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Short Communication

Detection of rhabdovirus viral RNA in oropharyngeal swabs and ectoparasites of Spanish bats

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Rhabdoviruses infect a variety of hosts, including mammals, birds, reptiles, fish, insects and plants. As bats are the natural host for most members of the genus *Lyssavirus*, the specificity of the amplification methods used for active surveillance is usually restricted to lyssaviruses. However, the presence of other rhabdoviruses in bats has also been reported. In order to broaden the scope of such methods, a new RT-PCR, able to detect a diverse range of rhabdoviruses, was designed. The method detected 81 of 86 different rhabdoviruses. In total, 1488 oropharyngeal bat swabs and 38 nycteribiid samples were analysed, and 17 unique rhabdovirus-related sequences were detected. Phylogenetic analysis suggested that those sequences detected in bats did not constitute a monophyletic group, even when originating from the same bat species. However, all of the sequences detected in nycteribiids and one sequence obtained from a bat did constitute a monophyletic group with *Drosophila melanogaster* sigma rhabdovirus.

The family *Rhabdoviridae* is one of the most host-range-diverse families of RNA viruses, with members infecting a wide range of organisms (Kuzmin *et al.*, 2009) of marine, freshwater and terrestrial environments (Walker *et al.*, 2011). It currently comprises six genera (*Lyssavirus*, *Vesiculovirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus*) containing about 50 species, and more than 150 viruses that have not been formally classified (Walker *et al.*, 2011).

Within the family *Rhabdoviridae*, the lyssaviruses are the most significant for public health, and most of them have been detected in bats, like other rhabdoviruses such as Oita virus (OITAV) (Iwasaki *et al.*, 2004), Mount Elgon bat virus (MEBV) (Metselaar *et al.*, 1969) and Kern Canyon virus (KCV) (Murphy & Fields, 1967). Interestingly, although not implicated as a cause of disease in humans or animals, both OITAV and MEBV cause fatal encephalitis when inoculated experimentally into newborn mice (Iwasaki *et al.*, 2004; Metselaar *et al.*, 1969; Patel, 1979). In contrast, the vesiculovirus Chandipura virus has caused a number of arthropod-borne epidemic outbreaks of severe febrile encephalitis in different parts of India (Basak *et al.*, 2007). Some cases of mild human disease due to the arthropod-borne vesicular stomatitis virus (VSV) have been described (Quiroz *et al.*, 1988). Some studies have suggested the presence of VSV-specific antibodies in bats (Ubico & McLean, 1995; Zuluaga & Yuill, 1979), although virus has not been detected. Finally, Le Dantec virus was first isolated in 1965 in Senegal from a 10-year-old girl with fever and hepatosplenomegaly (Buck, 1961; Woodruff *et al.*, 1977).

Generic amplification methods able to detect a wide range of rhabdoviruses in cell-culture supernatants (Bourhy *et al.*, 2005; Kuzmin *et al.*, 2006), as well as a microarray

The GenBank/EMBL/DDBJ accession numbers for the sequences obtained in this study are available in Tables 1 and S1.

Two supplementary tables are available with the online version of this paper.

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(Dacheux *et al.*, 2010), have been described. Nevertheless, both the PCR and microarray approaches have been limited in their ability to detect rhabdovirus RNA from natural samples, such as ectoparasites and oropharyngeal swabs from bats. In this study, we describe a new generic nested RT-PCR method named DimLis PCR, able to detect most of the viruses included on a panel of 86 rhabdoviruses belonging to different genera associated with mammals, birds, reptiles and arthropods (Calisher *et al.*, 1989), as well as other rhabdovirus-related sequences from samples taken from bats and their ectoparasites. The RT-PCR primers (Fig. 1) were designed to target the polymerase (L) gene because it is responsible for the majority of enzymic activities involved in viral transcription and replication and exhibits a high degree of conservation amongst the rhabdoviruses, with strongly invariant amino acids embedded in conserved blocks separated by variable regions (Poch *et al.*, 1990). A similar approach has recently been applied to the development of a pan-paramyxovirus RT-PCR that targets conserved regions within the viral polymerase gene (van Boheemen *et al.*, 2012). Although the nucleoprotein (N) gene has previously been used to determine the phylogenetic relationships within the family *Rhabdoviridae* (Kuzmin *et al.*, 2006), more recent studies have shown that L provides a much greater phylogenetic resolution than N, even when looking at closely related viruses (Bourhy *et al.*, 2005; Longdon *et al.*, 2010).

Forty available rhabdovirus complete L gene sequences, from viruses belonging to the genus *Lyssavirus* and the dimarhabdovirus supergroup, were obtained from GenBank and aligned using CLUSTAL_X (Fig. 1). The selected external primers were DimLis1F, 5′-GGKMGRTTYTTYKCHYTDATG-3′ (position 7063–7083), and DimLis1R, 5′-CARAARGGNTGGASYNTHBT-3′ (position 7510–7529), which generate a 461–467 nt amplicon depending on the rhabdovirus sequence being amplified. Nested primers DimLis2F, 5′-YTNTTYVANGSVYTRACNATG-3′ (position 7147–7167), and DimLis2R, 5′-TGGAAYAAYCAYCARMGRHWD-3′ (position 7267–7296), were used to generate a 150 nt second-round amplicon. All genome positions were calculated using the rabies virus CVS strain (GenBank accession no. GQ918139). Control RT-PCRs were performed using an Access RT-PCR kit internal control template and primers as described in the manufacturer's instructions (Promega). Single-step reverse transcription and primary amplification were performed by using an Access RT-PCR kit (Promega). Briefly, 5 µl nucleic acid extract was added to 45 µl reaction mixture containing: 10 µl 5× reaction buffer; 3 mM magnesium sulphate; 500 µM each dNTP; 1.5 µM DimLis1F and DimLis1R primers; 5 µM internal control kit primers; 5 U avian myeloblastosis virus reverse transcriptase; and 5 U *Thermus flavus* DNA polymerase. Amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) programmed for a first reverse-transcription step of 45 min at 48 °C, followed by 94 °C for 2 min, then 30 cycles of 93 °C for 1 min, 49 °C for 4 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. For nested PCR, 1 µl of the primary amplification product was added to the nested PCR mixture containing: 5 µl 10× buffer II (Roche Molecular Systems); 4 mM magnesium chloride; 500 µM each dNTP; 1.5 µM DimLis2F and DimLis2R primers; 5 µM internal control kit primers; and 1.25 U AmpliTaq DNA polymerase (Roche Molecular Systems). Reaction conditions were as follows: 94 °C for 2 min, then 30 cycles of 93 °C for 1 min, 55 °C for 3 min and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. PCR products were visualized by UV excitation following gel electrophoresis in 2 % agarose containing 0.5 g ethidium bromide ml^{-1} in TBE buffer. All PCR products with the expected size were sequenced on an ABI PRISM 377 DNA sequencer (Applied Biosystems) using each of the nested primers. In the first approach, all edited consensus sequences were submitted manually to the nucleotide–nucleotide BLASTN query (http://www.ncbi.nlm.nih.gov/BLAST), to determine the closest match to sequences available in GenBank. All sequences with a degree of similarity to pre-existing rhabdovirus sequences, together with all representative strains of rhabdoviruses obtained from genomic databases, were aligned using the CLUSTAL_X 1.81 program. Before conducting further analyses, jModelTest (https://code.google.com/p/jmodeltest2/) was used to select the best-fitting substitution model for our sequences according to the corrected Akaike information criterion. A Bayesian phylogenetic inference (BPP) was then obtained by using MrBayes version 3.1 (http://mrbayes.sourceforge.net/) with random starting trees without constraints. Four simultaneous runs of 5 million generations were conducted, each with five Markov chains, and trees were sampled every 300 generations. Maximum-likelihood (ML) reconstruction and bootstrap support to the nodes after 1000 iterations were obtained in PhyML (http://www.atgc-montpellier.fr) (Guindon *et al.*, 2010).

Results of the 86 rhabdoviruses analysed with the DimLis PCR are shown in Table S1 (available in JGV Online). Products of the expected size were obtained in 81 of 86 strains analysed (94 %). The use of nested amplification seems to be necessary when using highly degenerated primers like those used in this study, as 30 viruses were only detected following second-round amplification. This is likely to be a factor of very low levels of target viral RNA, as well as potential low-level first-round amplification

Fig. 1. Alignment of the external (DimLis1F and DimLis1R) and internal (DimLis2F and DimLis2R) primers with the sequences of the 40 different rhabdoviruses available. Matches in non-degenerated positions are displayed as dashes. The first column shows the virus abbreviation, the classification (L, *Lyssavirus*; V, *Vesiculovirus*; E, *Ephemerovirus*; H, Hart Park group; T, Tibrogargan group; U, unassigned) and the GenBank accession no. All virus abbreviations are as detailed in Table S1.

	DimLis1F	DimLis1R
	G G K M G R T T Y T T Y K C H Y T D A T G 3′	C A R A A R G G N T G G A S Y N T H B T 3′
VSIV (V) X00939	- -	- - - - - - - - - - - - - - - - - - - -
ISFV (V) AJ810084	- -	- - - - - - - - - - - - - - - - - - - -
CHPV (V) AJ810083	- -	- - - - - - - - - - - - - - - - - - - -
SVCV (V) NC_002803	- -	- - - - - - - - - - - - - - - - - - - -
PFRV (V) FJ872827	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - G - -
COCV (V) EU373657	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
BEFV (E) NC_002526	- -	- - - - - - - - - - - - - - - - - - - -
ARV (E) AF234534	- -	- - - - - - - - - - - - - - - - - - - -
RABV (L) NC_001542	- -	- - - - - - - - - - - - - - - - - - - -
ABLV (L) NC_003243	- -	- - - - - - - - - - - - - - - - - - - -
MOKV (L) NC_006429	- -	- - - - - - - - - - - - - - - - - - - -
LBV (L) GU170202	- - A - - T - - - - - - - - G - - - - - -	- - - - - - - - - - - - - - - - - G - -
DUVV (L) EU293120	- - C - - - - - - - - - - - - - - C - - -	- - - - - - - - - - - - - - - - - - - -
EBLV 1 (L)EF157976	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - G - -
EBLV 2 (L)EF157977	- - - - - - - - - - - - - - G - - - - - -	- - - - - - - - - - - - - - - - - - - -
ARAV (L) EF614259	- - A - - T - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - G - -
KHUV (L) EF614261	- -	- - - - - - - - - - - - - - - - - - - -
IRKV (L) EF614260	- -	- - - - - - - - - - - - - - - - - G - -
WCBV (L) EF614258	- - - - - - - - - - - - - - - - - C - - -	- - - - - - - - - - - - - - - - - G A -
Ozernoe Virus (U) FJ905105	- -	- - - - - - - - - - - - - - - - - - - -
SHIBV (L) GU170201	- - C - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
FLANV (H) AF523199	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - T - A - - - - -
NGAV (U) NC_013955	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - T - - - G - - -
MOUV (U) FJ985749	- -	- - - - - - - - - - - - - - - - - G - -
DAffSV (U) GQ410980	- - A - - - - - - - A - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
DObsSV (U) GQ410979	- -	- - - - - - - - - - - - - - - - - - - -
DMelSV (U) GQ375258	- -	- - - - - - - - - - - - - - - - - - - -
OFV (U) AB516441	- C C - - - - - - - - - - - - - - - - - -	- - - - - - T A - - - - - - - - - - A -
SCRV (U) NC_008514	- - A - - T - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
WONV (U) NC_011639	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - T - A - - - - -
TUPV (U) NC_007020	- -	- - - - - - - - - - - - - - - - - - - -
VSAV (V) EU373658	- - C - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
OVRV (U) JF705876	- - C - - - - A - - - - - - - - - - - - -	- - - - - - - - - - - - - - A - - - A C
SMRV (U) HQ003891	- - - - - - - - - - - - - - G - - - - - -	- - - - - - - - - - - - - - - - - - - -
TIBV (T) GQ294472	- - - - - T - - - - - - - - - - - - - - -	- - - - - - - - - - - - T - A - - - - -
CPV (T) GQ294473	- - A - - - - A - - - - - - - - - - - - -	- - - - - - - - - - - - T - A - - - - -
BBLV (L) JF311903	- -	- - - - - - - - - - - - - - - - - - - -
EVEX (U) FN557213	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
MARAV (V) HQ660076	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - - - - - - - - -
DURV (U) FJ952155	- - A - - - - - - - - - - - - - - - - - -	- - - - - - - - - - - - T - - - - - - -

	DimLis 2F	DimLis 2R
	Y T N T T Y V A N G S V Y T R A C N A T G 3′	T G G A A Y A A Y C A Y C A R M G R H W D 3′
VSIV (V) X00939	A -	- -
ISFV (V) AJ810084	- -	- -
CHPV (V) AJ810083	- -	- -
SVCV (V) NC_002803	- -	- -
PFRV (V) FJ872827	- - - - - - - - - - - - - - C - - - - - -	- -
COCV (V) EU373657	A -	- - - - - - - - - - - - - - - - - C - - -
BEFV (E) NC_002526	- -	- - - - - - - - - T - - - - - - - - - - -
ARV (E) AF234534	- -	- - - - - - - - - T - - - - - - - - - G -
RABV (L) NC_001542	- -	- -
ABLV (L) NC_003243	- -	- -
MOKV (L) NC_006429	- -	- C
LBV (L) GU170202	- -	- -
DUVV (L) EU293120	- - - - - - - - - T - T - - - - - - - - -	- -
EBLV 1 (L)EF157976	- -	- -
EBLV 2 (L)EF157977	- - - - - - - - - - - T - - - - - - - - -	- -
ARAV (L) EF614259	- - - - - - - - - - - T - - - - - - - - -	- -
KHUV (L) EF614261	- -	- -
IRKV (L) EF614260	- -	- -
WCBV (L) EF614258	- -	- -
Ozernoe Virus (U) FJ905105	- -	- -
SHIBV (L) GU170201	- - - - - - - - - - - T - - - - - - - - -	- -
FLANV (H) AF523199	- -	- -
NGAV (U) NC_013955	- -	- -
MOUV (U) FJ985749	A -	- - - - - - - - - A - - - - - - - C - - -
DAffSV (U) GQ410980	- -	- - - - - - - - - - - - - - - - - - G C -
DObsSV (U) GQ410979	- -	- -
DMelSV (U) GQ375258	- -	- - - - - - - - - - - - - - - - - - G G -
OFV (U) AB516441	- A - - - - - C - A T - G - C - - - - - -	- - - - - - C - A - - G A T - - - - G - -
SCRV (U) NC_008514	- -	- - - - - - - - - - - - - - - - - - - G -
WONV (U) NC_011639	- -	- -
TUPV (U) NC_007020	- -	- -
VSAV (V) EU373658	A -	- -
OVRV (U) JF705876	- - - - - - - - - - - - A - - - - - - - -	- - - - - - - - - - - - - - - - - - G G -
SMRV (U) HQ003891	- - - - - - - - - - - - - - - G - - - - -	- -
TIBV (T) GQ294472	- - - - - - - - - - - T - - - - - - - - -	- - - - - - - - - T - - - - - - - - - - -
CPV (T) GQ294473	- - - - - - T C - - - - - - - - - - - - -	- - - - - - - - - T - - - - - - - - - G -
BBLV (L) JF311903	- -	- -
EVEX (U) FN557213	- -	- -
MARAV (V) HQ660076	A -	- -
DURV (U) FJ952155	- - - - - - - - - - - T - - C - - - - - -	- -

Table 1. DimLis RT-PCR results from samples of bats and bat parasites

Species	Negative	Inhibited	Positive	Total sample
Bat samples				
Barbastella barbastellus	16			16
Eptesicus isabellinus	365	48	5*	418
Hypsugo savii	28	4	1†	33
Miniopterus schreibersii	119	8	1‡	128
Myotis bechsteinii	17	1		18
Myotis blythii	19			19
Myotis capaccinii	26			26
Myotis daubentonii	214	4		218
Myotis emarginatus	49			49
Myotis escalerai	10			10
Myotis myotis	55	2		57
Myotis mystacinus	5			5
Myotis cf. *nattereri*	34			34
Nyctalus lasiopterus	128	5		133
Nyctalus leisleri	14	1		15
Nyctalus noctula	31			31
Pipistrellus kuhlii	9	3		12
Pipistrellus pipistrellus	8			8
Pipistrellus pygmaeus	14	4		18
Pipistrellus sp.	18			18
Plecotus auritus	25	1	1§	27
Plecotus austriacus	17			17
Rhinolophus euryale	82	6		88
Rhinolophus ferrumequinum	68	4	2‖	74
Rhinolophus hipposideros	9			9
Rhinolophus mehelyi	5	1		6
Tadarida teniotis		1		1
Total bat samples	1385	93	10	1488
Bat parasite samples				
Nycteribia kolenatii	0		1¶	1 (7 individuals)
Penicillidia conspicua	0		4#	4
Nycteribia schmidli	0		4**	4
Cimex pipistrelli	3			3
Argas vespertilionis	20			20
Total bat parasite pool samples (individual number)	23	0	9	32 (38)

*The five sequences detected were named *Eptesicus isabellinus* rhabdovirus 1–5 (EIR1, EIR2, EIR3, EIR4 and EIR5) and their respective GenBank accession numbers are JX276976, JX276977, JX276978, JX276961 and JX276974.
†*Hypsugo savii* rhabdovirus 1 (HSR1, JX276962).
‡*Miniopterus schreibersii* rhabdovirus 1 (MSR1, JX276981).
§*Plecotus auritus* rhabdovirus 1 (PAR1, JX276980).
‖*Rhinolophus ferrumequinum* rhabdovirus 1 (RFR1, JX276975) and RFR2, JX276979.
¶*Nycteribia kolenatii* rhabdovirus 1 (NKR1, JX276967).
#*Penicillidia conspicua* rhabdovirus 1 and 2 (PCR1, JX276972 and PCR2, JX276973).
***Nycteribia schmidli* rhabdovirus 1–4 (NSR1, JX276968; NSR2, JX276969; NSR3, JX276970; NSR4, JX276971).

using highly degenerate primers. The nucleotide sequences of the genomic fragments obtained from the 53 rhabdoviruses without previously published sequences are available in GenBank (Tables 1 and S1).

In total, 1488 oropharyngeal samples were taken from bats throughout Spain between 2004 and 2010 as described previously (Echevarría *et al.*, 2001; Vázquez-Morón *et al.*, 2008), stored in lysis buffer and kept at −80 °C until RNA extraction (Casas *et al.*, 1995). An additional swab on viral transport medium was taken from the majority of bats. Ten (0.7 %) of them, from five of the 27 bat species sampled (Table 1), were positive and, after sequencing, BLAST analysis (Altschul *et al.*, 1990) showed similarity to existing

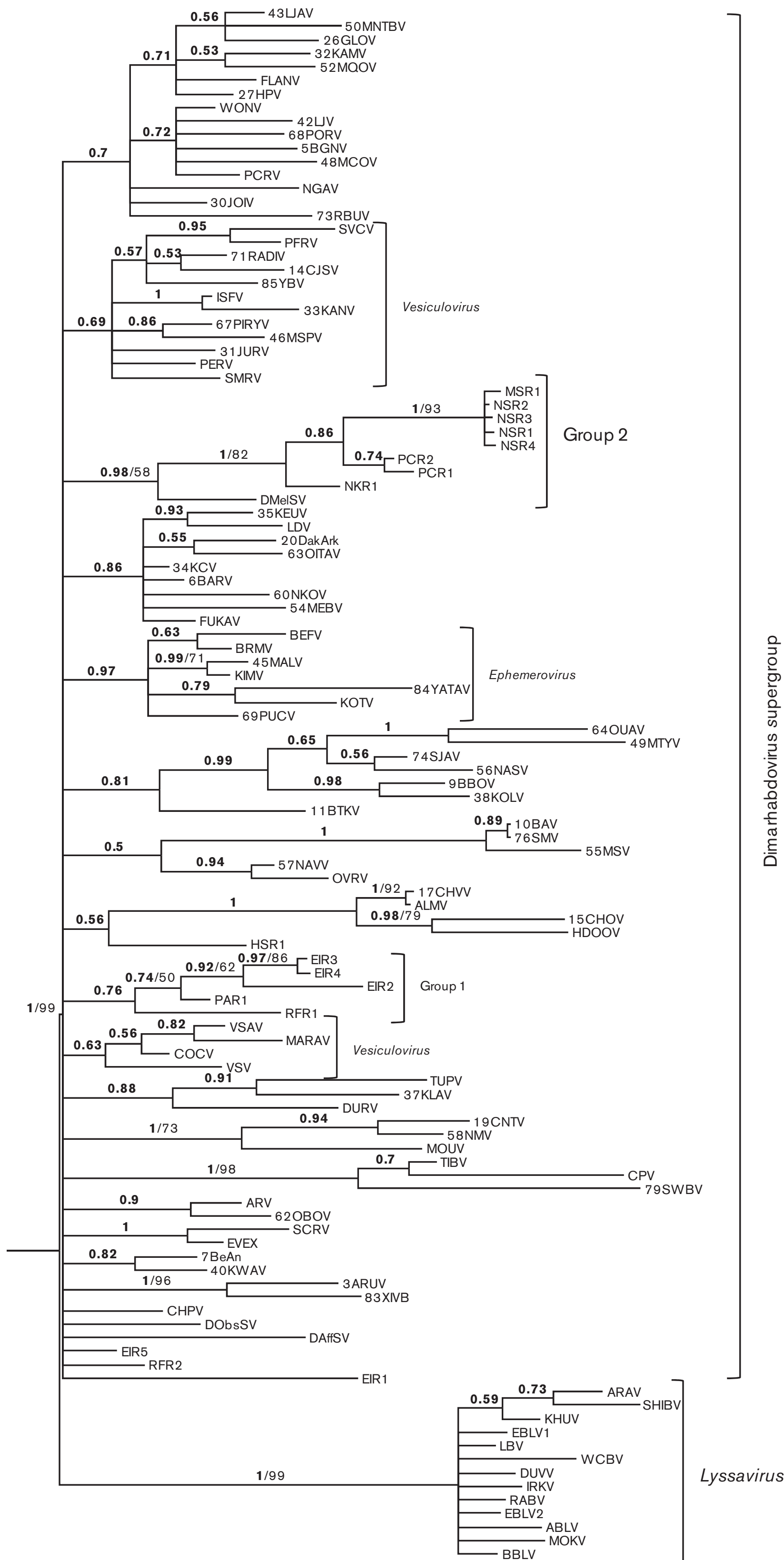

Fig. 2. Phylogenetic hypothesis for the sequences detected in this work, as well as those available in GenBank of members of the genus *Lyssavirus* and the dimarhabdovirus supergroup, obtained from an L-gene fragment using BPP and ML criteria. Bayesian posterior probabilities of clusters are shown in bold, and ML bootstrap values for clusters are given to the right, separated by /.

members of the family *Rhabdoviridae*. Five of these 10 samples with an available aliquot on viral transport medium were inoculated intracranially into mice, which survived and remained healthy for 28 days post-inoculation. No successful amplifications were obtained from the brain, saliva swabs or salivary glands collected post-mortem. It is important to note that the samples were taken from healthy bats, while other bat rhabdoviruses inducing neurological disease in mice were isolated from bats showing clinical signs (Iwasaki *et al.*, 2004; Metselaar *et al.*, 1969).

Thirty-eight bat parasites, corresponding to 32 pools, were collected during 2009–2011 from different bats from Andalusia (southern Spain). All samples (either individual or pooled) of nycteribiids were positive using the DimLis RT-PCR, while all three bat true bugs (*Cimex pipistrelli*) and bat ticks (*Argas vespertilionis*) were negative (Table 1). Such a high frequency observed in insects could reflect either infection with rhabdoviruses or amplification of genomic DNA fragments of rhabdoviral origin, which have recently been reported to be present in insect DNA (Fort *et al.*, 2012). The only sample from *Nycteribia kolenatii* consisted of a pool of seven individuals from Daubenton's bats (*Myotis daubentonii*) and contained a rhabdoviral sequence that we have designated NKR1. The four samples of *Penicillidia conspicua* that were taken from *Miniopterus schreibersii* tested positive for two different rhabdovirus-related sequences, designated PCR1 (detected in three samples) and PCR2 (detected in one sample). The four samples of *Nycteribia schmidli* that were also taken from *Miniopterus schreibersii* tested positive for four different rhabdovirus-related sequences designated NSR1, NSR2, NSR3 and NSR4.

Despite the fact that the majority of rhabdoviruses detected in bats worldwide are lyssaviruses (Banyard *et al.*, 2011), no lyssavirus-related sequences were found in the bat oropharyngeal swabs tested in this study. In contrast to previous studies (Kuzmin *et al.*, 2009), these bat-associated sequences did not form a monophyletic group (Fig. 2). The short sequence fragment generated in this study is unsuitable for use as the basis for a solid phylogenetic hypothesis, as it was not our purpose when designing the method. In spite of this fact, five of the rhabdovirus-related sequences found in bat oropharyngeal swabs grouped together in a distinct cluster (group 1, Fig. 2), supported by a posterior probability of 0.76, but they could not be related to any previously described rhabdovirus. Another four bat-associated sequences did not group with any other virus. However, one sequence detected in *Miniopterus schreibersii* (MSR1) grouped into a well-supported cluster (group 2, Fig. 2) together with the seven sequences associated with bat nycteribiids (NSR1–4, PCR1–2 and NKR1) and *Drosophila melanogaster* sigma rhabdovirus (DMelSV), which is also a dipteran rhabdovirus (Longdon *et al.*, 2010). Interestingly, this bat-associated sequence was almost identical to the sequences NSR1, 2, 3 and 4 (Table S2), which were amplified from *Nycteribia schmidli* collected from the same bat species. Whether the sequence found in the mouth of the bat came from parasites eaten just before sampling or from rhabdoviruses that were infecting the bat remains unclear. Although there is no direct evidence of nycteribiid consumption by bats, feeding on other ectoparasites has been demonstrated (Goiti *et al.*, 2003; Jones *et al.*, 2006). The second option would reflect the ability of rhabdoviruses to adapt to new hosts through parasitic relationships.

In conclusion, the RT-PCR assay described here was able to detect a wide range of rhabdoviruses and has been used successfully to detect new rhabdoviruses in bats and bat parasites. We believe that this molecular assay can be applied to the detection of novel rhabdoviruses from different hosts and can serve as an effective tool for future research to increase our knowledge of the complex diversity of the family *Rhabdoviridae*, which may help our understanding of the natural mechanisms of viral spillover and adaptation that might underlie the emergence of viral diseases.

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