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Minireview

Phylogeny and evolution of old world arenaviruses

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Abstract

The intention of this study was to investigate the genomics, phylogeny and evolution of the Old World arenaviruses based on sequence data representing the four viral genes. To achieve this aim, we sequenced the complete S and L RNA segments of *Ippy virus* (IPPYV), *Mobala virus* (MOBV) and *Mopeia virus* (MOPV). Full-length sequences of the NP, GPC, Z and L genes were used to reconstruct phylogenetic relationships and to compare resulting tree topologies. Each of the five Old World arenavirus species (namely *Lassa virus* [LASV], IPPYV, MOBV, MOPV and *Lymphocytic choriomeningitis virus* [LCMV]) are monophyletic; seven selected strains of LASV showed a similar topology regardless of the gene under analysis; IPPYV rooted the three other African arenaviruses; the four African arenaviruses are rooted by the ubiquitous LCMV; and the tree topologies of the three African arenaviruses other than LASV are identical regardless of the gene used for analysis. No evidence for significant evolutionary events such as intra- or intersegmental recombination was obtained.

Keywords: Arenavirus; Evolution; Recombination; Phylogeny; Viral hemorrhagic fever; *Lassa virus*; BSL-4; Africa; *Ippy virus*; *Mobala virus*; *Mopeia virus*

Introduction

The family Arenaviridae comprises a unique genus (*Arenavirus*) that currently contains 22 recognized viruses. Arenaviruses possess single stranded bi-segmented RNA genomes. The large (L) genomic segment (~7,200 nt) encodes the viral RNA-dependent RNA polymerase and a zinc-binding matrix protein, acting as a bona fide matrix protein (Perez et al., 2003; Strecker et al., 2003) The small (S) genomic segment (~3500 nt) encodes the nucleocapsid protein (NP) and the glycoprotein precursor (GPC) in two non-overlapping reading frames of opposite polarities. The GPC is secondarily cleaved into the envelope proteins G1 and G2. The genes on both S and L segments are separated by an intergenic non-coding region with the potential to form one or more hairpin configurations. The 5′ and 3′ ends of each RNA segment possess a relatively conserved reverse complementary sequence spanning 19 nucleotides at each extremity. The arenaviruses have been classified according to their antigenic properties into two groups: the Tacaribe serocomplex (including viruses indigenous to the New World) and the Lassa–Lymphocytic choriomeningitis serocomplex (including the viruses indigenous to Africa and the ubiquitous *Lymphocytic choriomeningitis virus* [LCMV], recognized as the Old World group) (Charrel and de Lamballerie, 2002; Clegg et al., 2000). Specific rodents are the principal hosts of the arenaviruses. Humans usually become infected through contact with infected rodents or inhalation of infectious rodent excreta or secreta. At least 9 arenaviruses are associated with human disease, of which five – *Lassa* (LASV), *Junin*, *Machupo*, *Guanarito* and *Sabia* – are known to cause severe hemorrhagic fever in western Africa, Argentina, Bolivia, Venezuela and Brazil, respectively. The other four arenaviruses are LCMV (causing acute central nervous system disease (Barton, 1996) and congenital malformations (Barton and Mets, 2001)), Flexal and Tacaribe viruses (febrile illnesses in laboratory workers (Peters et al., 1996; Buchmeier et al., 1974)) and, more recently, Whitewater Arroyo virus associated with fatal cases of infection in California (CDC, 2000). The five arenaviruses causing viral hemorrhagic fever are included in the

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Category A Pathogen List, considered as Select Agents as defined by the CDC, and listed as Biosafety Level 4 agents. In the past 5 years, our understanding of the phylogeny and evolution of arenaviruses has made considerable progress due to the availability of complete genomic sequences; evidence for recombination has resulted from the analysis of full-length genomes and showed that significant evolution events may play a substantial part in arenavirus evolution (Archer and Rico-Hesse, 2002; Charrel et al., 2001, 2002). To date, most studies have focused on New World arenaviruses and neglected Old World arenaviruses. Our objective was to study the evolution of these viruses with a strategy similar to that previously used for New World arenaviruses (Charrel et al., 2003) to explore the respective role of the 3 mechanisms driving the genetic diversity of Arenaviridae (specifically, the accumulation of mutations, intrasegmental recombination and intersegmental recombination or reassortment). To achieve this aim, we first determined the full-length genome sequences for *Ippy* (IPPYV), *Mobala* (MOBV) and *Mopeia* (MOPV) viruses in order to complete sequence data sets for African arenaviruses; we then used a comprehensive set of sequences for phylogeny reconstruction and compared tree topologies obtained independently from the four genes. Finally, different methods were used to search for significant evolutionary events such as recombination and reassortment.

Results and discussion

To date, little data were available for the Old World arenaviruses, and the most comprehensive phylogenetic study was based on analysis of a partial region of the nucleoprotein gene (Bowen et al., 1997, 2000). Therefore, it was essential to obtain a complete genetic data set in order to examine the phylogenetic relationships of arenaviruses within the Old World complex. Here, we report the first comprehensive phylogenetic study based on independent analysis of full-length sequences of

Table 1
Characteristics of complete sequences of Old World Arenaviruses S (A) and L RNA (B)

(A)

Virus species	Acronym	Strain	S RNA						
			GenBank accession nos.	5′ NCR (nt)	GPC (aa)	IGR (nt)	NP (aa)	3′ NCR (nt)	Length (nt)	GC (%)
Lassa virus	LASV	Josiah	NC_004296	55	491	67	569	100	3402	44.15
		Josiah	AY628203	55	491	67	569	100	3402	44.12
		NL	AY179173	nc	491	67	569	nc	nc	44.62
		z148	AY628205	54	491	66	569	96	3396	43.96
		Macenta	AY628201	54	491	66	569	99	3399	43.98
		AV	AF246121	53	491	67	569	91	3391	44.35
		CSF	AF333969	nc	490	65	569	nc	nc	44.05
Lymphocytic choriomeningitis virus	LCMV	WE	M22138	77	498	70	558	60	3375	44.77
		Armstrong	NC_004294	77	498	70	558	61	3376	46.12
Mopeia virus	MOPV	AN20410	AY772170	53	489	129	570	68	3427	45.55
		Mozambique	**DQ328874**	53	489	86	570	68	3384	45.39
Mobala virus	MOBV	Acar 3080	**AY342390**	84	491	69	568	50	3380	43.82
Ippy virus	IPPYV	Dak An B 188 d	**DQ328877**	nc	495	64	570	58	nc	45.07

(B)

Virus species	Acronym	Strain	L RNA						
			GenBank accession nos.	5′ NCR (nt)	Z (aa)	IGR (nt)	L (aa)	3′ NCR (nt)	Length (nt)	GC %
Lassa virus	LASV	Josiah	NC_004297	65	99	106	2218	157	7279	41.12
		Josiah	AY628202	65	99	106	2220	157	7285	40.99
		NL	AY179172	nc	99	105	2220	139	nc	41.3
		z148	AY628204	66	99	104	2219	156	7280	42.2
		Macenta	AY628200	66	99	104	2219	156	7280	42.2
		AV	AY179171	nc	99	105	2220	nc	nc	40.27
		CSF	L: AY179174	nc	99	nc	2217	nc	nc	L: 40.31
			Z: AY179175							Z: 50.51
Lymphocytic choriomeningitis virus	LCMV	WE	AF004519	88	90	202	2209	32	7219	42.06
		Armstrong	L: J04331	88	90	200	2210	32	7220	L: 40.36
			Z: M27693							Z: 53.06
Mopeia virus	MOPV	AN20410	AY772169	79	103	116	2237	56	7271	42.5
		Mozambique	**DQ328875**	80	103	111	2229	55	7242	40.87
Mobala virus	MOBV	Acar 3080	**DQ328876**	116	99	102	2220	150	7325	39.59
Ippy virus	IPPYV	Dak An B 188 d	**DQ328878**	148	101	170	2208	71	7316	39.76

Accession numbers in bold refer to sequences determined in this study.

the four genes of all recognized Old World arenaviruses. This allowed the comparative analysis of evolutionary information between all viral genes and the existence of inter- or intrasegmental recombination to be addressed. The characteristics of the sequences determined in this study are presented in Table 1. Nucleotide composition analysis showed that: (i) GC % was statistically different from gene to gene, but not between viruses (two way ANOVA, $P < 0.001$ and $P = 0.744$, respectively); (ii) G + C content at the third nucleotide position reflected the overall G + C content; (iii) a clear bias towards under-representation of the dinucleotides CpG and UpA was found, as demonstrated by Arg coding: this amino acid is potentially encoded by 6 different codons (AGr and CGn), but AGA and AGG represented approximately 90% of the Arg codons, irrespective of virus or gene. Similar results were previously reported in the literature for viruses and eukaryotic organisms (Karlin and Burge, 1995).

Phylogeny

Phylogenetic data provided by previous studies concerning Old World arenaviruses were in agreement with regard to three points: (i) the Old World complex is monophyletic, (ii) LCMV roots other Old World arenaviruses and (iii) IPPYV is the most

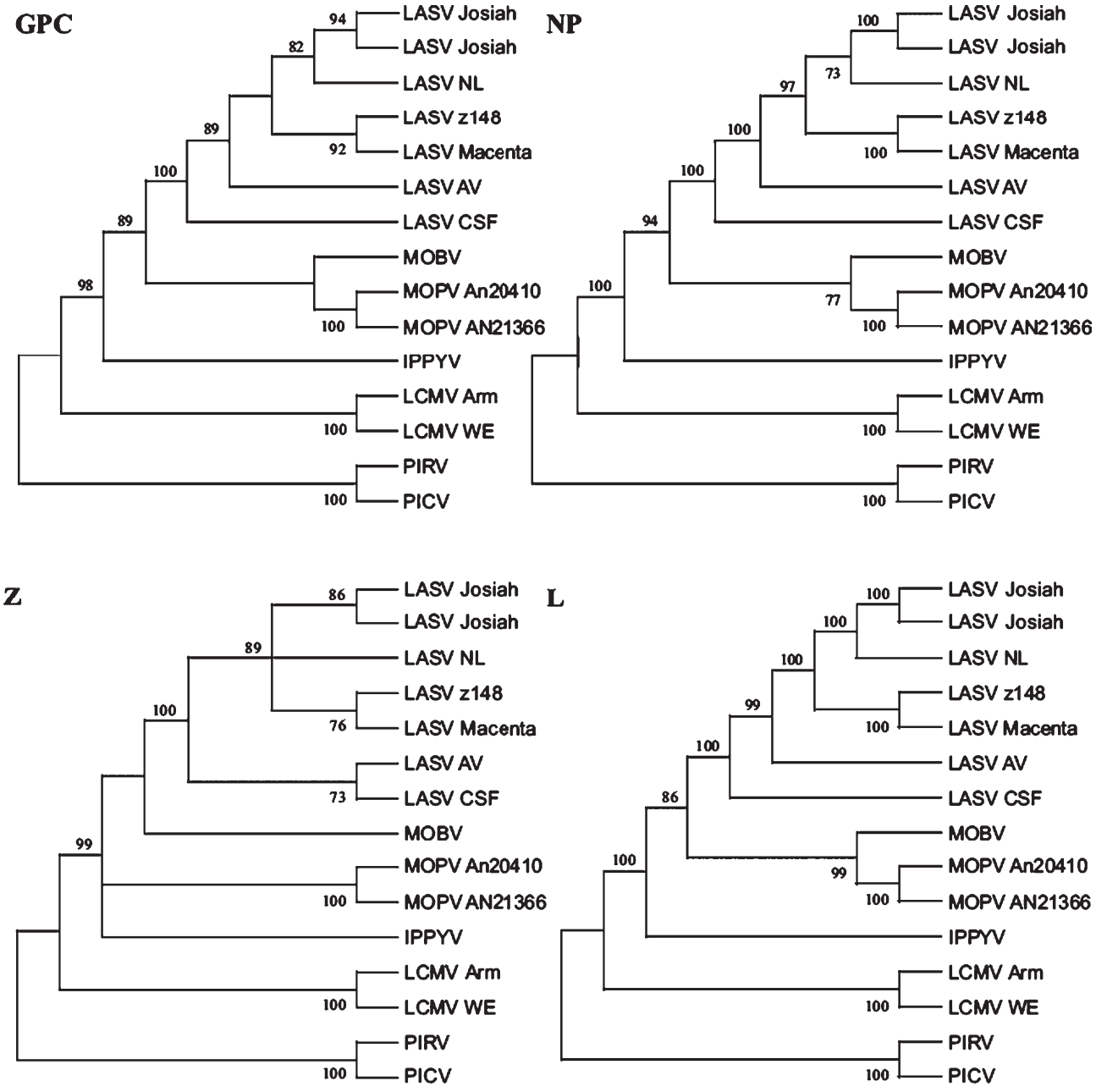
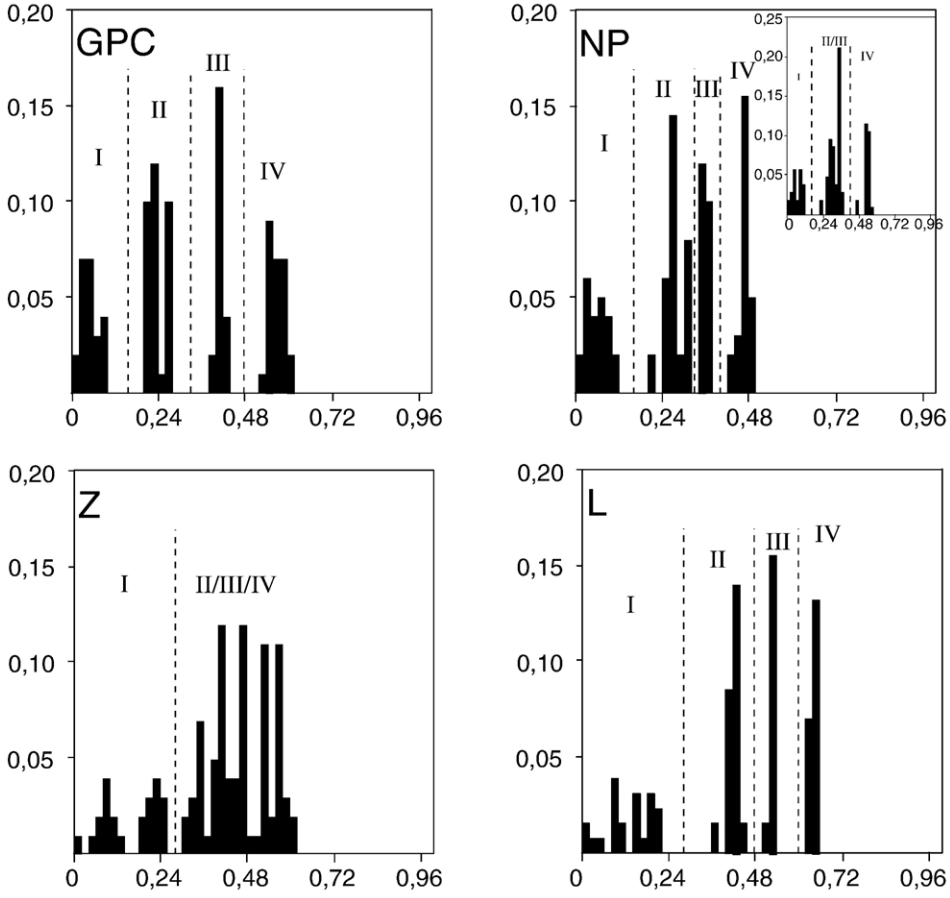


Fig. 1. Phylogeny of Old World arenaviruses based on the analysis of complete sequences of the GPC, NP, Z and L genes. Trees were estimated by maximum parsimony analysis using the PROTPARS stepmatrix of amino acid substitution and a branch-and-bound search for the most parsimonious tree. For Z gene, the cladogram shown is the strict consensus of the 3 trees retained. Numbers represent percentage bootstrap support (500 replications).

basal of African arenaviruses (Bowen et al., 1996, 1997, 2000; Charrel and de Lamballerie, 2003; Charrel et al., 2002; Vieth et al., 2004). However, the position of MOBV remained unresolved. For example, MOBV grouped with either LASV (Bowen et al., 1997) or MOPV (Bowen et al., 2000) depending on the phylogenetic method used for analysis. Comparative analysis of the phylograms corresponding to the four genes suggested the following conclusions regardless of the method used to reconstruct evolutionary relationships (Fig. 1): (i) the arenaviruses from the Old World serocomplex formed a monophyletic group; (ii) each of the five Old World species is monophyletic, with bootstrap support of 100%; (iii) the seven strains of LASV for which sequence data are available for the four genes showed a similar topology (bootstrap value equal or higher than 73%), with results similar to those reported for four strains by Vieth et al. (2004); (iv) IPPYV rooted the three other African arenaviruses (LASV, MOBV and MOPV), confirming previous results (Bowen et al., 1997, 2000); (v) these four African arenaviruses were rooted by the ubiquitous LCMV as reported by Bowen et al. (1997, 2000); (vi) MOPV and MOBV virus exhibited a common ancestor supported by 77% and 99% in NP and L, respectively. A 45% value was observed in the GPC-based parsimony tree, but a 79% bootstrap value was obtained by analysis derived using the distance method (data not shown). The only discrepancy was observed with Z sequences irrespective of the algorithm (neighbor-joining or maximum parsimony). Although this may reflect an intrasegmental recombination event (see below), this most likely results

A



B

Gene	intra-OW species distances				inter-OW species distances				inter-African species distances				between African species and LCMV distances			
	N	Mean	Max	Min	N	Mean	Max	Min	N	Mean	Max	Min	N	Mean	Max	Min
GPC	23	0,0493	0,086	0	55	0,3057	0,427	0,204	33	0,2367	0,277	0,204	22	0,4093	0,427	0,398
NP	23	0,0567	0,102	0	55	0,3077	0,369	0,215	33	0,274	0,31	0,215	22	0,3582	0,369	0,345
Z	23	0,1521	0,242	0	55	0,418	0,533	0,302	33	0,3807	0,429	0,302	22	0,474	0,533	0,427
L	23	0,128	0,208	0,01	55	0,465	0,537	0,366	33	0,423	0,448	0,366	22	0,527	0,537	0,517

Fig. 2. (A) Distribution of evolutionary distances upon pairwise comparison observed between Old World arenaviruses. Distances were calculated from complete GPC, NP, Z and L proteins. Genetic distance is reported on the *x* axis. Frequency of genetic distance is recorded on the *y* axis. In the upper right corner of NP gene is the distribution obtained when the region used by Bowen et al. (1997) is analyzed. Groups I–IV correspond to intraspecies, interspecies within African arenaviruses, interspecies between African arenaviruses and LCM virus and interspecies between Old World and the two representatives of New World arenaviruses (PIR and PIC viruses). (B) Genetic diversity between groups at the amino acid level for the four genes. *N*, number of pairwise comparison; Mean, Max, Min, mean, maximum and minimum values of pairwise comparisons for each gene.

from the small size of Z sequences combined with high interspecies heterogeneity.

Mechanisms driving the evolution of Old World arenavirus

As shown by the recent discovery of recombinant viruses (i.e. viruses whose genome is derived from intrasegmental recombination) in various genera of RNA viruses (and specifically in arenaviruses), detection of recombination is primarily based on the comparative analysis of phylograms reconstructed from different genes or portions of genes, later confirmed by specific programs. Previous studies demonstrated a potential for major evolutionary events such as intrasegmental recombination or segmental reassortment, either via natural or experimental processes in arenaviruses (Archer and Rico-Hesse 2002; Charrel et al., 2001; Lukashevich, 1992; Riviere and Oldstone, 1986; Riviere et al., 1986). There is currently no evidence for naturally occurring reassortant viruses in the Arenaviridae family. Comparative analysis of the four phylograms reconstructed from full-length gene sequences showed a global homogeneity in the topology, allowing reassortment to be excluded. The only discrepancy comprised the position of MOBV, which shifted from a sister group position in the GPC, NP and L trees to root LASV complex in the Z-based tree. This divergence in tree topology could reflect the fact that the evolutionary history of the Z gene is different from that of other genes, thus suggesting that a significant evolutionary event such as an intrasegmental recombination may have occurred in the case of MOBV or an ancestor of MOBV. However, evidence for recombination involving MOBV was investigated using other methods based on distance comparison (Simplot 2.5 software written by S. Ray and distributed by the author at http://med.jhu.edu/deptmed/sray/, and Recombination Detection Program) (Martin and Rybicki, 2000) and bootscanning analysis (Charrel et al., 2001; Salminen et al., 1995), and no support for recombination was obtained. Accordingly, the divergence in the Z-based topology most likely resulted from the relative short length of Z gene sequences (111 aa), the high genetic heterogeneity of the data set (up to 62%) and the presence of many insertions and deletions.

Distance analysis

Between genes

Maximal intraspecies distances observed for the GPC and NP genes were 8.6–10.2% and 20.8–24.2% for the Z and L genes. Maximal interspecies distances observed for the GPC and NP genes were 27.7–42.7% and 42.9–53.7% for the Z and L genes. Distances between Old World arenaviruses from different species but also between different representatives of the same species showed that structural genes in the S RNA segment exhibited a lower variability than the polymerase gene (Fig. 2). Similar analyses conducted in other orders or families of animal RNA viruses (Filoviridae, Bunyaviridae, Paramyxoviridae, Orthomyxoviridae, Pneumoviridae, Reoviridae, Flaviviridae) demonstrated that distances between structural genes are always higher than that observed between genes implicated in viral replication. In light of these

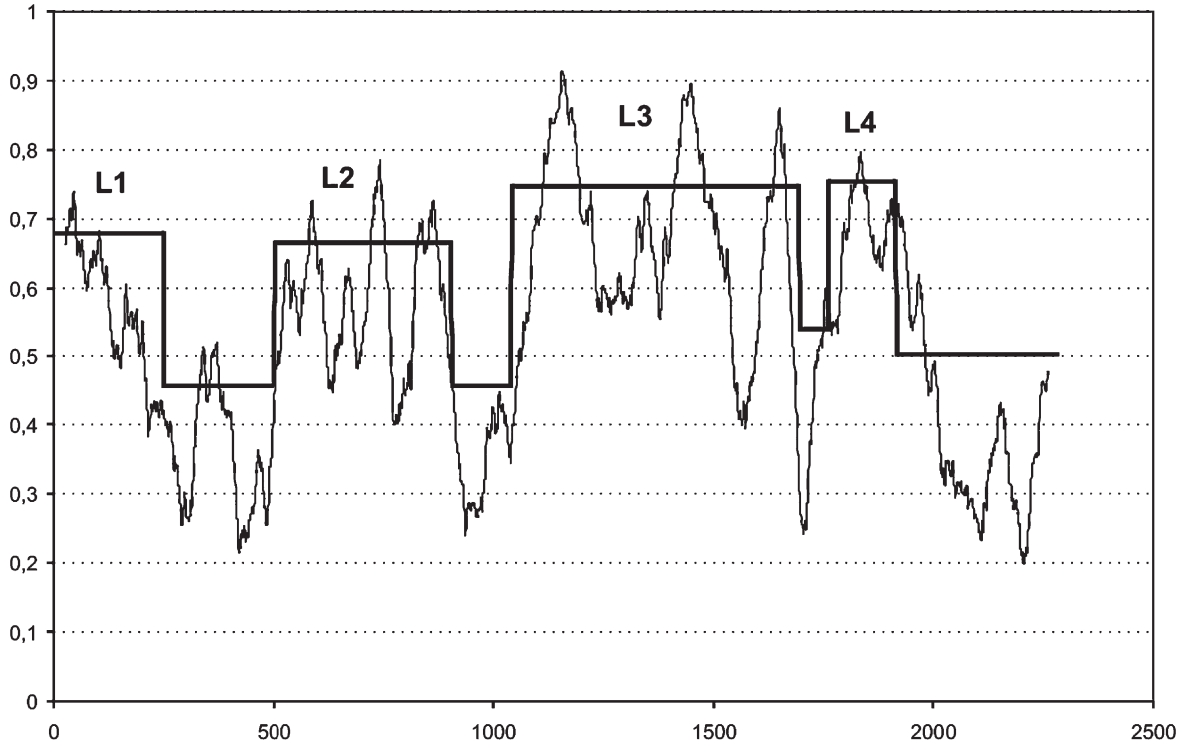


Fig. 3. Sequence homology across Old World virus L proteins. Pairwise identities were calculated for all possible pairs of Old World arenavirus species sequences with a sliding window of 50 amino acids. Mean values of pairwise identities were reported across the L protein. The four regions previously identified by Vieth et al. (2004) are reported (L1 to L4). Pairwise homology was calculated using MEGA for regions and interdomains (bold line).

interesting results, we postulated that the high genetic diversity within the polymerase encoding gene might be due to an extreme heterogeneity between interdomains, whereas functional domains remain relatively conserved across species. To address this point, we calculated the mean values of pairwise distances within incremented subsets of the amino acid alignments for all couples of species. Fig. 3 showed that the heterogeneity was not spread homogenously along the L protein. The most conserved regions (Region I: AA1–AA250, Region II: AA480–AA865, Region III: AA996–AA1650, Region IV: AA1706–AA1858, for LCMV Armstrong L gene) were previously defined as functional domains (L1–L4) implicated in viral replication (Vieth et al., 2004). In contrast, higher diversity (between L1 and L2: 0.458, between L2 and L3: 0.458, between L3 and L4: 0.541, after L4: 0.503) was observed in regions defined as interdomains. A similar analysis was conducted into the hantaviruses, which share close ecological and genetic characteristics with the arenaviruses. Genetic divergence between functional domains and interdomains was found to be considerably lower than that observed with Old World arenaviruses (data not shown). Preliminary data suggest that this may be confirmed for New World arenaviruses, and a comprehensive study of this issue is currently underway.

Between viruses

Amino acid sequences of the full-length GPC, NP, Z and L genes were used to study the distribution of evolutionary distances upon pairwise comparison (Fig. 2). Four groups were distinguished and corresponded to intraspecies distances [group I], interspecies distances within African arenaviruses [group II], interspecies distances between African arenaviruses and LCMV [group III] and interspecies distances between Old World arenaviruses and the outgroup consisting of two New World arenaviruses [group IV]. The distribution presented in Fig. 2 clearly delineated these four categories when analysis was performed using the complete GPC and L gene proteins. The situation was less clear with the NP and Z gene protein since delineation was either not possible or problematic between groups II and III, and II, III and IV, respectively. To date, the definition of species in the genus *Arenavirus* is polythetic, based on the nature of the host species (rodent or bat), the geographic distribution, the existence of human pathogenicity, the antigenic cross-reactivity and result of cross-neutralization tests and significant amino acid sequence divergence (Clegg et al., 2000); for the latter, no additional information is provided regarding either the gene to be used or the cut-off value to adopt. In the case of Old World arenaviruses, the cut-off value suitable for species definition must distinguish between group I and groups II–IV. Possible cut-off value boundaries suitable to delineate the five species were 8.6–20.4% (GPC), 10.2–21.5% (NP), 24.2–30.2% (Z) and 20.8–36.6% (L). Therefore, sequences of the four gene proteins were candidates for this purpose. The 12% criterion in the partial NP, as previously defined (Bowen et al., 2000), was also utilized when complete NP protein sequences were used. However, extending this analysis to New World arenaviruses is essential before any conclusion can be drawn concerning the possibility of using genetic criteria for arenavirus taxonomy.

Materials and methods

Virus strains, culture and sequencing

Virus strains used in this study are presented in Table 1. Viral culture was performed in a biosafety level 2 laboratory (Unité des Virus Emergents, Marseilles, France) either in Vero cells (MOBV and MOPV) (Charrel et al., 2001) or by intracerebral inoculation to newborn mice (IPPYV). Viral RNA was purified using RNA NOW (Biogentex), resuspended in 50 µl of water and quantified by spectrophotometer at $OD_{260\ nm}$ to adjust total RNA concentration to between 150 and 250 µg/mL. RT-PCR was performed with the cMaster RT_{plus}PCR System (Eppendorf) following manufacturer's recommendations using 0.3–0.5 µg of RNA, a concentration range experimentally determined to provide optimal efficiency for that purpose. PCR products of the expected size were purified and sequenced directly or cloned into PGEM-T System I (Promega) and sequenced further with M13 primers. Sequences were determined from PCR products obtained with primers designed to conserved regions from the alignment of other arenaviruses (primers are available upon request to the corresponding author) in combination with primers previously described (Bowen et al., 1997; Charrel et al., 2003).

Sequence data analysis (alignment, phylogeny, evolution)

Amino acid sequences were aligned using ClustalX 1.81 (Thompson et al., 1997) using default parameters. Sequences of African arenaviruses were aligned first to constitute profile #1; then the two sequences of arenaviruses selected as outgroups were aligned onto profile #1 using the "Profile Alignment Mode". Due to the high genetic distances between Old World and South American arenaviruses, this strategy prevented the incorporation of gaps within sequences of African arenaviruses for optimal alignment with outgroup sequences. Distances were calculated with pairwise distance algorithms, and groupings were computed with neighbor-joining and maximum parsimony methods implemented in the MEGA 2.1 software package (Kumar et al., 2001) and PAUP* version 4b10 (Swofford, 2002), with the PROTPARS stepmatrix of amino acid substitution and a branch-and-bound search for the most parsimonious tree. Support for nodes was tested by bootstrapping using 500 pseudoreplications. Amino acid sequence diversity was calculated by pairwise comparison using p-distance. Identities were then plotted on a density histogram representing the distribution of evolutionary distance (x axis) upon pairwise comparison (y axis) (Fig. 2). A systematic search for intrasegmental recombination was performed using different recognized methods such as bootscanning, recombination detection program (Martin and Rybicki, 2000) and manual analysis of aa patterns. Bootscanning produces a phylogenetic tree from a window (subset) of the multiple sequence alignment

(Salminen et al., 1995; Charrel et al., 2001), which is incrementally shifted along the alignment and used to reconstruct a phylogenetic tree from each set; sliding windows of varying size were tested (100, 200 and 500 aa) and overlapped the precedent window by 50, 100 and 250 aa, respectively. The Recombination Detection Program utilizes a pairwise scanning approach combined with a UPGMA-based reconstruction algorithm which tests the probability that the nucleotide arrangement in a recombinant region occurred by chance (Charrel et al., 2001).

Diversity along the polymerase gene was analyzed using pairwise distances within a subset of the alignment between two sequences of Old World arenaviruses, using incremental shifts of a 50 amino acid window and distances reported to an Excel file. This procedure was repeated for each pair of viral species (with LCMV strain Armstrong, LASV strain Josiah AY628203 and MOPV strain AN21366 being used for their respective species). The mean distance for each window was calculated and used to plot a curve representing genetic heterogeneity along the L gene protein (Fig. 3).

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