenaviruses in Africa based on isolation. MOBV, MOPV and IPPYV, which have not been associated with disease in humans, are shown by ◆. LASV (◆) and LUJV (◇) are associated with haemorrhagic fever. The arrow indicates the approximate location where MWV was recovered (●).

METHODS

Source of virus. The prototype strain of MWV, SPU 353-85, was isolated in 1985 from a bush Karoo rat, *Myotomys unisulcatus*, captured at Merino Walk, Eastern Cape, South Africa (Fig. 5). *Myotomys unisulcatus* is endemic to southern Africa (Skinner & Smithers, 1990), confined primarily to the semi-arid Karoo region of the South-West Arid Zone (Davis, 1962; Skinner & Smithers, 1990), but occasionally found in the South-West Cape Zone (Davis, 1962). *Myotomys unisulcatus* (previously known as *Otomys unisulcatus*) is a member of the family Muridae (Musser & Carleton, 2005).

Virus isolation and immunological characterization. Tissue samples were homogenized mechanically in 650 μl virus isolation medium [1 × minimum essential medium (MEM), 2.2 g $NaHCO_3$ l^{-1}, 10 % fetal bovine serum (FBS) and 2 × antibiotic/antimycotic solution (400 U penicillin ml^{-1}, 400 μg streptomycin ml^{-1}, 1 μg amphotericin B ml^{-1})]. Homogenized tissues were centrifuged (6700 ***g*** for 10 min) to pellet debris, and an aliquot (100 μl) of the clarified supernatant was used to inoculate Vero cells. Cells were maintained in MEM supplemented with 5 % FBS and 2 × antibiotic/antimycotic solution, and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2.

Antigens and immunological reagents. In addition to MWV, the following viruses were used to prepare the antigens and immune reagents: IPPYV (strain Dak An B 188d), MOPV (strain AN 20410) and MOBV (strain ACAR 3080-MRC 5P2). Antigens for use in CF tests were prepared from infected newborn mouse brain by the sucrose–acetone extraction method (Beaty *et al.*, 1995). Immunizing antigens were 10 % crude brain suspensions of infected mice in PBS.

Specific hyperimmune mouse ascitic fluids (MAF) were prepared against the arenavirus prototype strains listed above. The immunization schedule consisted of four weekly intraperitoneal injections of mouse brain antigen mixed with Freund's adjuvant, as described previously (Fulhorst *et al.*, 1997). Following the fourth injection, sarcoma 180 cells were also injected intraperitoneally to induce ascites formation. The lymphocytic choriomeningitis MAF was produced by the same method, but was obtained from the NIAID Reference Reagents Program (V-580-701-562). All animal work was carried out under an animal protocol approved by the University of Texas Medical Branch.

Serology. CF tests were performed, using a microtechnique (Beaty *et al.*, 1995), with two full units of guinea pig complement. Titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 (complete haemolysis) to 4+ (no haemolysis).

Transmission electron microscopy. For ultrastructural analysis, infected cells were fixed in 2 % paraformaldehyde/2.5 % glutaraldehyde (Polysciences Inc.) in 100 mM phosphate buffer, pH 7.2, for 1 h at room temperature. Samples were washed in phosphate buffer and post-fixed in 1 % osmium tetroxide (Polysciences Inc.) for 1 h. Samples were then rinsed extensively in distilled H_2O (dH_2O) prior to en bloc staining with 1 % aqueous uranyl acetate (Ted Pella Inc.) for 1 h. Following several rinses in dH_2O, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.).

Genomic sequencing. The protocol for preparation of genomic material for pyrosequencing has been published in detail elsewhere (Palacios *et al.*, 2008). MWV was extracted by using TRIzol LS (Invitrogen). Total RNA extracts were treated with DNase I (DNA-free; Ambion) and cDNA was subjected to a modified DOP-PCR procedure (Palacios *et al.*, 2007). Products >70 bp were selected by column purification (MinElute; Qiagen) and ligated to specific adapters for sequencing on a 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 removal of primer sequences, redundancy filtering and sequence assembly. With primers designed by using pyrosequence data, gaps between the aligned fragments were rapidly filled by specific PCR amplification. Conventional PCRs were performed with HotStar polymerase (Qiagen) 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 72 °C for 1–3 min depending on the expected amplicon size. Specific primer sequences are available upon request. Amplification products were run on 1 % agarose gels, purified (MinElute; Qiagen) and sequenced directly in both directions with ABI PRISM Big Dye Terminator 1.1 Cycle Sequencing kits on ABI PRISM 3700 DNA Analyzers (Perkin-Elmer Applied Biosystems). Terminal sequences were generated by using a universal arenavirus primer targeting the conserved viral termini (5′-CGCACMGDGGATCCTAGGC), combined with four specific primers positioned near the ends of each segment. Sequence was verified by classical dideoxy sequencing using primers designed along the draft sequence. The assembled data revealed a classical arenavirus genome structure (GenBank accession nos GU078660 and GU078661).

Phylogenetic analysis. A set of OW and NW arenavirus sequences (50 for the L segment; 95 for the GPC gene; 116 for the NP gene) comprising all arenavirus sequences available from GenBank (June 2009) were used to assess the phylogenetic history of MWV. All arenavirus sequences were aligned by using the CLUSTAL algorithm [as implemented in the MEGA package (version 3); Kumar *et al.*, 2004] at the amino acid level, with additional manual editing to ensure the highest possible quality of the alignment. Bayesian phylogenetic analyses of the sequence differences among the S and L segments of arenaviruses were conducted by using the BEAST, BEAUti and Tracer analysis software packages (Drummond & Rambaut, 2007). Preliminary analyses were run for 10 000 000 generations with the HKY + G nucleotide-substitution model to select the clock and demographic models most appropriate for the GPC, NP and L datasets. An analysis of the marginal likelihoods indicated that the relaxed lognormal molecular clock and constant population size model was decisively chosen ($\log_{10}$ Bayes factors=78.416 for GPC ORF, 11.127 for NP and 25.097 for L). Final data analyses included Markov chain Monte Carlo chain lengths of 10 000 000 generations, with sampling every 1000 states.

Reference arenavirus G1 sequences were aligned by using PROMALS 3D software and 3D T-Coffee to obtain a secondary-structure alignment. Resulting alignments were analysed with the same parameters as described above.

Sequence analysis. Programs of the Geneious package (Biomatters, NZ) were used for sequence assembly and analysis; percentage sequence identities were calculated by using MEGA. 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 Phobius (http://phobius.sbc.su.se/) (Bendtsen *et al.*, 2004; Claros & von Heijne, 1994; Käll *et al.*, 2004; Krogh *et al.*, 2001).

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