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Phylogenetic relationships among rhabdoviruses inferred using the L polymerase gene

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RNA viruses of the family *Rhabdoviridae* include arthropod-borne agents that infect plants, fish and mammals, and also include a variety of non-vector-borne mammalian viruses. Herein is presented a molecular phylogenetic analysis, the largest undertaken to date, of 56 rhabdoviruses, including 20 viruses which are currently unassigned or assigned as tentative species within the *Rhabdoviridae*. Degenerate primers targeting a region of block III of the L polymerase gene were defined and used for RT-PCR amplification and sequencing. A maximum-likelihood phylogenetic analysis of a 158-residue L polymerase amino acid sequence produced an evolutionary tree containing the six recognized genera of the *Rhabdoviridae* and also enabled us to identify four more monophyletic groups of currently unclassified rhabdoviruses that we refer to as the 'Hart Park', 'Almpiwar', 'Le Dantec' and 'Tibrogargan' groups. The broad phylogenetic relationships among these groups and genera also indicate that the evolutionary history of rhabdoviruses was strongly influenced by mode of transmission, host species (plant, fish or mammal) and vector (orthopteran, homopteran or dipteran).

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INTRODUCTION

Of the currently described RNA viruses, more than 400 are primarily, though not exclusively, transmitted by arthropod vectors, such as mosquitoes, sandflies, fleas, ticks and lice. These viruses have complex life cycles, with many replicating in both primary and secondary hosts (although the latter may often be dead-ends for transmission) as well as in their arthropod vectors. Before the availability of molecular phylogenetic analysis these viruses were grouped together under the term 'arboviruses', although it is now known that they fall into five phylogenetically distinct viral families, the *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae* and *Rhabdoviridae*. The *Rhabdoviridae* currently comprises six genera, and members of three of these genera – *Vesiculovirus*, *Lyssavirus* and *Ephemerovirus* – have been obtained from a variety of animal hosts and vectors, including mammals, fish and invertebrates (Tordo *et al.*, 2004). The remaining three rhabdovirus genera are more taxon-specific in their host preference. Novirhabdoviruses infect numerous species of fish, while cytorhabdoviruses and nucleorhabdoviruses are arthropod-borne and infect plants.

All rhabdoviruses contain a single-stranded (−) RNA genome which encodes five virion structural proteins: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G) and the polymerase (L) (Dale & Peters, 1981). An added layer of complexity is present in the genus *Ephemerovirus*, as these viruses contain several additional open reading frames (ORFs) between the G and L genes which encode a second glycoprotein (G_{NS}) and several other non-structural proteins (Walker *et al.*, 1992, 1994; Wang *et al.*, 1994; McWilliam *et al.*, 1997). Similarly, in the genus *Novirhabdovirus*, a sixth functional cistron between the G and L genes encodes a non-structural protein (NV) of unknown function (Basurco & Benmansour, 1995). The unclassified rhabdovirus Sigma virus of *Drosophila* and plant rhabdoviruses in the genera *Cytorhabdovirus* and *Nucleorhabdovirus* also contain an additional ORF, which is located between the P and M genes (Heaton *et al.*, 1989; Landes-Devauchelle *et al.*, 1995; Wetzel *et al.*, 1994).

The available gene-sequence data from rhabdoviruses has increased considerably in recent years and this, in conjunction with data on genome organization and a variety of other biological characteristics, has been used for taxonomic classification and species demarcation among the *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus* and *Novirhabdovirus*

genera. In particular, the subdivision of each genus into species is supported by the comparison of nucleotide and deduced amino acid sequences of one (N gene) or several (N and G) common genes (Badrane & Tordo, 2001; Barr *et al.*, 1991; Basurco *et al.*, 1995; Bourhy *et al.*, 1993; Crysler *et al.*, 1990; Kissi *et al.*, 1995; Masters & Banerjee, 1987; Walker *et al.*, 1994; Wang *et al.*, 1995). However, complete genome sequences are available for only a few type species and it is unlikely that such data will be sought for the vast number of unclassified rhabdoviruses: a list of 63 unassigned animal rhabdoviruses is presented in the eighth International Committee on Taxonomy of Viruses (ICTV) report, a further 29 have been only tentatively assigned to genera due to inadequate data (Tordo *et al.*, 2004), and many more are awaiting classification.

One approach to the determination of the phylogenetic relationships among the *Rhabdoviridae*, as well as the identification of new viral species, is to utilize the conserved amino acid sequence blocks and/or motifs that have been identified in alignments of the RNA-dependent RNA polymerase (L protein) (Bock *et al.*, 2004; Delarue *et al.*, 1990; Dhillon *et al.*, 2000; Elliott *et al.*, 1992; Müller *et al.*, 1994; Le Mercier *et al.*, 1997; Poch *et al.*, 1989, 1990; Tordo *et al.*, 1988; Vieth *et al.*, 2004). Block III of the L polymerase is predicted to be essential for RNA polymerase function because it is conserved among all RNA-dependent RNA polymerases (Delarue *et al.*, 1990; Poch *et al.*, 1989; Xiong & Eickbush, 1990) and mutations in this region abolish polymerase activity (Schnell & Conzelmann, 1995; Sleat & Banerjee, 1993; Jin & Elliott, 1991, 1992). The sequence conservation displayed by this region suggests that it may be a useful target for the exploration of distant evolutionary relationships among the vast array of unclassified rhabdoviruses.

In this study, we inferred the phylogenetic relationships among 56 rhabdoviruses, 20 of which are currently tentative species or unassigned within the *Rhabdoviridae*. This represents the largest phylogenetic study of the *Rhabdoviridae* undertaken to date. Degenerate primers targeting block III of the L gene were defined and used for RT-PCR and sequence analysis, providing a rapid and expansive method to investigate the phylogenetic relationships. The broader goal of this research is to merge phylogenetic and epidemiological information, such as the host and vector species, to provide a more accurate and complete picture of the evolution of key biological characteristics within the *Rhabdoviridae*.

METHODS

Virus collection. A total of 38 rhabdoviruses isolated from various mammalian and insect species were selected from virus collections maintained at CSIRO in Australia and by the Pasteur Institutes in France and Senegal (Table 1). Of these, 15 were unassigned at the species level and five were tentatively classified to a particular rhabdovirus genus. Original virus isolations were predominantly made by intra-cerebral injection in suckling mice (Bourhy & Sureau, 1991) or passage in C6-36 mosquito cells followed by two to three passages in BHK-21 cells. With many of the viruses, frozen BHK-21 cells or suckling mouse brain stocks were used directly to prepare RNA. Some viruses were additionally grown in 25 mm^2 dishes of BHK-21 cells for 1 to 4 days until a cytopathic effect was recorded.

RT-PCR, cloning and sequencing methods. Total RNA was isolated from virus-infected cells (C6-36 or BHK-21) or virus-infected mouse brain using RNAzol B (Tel-Test Inc.). cDNA was synthesized using 2·5 μg total RNA, 250 ng of each primer and 10 U AMV reverse transcriptase (Promega) in a 20 μl reaction volume using standard methods. Virus L gene fragments were amplified by RT-PCR using the AmpliTaq buffer [2·5 mM $MgCl_2$, 200 nM each dNTP, 100 pmol each primer and 2·5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus)] in a 50 μl reaction volume. Amplified DNA was resolved in 2 % Ultrapure low-melting-point (LMP) agarose (Gibco-BRL) gels, and DNA products of the expected size were purified using the BresaClean kit (Bresatec). PCR products were either directly sequenced or cloned into the pGEM-T vector and cycle-sequenced using universal pUC forward and reverse primers and fluorescent dye terminator FS reagent (ABI). Several independent PCR clones (3–5 clones) were analysed to produce a consensus nucleotide sequence for each virus. Due to genetic variation within the degenerate primer sequences, the primer sequences were excluded from the phylogenetic analysis.

Phylogenetic analysis. The dataset of 38 rhabdovirus sequences newly determined here was compared with the corresponding block III L polymerase amino acid sequences of 18 rhabdoviruses collected from GenBank (Table 1). All amino acid sequences were aligned using the CLUSTAL W programme (Thompson *et al.*, 1994) and then checked for accuracy by eye. This resulted in a final alignment of sequences of 158 amino acid residues in length (Fig. 1).

A phylogenetic tree from these data was inferred using the maximum-likelihood method available in TREE-PUZZLE (Schmidt *et al.*, 2002). The WAG model of amino acid substitution was employed along with a gamma (Γ) distribution of rate heterogeneity among sites, with the value of the shape parameter ($\alpha = 1$, for eight rate categories) estimated from the empirical data during tree reconstruction (other parameter values available from the authors on request). Support for each node in the tree was obtained by examining quartet puzzling support values.

RESULTS AND DISCUSSION

Design of primers

On initiation of this study, nucleotide sequence data from the L polymerase gene was only available for 10 members of the genera *Lyssavirus*, *Vesiculovirus* and *Ephemerovirus*. This sequence dataset was first examined to predict degenerate primers that would be broadly reactive among the animal rhabdoviruses. Several degenerate primers were designed to functional L-gene sequences conserved among rhabdoviruses and other negative-sense RNA viruses. No mismatches were tolerated at the 3′ end, and less than three mismatches were accepted in the entire sequence, with the exception of *Infectious haematopoietic necrosis virus* (IHNV). Primers PVO3 [5′-CCADMCBTTTGYCKYARRCCTTC-3′, genome position 7526–7503 in *Rabies virus* (RV) PV strain] and PVO4 (5′-RAAGGYAGRTTTTTYKCDYTR-ATG-3′, position 7068–7088), designed to conserved pre-motif A and to motif B in block III of the L gene, respectively, were found to perform best in preliminary RT-PCR tests

Table 1. Isolates of rhabdovirus analysed in this study

UA, Unassigned species and unclassified viruses; TS, tentative species. The abbreviation of the viral name is according to Tordo *et al.* (2004).

Genus and name	UA/TS	Abbreviation	Reference no.	Host species/vector	Origin	Year of first isolation	GenBank accession no.
Nucleorhabdovirus							
Rice yellow stuntvirus		RYSV	RYSVN	Leafhopper			AB011257
Sonchus yellow net virus		SYNV	SYNV	Aphid			L32603
Maize mosaicvirus		MMV	MMV	Leafhopper (*Peregrinus maidis*)			NC 005975
Cytorhabdovirus							
Northern cereal mosaic virus		NCMV	NCMV	Leafhopper (*Laodelphax striatellus*)	Japan		AB030277
Strawberry crinkle virus		SCV	SCV	Aphid (*Fragaria* species)	Chile		AY331385
Taastrup virus	UA	TaasV	TV	Leafhopper (*Psammotettix alienus*)	Denmark		AY423355
Novirhabdovirus							
Infectious hematopoietic necrosis virus		IHNV	IHNV	Rainbow trout (*Onchorynchus mykiss*)/invertebrate reservoirs?			X89213
Viral haemorrhagic septicaemia virus		VHSV	VHSV	Rainbow trout (*Onchorynchus mykiss*)/invertebrate reservoirs?			Y18263
Snakehead rhabdovirus	TS	SHV	SR	Snakehead fish (*Ophicephalus striatus*)			AF147498
Hiramerhabdovirus	UA	HirR	HR				AF104985
Ephemerovirus							
Adelaide Rivervirus		ARV	DPP 61	*Bos taurus*	Australia	1981	AY854635
Berrimah virus		BRMV	DPP 63	*Bos taurus*	Australia	1981	AY854636
Kimberley virus	TS	KIMV	CS 368	*Bos taurus*	Australia	1980	AY854637
Kotonkon virus	UA	KOTV	IbAr23380	*Culicoides* species	Nigeria	1967	AY854638
Bovine ephemeral fever virus		BEFV	BB7721	*Bos taurus*	?	1968	AY854671
Bovine ephemeral fever virus		BEFV	CS 42	*Anopheles bancrofti*	Australia	1975	AY854639
Bovine ephemeral fever virus		BEFV	CS 53	Mixed species	Australia	1974	AY854640
Bovine ephemeral fever virus		BEFV	CS 1933	*Bos taurus*	Australia	1973	AY854641
Bovine ephemeral fever virus		BEFV	Beijing 1	*Bos taurus*	China	1976	AY854642
Almpiwar group							
Humpty Doo virus	UA	HDOOV	CS 79	*Lasiohelea* species	Australia	1975	AY854643
Charleville virus	UA	CHVV	Ch 9824	*Phlebotomus* species, lizard (*Gehyra australis*)	Australia	1969	AY854644
Charleville virus	UA	CHVV	Ch 9847	*Phlebotomus* species		1979	AY854672
Almpiwar virus	UA	ALMV	MRM4059	*Ablepharus boutonii virgatus*	Australia	1966	AY854645
Oak-Vale virus	UA	OVRV	CS 1342	*Culex* species	Australia	1981	AY854870
Tibrogargan group							
Tibrogarganvirus	UA	TIBV	CS 132	*Culicoides brevitarsis*, water buffaloes, cattle	Australia	1976	AY854646
Hart Park group							
Parry Creek virus	UA	PCRV	OR 189	*Culex annulirostris*	Australia	1972	AY854647
Wongabel virus	UA	WONV	CS 264	*Culicoides austropalpalis*	Australia	1979	AY854648
Flanders virus	UA	FLANV	61-7484	*Culiseta melanura*, *Culex pipiens quinquefasciatus*, *Culex salinarus*, *Culex territans*, *Culex tarsalis*, *Seiurus aurocapillus*	New York, USA	1961	AF523199
Ngaingan virus	UA	NGAV	NRM14556	*Culicoides brevitarsis*, wallabies, kangaroos, cattle	Australia	1970	AY854649

Table 1. cont.

Genus and name	UA/TS	Abbreviation	Reference no.	Host species/vector	Origin	Year of first isolation	GenBank accession no.
Le Dantec and Kern Canyon group							
Le Dantec virus	UA	LDV	DakHD 763	Human	Senegal	1965	AY854650
Fukuoka virus	UA	FUKV	FUK-11	*Culicoides punctatus*	Japan	1982	AY854651
Vesiculovirus							
Perinet virus	TS	PERV	Ar Mg 802	Mosquitoes: *Anopheles coustani*, *Culex antennatus*, *Culex* gr. *pipiens*, *Mansonia uniformis*; other: *Phlebotomus berentensis*	Madagascar	1978	AY854652
Vesicular stomatitis New Jersey virus		VSNJV	VSV NJ-H	*Sus scrofa*/*Culex nigripalpus*, *Culicoides* species, *Mansonia indubitans*	Georgia, USA	1952	AY074803
Vesicular stomatitis New Jersey virus		VSNJV	VSV NJ-O	*Bos taurus*, equine/*Culex nigripalpus*, *Culicoides* species, *Mansonia indubitans*	Utah, USA	1949	AY074804
Vesicular stomatitis Indianavirus		VSIV	VSV IND	*Bos taurus*	Indiana, USA	1925	J02428
Spring viraemia of carp virus	TS	SVCV		*Cyprinus carpio*			U18101
Spring viraemia of carp virus	TS	SVCV		*Cyprinus carpio*	Yugoslavia	1971	AJ318079
Lyssavirus							
Mokola virus		MOKV	Mok	Cat	Zimbabwe	1981	AY854653
Lagos bat virus		LBV	8619NGA	Bat (*Eidolon helvum*)	Nigeria	1956	AY854654
European bat lyssavirus 1		EBLV-1	8918FRA	Bat (*Eptesicus serotinus*)	France	1989	AY854655
European bat lyssavirus 1		EBLV-1	9480HOL	Bat (*Eptesicus serotinus*)	The Netherlands	1987	AY854656
European bat lyssavirus 2		EBLV-2	9337SWI	Bat (*Myotis daubentonii*)	Switzerland	1993	AY854657
European bat lyssavirus 2		EBLV-2	94112HOL	Bat (*Myotis dasycneme*)	The Netherlands	1989	AY854658
Duvenhage virus		DUVV	94286SA	Human	South Africa	1986	AY854659
Australian bat lyssavirus		ABLV	ABLh	Human	Australia	1998	AF418014
Australian bat lyssavirus		ABLV	ABLb	Bat (*Pteropus* species)	Australia	1996	AF081020
Rabies virus		RABV	9911	Dog	Cambodia	1998	AY854660
Rabies virus		RABV	PV	Vaccine			AY854661
Rabies virus		RABV	SADB19	Vaccine			AY854662
Rabies virus		RABV	9706	Vaccine AG	China		AY854663
Rabies virus		RABV	8743	Human	Thailand	1983	AY854664
Rabies virus		RABV	9702	Human	India	1997	AY854665
Rabies virus		RABV	9106	Human	Morocco	1990	AY854666
Rabies virus		RABV	9704	Bat (*Tadarida brasiliensis*)	Argentina	1997	AY854667
Rabies virus		RABV	9147	Fox	France	1991	AY854668
Rabies virus		RABV	02008	Bat	USA	1994	AY854669

with selected viruses and were thus used in subsequent tests. Amino acid and nucleotide sequence alignments from which the primer sequences were designed are shown in Fig. 1.

Primers PVO3 and PVO4 produced PCR products for 38 animal rhabdoviruses, 20 of which are not currently assigned to a particular genus (Table 1). These primers amplified a 456–462 nucleotide region, conforming to the expected size. Internal primers PVO5 (5′-ATGACGG-ACAAYCTGAACAA-3′, position 7170–7189) and PVO6 (5′-CCRTTCCARCAGGTAGGDCC-3′, position 7486–7467) were used for sequencing some PCR products. These primers amplified a 317 nucleotide region.

Sequence analysis of L polymerase block III

A total of 56 rhabdovirus L polymerase sequences were subjected to phylogenetic analysis (Table 1). These sequences encompass the three highly conserved segments (pre-motif A, motif A and motif B) of block III of the L polymerase (Fig. 2), which is present in all the

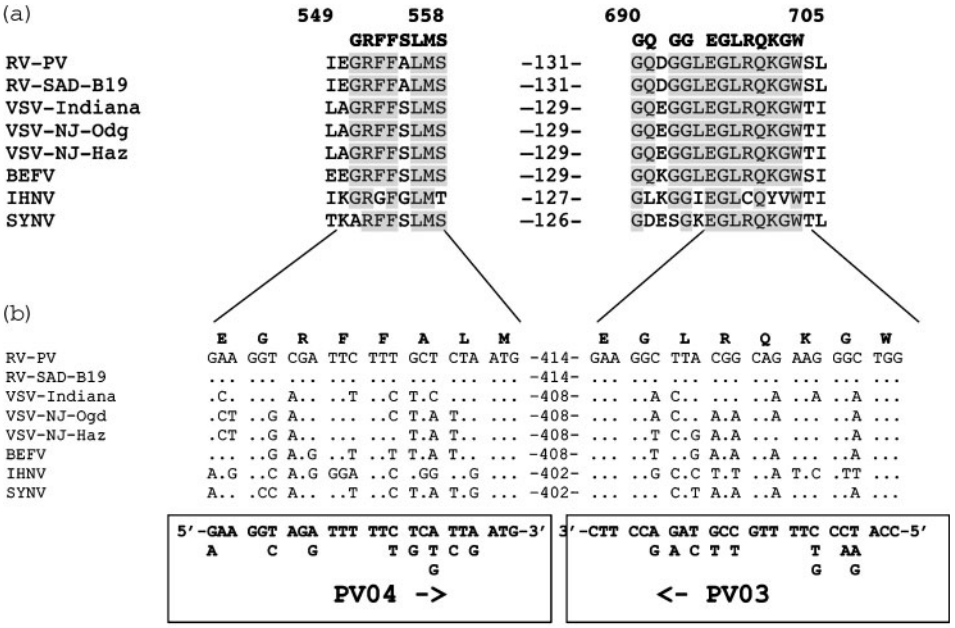


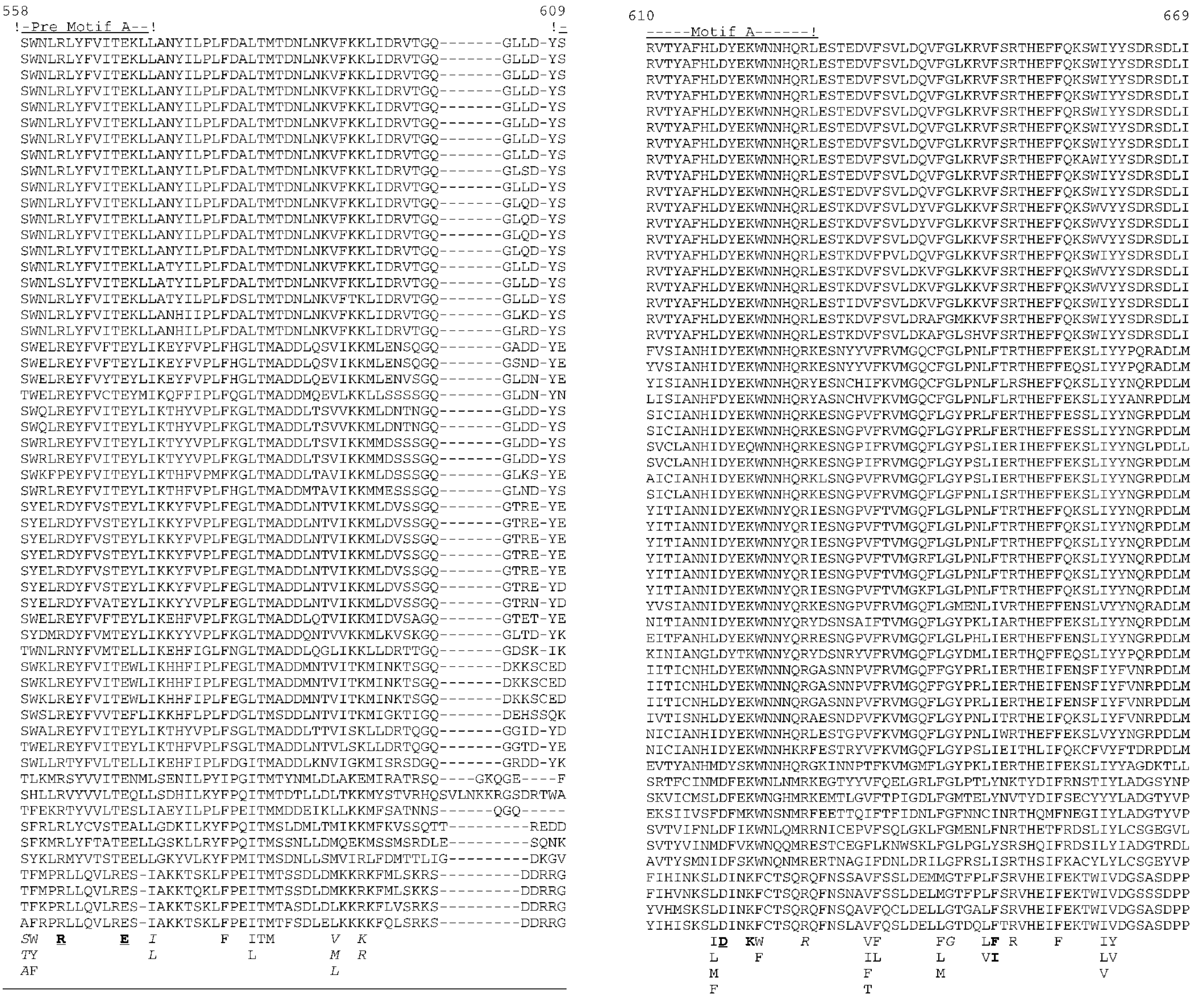
Fig. 1. Rationale for the design of primers. Conserved amino acid (a) and nucleotide sequences (b) in the L genes of RV strains PV (Genbank accession no. M13215) and SAB-B19 (M31046), VSV serotypes Indiana (K02378) and New Jersey, strains Ogden (M29788) and Hazelhurst (AY074803), bovine ephemeral fever virus (BEFV) (AF234533), IHNV (X89213) and *Sonchus yellow net virus* (SYNV) (M87829) to which degenerate PCR primers PV03 and PV04 were targeted. The 3′ termini of primers PV03 (384-fold degenerate) and PV04 (576-fold degenerate) were designed to invariant Met and Glu residues, respectively, and amino acids conserved in prototype viruses of at least four of the five genera compared are shaded. Amino acid positions are indicated according to the polymerase sequence of RV PV (Tordo *et al.*, 1988).

RNA-dependent RNA polymerases studied so far, including reverse transcriptase (Poch *et al.*, 1989; Xiong & Eickbush, 1990). Although these sequences are extremely divergent, sufficient sequence similarity exists in some domains of the rhabdovirus polymerases to make phylogenetic analysis possible (Dhillon *et al.*, 2000; Le Mercier *et al.*, 1997; Müller *et al.*, 1994; Vieth *et al.*, 2004). Importantly, the alignment confirmed the conservation of some residues among all the *Rhabdoviridae* (Le Mercier *et al.*, 1997; Müller *et al.*, 1994), whilst also identifying new residues that are conserved among the Mononegavirales (Fig. 2).

Structural conservation of residues in the aligned sequences was examined by using similarities in charge or polarity and matching aromatic residues (Poch *et al.*, 1990) as the primary criteria to define the following amino acid families: [P, G; S, T; A] [F, Y, W; I, L, M, V] [D, E; N, Q] [K, R, H] [C]. Only residues belonging to the same family and conserved in at least 54 of the 56 sequences and among all genera were considered as meaningful indicators of sequence homology. Ninety-nine amino acid residues were conserved among the entire *Rhabdoviridae* family (Fig. 2). Among these, 12 were conserved among the entire *Rhabdoviridae* and the *Paramyxovirinae* subfamily. Finally, five positions were conserved among the Mononegavirales and all known pre-motif A and motif A (Dhillon *et al.*, 2000; Le Mercier *et al.*, 1997; Müller *et al.*, 1994; Vieth *et al.*, 2004): R (562), E (569), D (618), K (621) and F/I (648).

Phylogenetic analysis of the *Rhabdoviridae* using the sequence of block III

Previously, taxonomic relationships among members of the *Rhabdoviridae* were primarily based on structural properties (genome size and complexity), large-scale biological properties (host range, epidemiological cycles, routes of transmission) and serological cross-reactions (immunofluorescence, complement fixation (CF), neutralization tests). Although serological data are useful taxonomic tools for closely related viruses, their interpretation in defining relationships among more distantly related viruses has proven complex (Calisher *et al.*, 1989; Shope, 1995; Wang *et al.*, 1995). More recently, the extent of sequence similarity within a given gene has largely been used for species demarcation in each genus of the *Rhabdoviridae*. In the *Lyssavirus* genus, for instance, percentage sequence similarity within the nucleoprotein gene has been used for the definition of different virus genotypes (Arai *et al.*, 2003; Bourhy *et al.*, 1993; Kuzmin *et al.*, 2003), and the same methodology has been used for the delineation of different species among the vesiculoviruses and ephemeroviruses (Barr *et al.*, 1991; Crysler *et al.*, 1990; Masters & Banerjee, 1987; Walker *et al.*, 1994; Wang *et al.*, 1995).



Our phylogenetic analysis of the 158-residue L polymerase sequence produced an evolutionary tree that generally, although not entirely, conformed to accepted serological groupings and taxa within the *Rhabdoviridae* (Calisher *et al.*, 1989; Shope, 1995; Tordo *et al.*, 2004). In particular, members of four genera – *Lyssavirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus* – obtained from a variety of host species, including mammals, fish, arthropods and plants, can be easily distinguished and fall into relatively well-supported clades (Fig. 3). Although the vesiculoviruses and ephemeroviruses also fell into clear monophyletic groups, they are less well supported by quartet puzzling, and each genus contained some unclassified viruses. Furthermore, Kotonkon virus, which causes clinical ephemeral fever in cattle (Kemp *et al.*, 1973; Tomori *et al.*, 1974), but which has previously been classified as a lyssavirus, very clearly clustered with members of the genus *Ephemerovirus*. Lastly, there is some evidence that the two groups of plant rhabdoviruses – the cytorhabdoviruses and nucleorhabdoviruses – form a distinct clade, although this has relatively low quartet puzzling support. Taastrup virus, which was unassigned (Bock *et al.*, 2004), is related to cytorhabdoviruses.

Strikingly, our phylogenetic analysis also identified four more monophyletic groups of currently unclassified rhabdoviruses which have variable support values. First, Wongabel, Parry Creek, Flanders and Ngaingan viruses formed a distinct cluster, with high levels (93 %) of quartet puzzling support. We refer to this as the 'Hart Park' group, based on the serologic grouping of Flanders virus in the Hart Park serological group (Boyd, 1972; Calisher *et al.*, 1989). Second, a tentatively named 'Almpiwar' group, containing *Almpiwar virus*, Humpty Doo virus, Charleville virus and Oak-Vale virus, was also identified. Although this grouping had only 65 % quartet puzzling support, *Almpiwar virus* and Charleville virus possessed almost indistinguishable sequences in the L-gene region, and both have been associated with infection in lizards. Another group, consisting of the Le Dantec and Fukuoka viruses, and herein referred

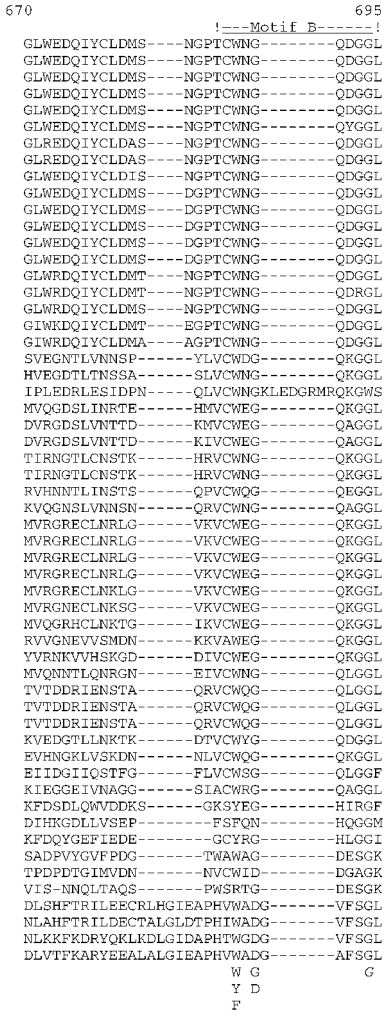


Fig. 2. Alignment of the conserved block III of 56 L polymerase sequences from the *Rhabdoviridae*. Amino acids belonging to the same residue family (Poch *et al*., 1990), conserved in at least 54 of the 56 sequences and among the different viral families, are described in the text. Residues which are conserved in the *Rhabdoviridae* are shown in roman type. Residues conserved in all the *Rhabdoviridae* and the *Paramyxovirinae* subfamily are shown in italics. Amino acids conserved in polymerases of other unsegmented (−) RNA viruses are shown in bold type. Residues conserved in other RNA polymerases are shown in bold and underlined type (Dhillon *et al*., 2000; Le Mercier *et al*., 1997; Müller *et al*., 1994; Vieth *et al*., 2004).

to as the 'Le Dantec' group, was also seen to form a distinct cluster, although with only 66 % quartet puzzling support. Finally, the phylogenetic position of Tibrogarganvirus was ambiguous. While there was weak support (54 % quartet puzzling) for this virus clustering with the Le Dantec group, the length of the branch leading to Tibrogarganvirus implies that it should be classified in its own group, although this contains only a single virus at present.

Perhaps the most notable result from our phylogenetic analysis was the strong support (98 %) for a virus 'supergroup', herein named 'dimarhabdovirus' (sigla for 'dipteran-mammal associated rhabdovirus'). This contained the four new groups of viruses described above, as well as the *Vesiculovirus* and *Ephemerovirus* genera. Despite major differences in genome organization, ephemeroviruses and vesiculoviruses share many similar biological characteristics. They are, together with the other dimarhabdoviruses, the only recognized rhabdovirus genera with viruses that replicate in both vertebrate and invertebrate hosts, and have biological cycles involving transmission by hematophagous dipterans. Although there is strong phylogenetic support for the dimarhabdovirus supergroup, the precise branching order within this group cannot be resolved on the L polymerase data. Indeed, there is a clear need for further phylogenetic studies within the dimarhabdovirus supergroup, particularly with respect to the demarcation of genera, which currently seems to be influenced more by genome structure than host–vector relationships. For example, compared to vesiculoviruses, ephemeroviruses contain multiple additional ORFs, including a second glycoprotein gene (G_{NS}) that appears to have been acquired by gene duplication (Wang & Walker, 1993). There is some evidence that Flanders virus may also have a complex pattern of gene expression (Boyd & Whitaker-Dowling, 1988). Although the functions of these additional proteins are not understood, revealing the evolution of genome complexity may be an important factor in resolving the taxonomy of this supergroup.

In sum, the sort of molecular phylogenetic analysis undertaken here, especially if combined with data on genome organization, is likely to provide a more useful guide to taxonomic classification, particularly for assignments above the species level and even among all (−) RNA viruses (Vieth *et al*., 2004). Indeed, our phylogenetic analysis of a conserved L-gene segment appears to provide a useful taxonomic tool for the rapid classification of rhabdoviruses.

Association between phylogenetic relationships and mode of transmission

A number of important biological conclusions can be drawn from the rhabdovirus phylogeny presented here. First, assuming a mid-point rooting of the tree, there is major split between those viruses that infect fish (novirhabdoviruses) and plants and which employ arthropods as vectors (cytorhabdoviruses and nucleorhabdoviruses), and those viruses that mainly infect mammals, lizards and dipterans (dimarhabdoviruses). Such a division illuminates the biology of a number of key rhabdoviruses. For example, although vesicular stomatitis virus (VSV) is responsible for a disease of horses, cattle and pigs and can be transmitted directly by transcutaneous or transmucosal routes (Stallknecht *et al*., 1999), there is good evidence that VSV may be an insect virus (Rodriguez, 2002). Indeed, it has been found to replicate in biting midges (*Culicoides*) and Simulium blackflies (Mead *et al*., 1999), and has been

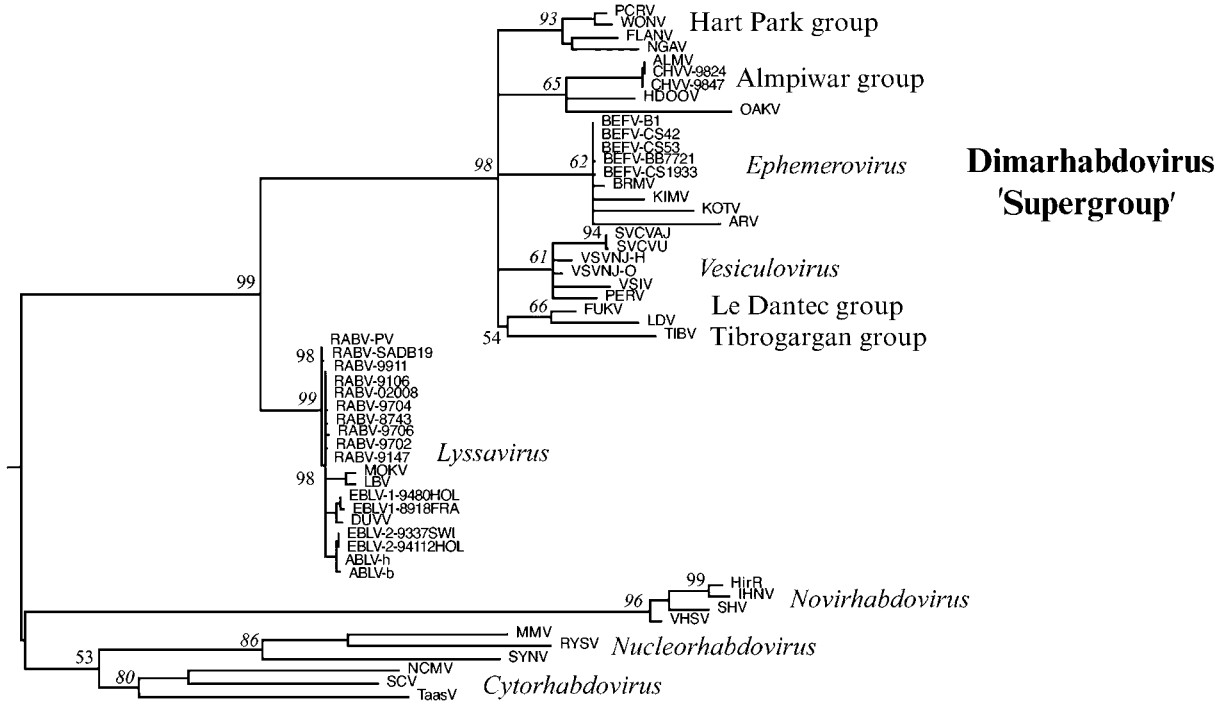


Fig. 3. Phylogenetic relationships of the *Rhabdoviridae* based on a maximum-likelihood analysis of a 158-residue alignment of the L polymerase region. The established rhabdovirus genera as well as the new groups proposed here are indicated. Horizontal branches are drawn to scale and quartet puzzling frequencies are shown for key nodes (values in italics are for genera, groups and supergroups, while all other quartet puzzling frequencies are shown in roman type). The tree is mid-point rooted for purposes of clarity only, and all potential outgroup sequences were deemed too divergent to include in the analysis.

isolated from sandflies, and epidemic and endemic bursts depend on region, season and the presence of dipterans (*Lutzomya*, *Simulidae*, *Culicoides* and *Musca domestica*) (Gard *et al.*, 1984; Walker & Cybinski, 1989). All these factors suggest that VSV may be insect-borne. Similarly, *Bovine ephemeral fever virus*, which is frequently found in Australasia, Asia and Africa, is also dipteran-transmitted, using vectors such as biting midges and culicine and anopheline mosquitos. Finally, viruses assigned by our phylogenetic analysis to the four new groups (the Le Dantec, Tibrogargan, Hart Park and Almpiwar groups) were all found to infect dipterans and in some cases mammals (Tibrogargan, Le Dantec and Ngaingan viruses) and lizards (Charleville virus) also.

Importantly, there is as yet no evidence for a virus that would constitute a link between plant and fish viruses and dimarhabdoviruses and the lyssaviruses. Furthermore, the uncertainty over branching order at the root of the tree makes it difficult to determine whether the ancestral mode of transmission in the rhabdoviruses was vector or non-vector transmission. A similar lack of resolution at the base of tree was found in a previous phylogenetic analysis of six genera of rhabdoviruses (Vieth *et al.*, 2004). However, the major phylogenetic division between these groups indicates that the biology of the rhabdoviruses could be strongly influenced by mode of transmission and by the host (plant, fish or mammal) and vector (orthopteran, homopteran or dipteran) species. Similar findings have been reported in other RNA viruses, such as the flaviviruses (Gaunt *et al.*, 2001) and the tick-borne nairoviruses (Honig *et al.*, 2004).

Finally, it is noteworthy that levels of genetic diversity vary substantially among genera. This is most apparent when comparing the tightly clustered lyssaviruses (the different genotypes of which our phylogenetic analysis cannot easily distinguish) with the cytorhabdoviruses and nucleorhabdoviruses, which are highly diverse. Indeed, the entire *Lyssavirus* genus, although clearly separate from the other rhabdoviruses, is less divergent than two serotypes (Indiana and New Jersey) of VSV. The most likely explanation for such differences is that these genera differ substantially in age, with the lyssaviruses evolving most recently. However, it is also possible that strong selective constraints acting against sequence change in the lyssaviruses also serve to limit amino acid variation (Guyatt *et al.*, 2003; Holmes *et al.*, 2002; Kissi *et al.*, 1999).

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