Short Communication

Molecular epidemiology of paramyxoviruses in Zambian wild rodents and shrews

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Rodents and shrews are known to harbour various viruses. Paramyxoviruses have been isolated from Asian and Australian rodents, but little is known about them in African rodents. Recently, previously unknown paramyxovirus sequences were found in South African rodents. To date, there have been no reports related to the presence and prevalence of paramyxoviruses in shrews. We found a high prevalence of paramyxoviruses in wild rodents and shrews from Zambia. Semi-nested reverse transcription-PCR assays were used to detect paramyxovirus RNA in 21 % (96/462) of specimens analysed. Phylogenetic analysis revealed that these viruses were novel paramyxoviruses and could be classified as morbillivirus- and henipavirus-related viruses, and previously identified rodent paramyxovirus-related viruses. Our findings suggest the circulation of previously unknown paramyxoviruses in African rodents and shrews, and provide new information regarding the geographical distribution and genetic diversity of paramyxoviruses.

Paramyxoviruses are well-known infectious agents of humans and animals. The family *Paramyxoviridae* contains non-segmented negative-strand RNA viruses, and can be divided into the subfamilies *Paramyxovirinae* and *Pneumovirinae*. The subfamily *Paramyxovirinae* contains the genera *Avulavirus, Rubulavirus, Respirovirus, Henipavirus* and *Morbillivirus*, along with some unclassified members. Rodents are classified in the order Rodentia and are the most diverse and abundant mammals worldwide. They harbour a wide range of viruses and can be reservoirs of zoonotic viruses such as hantavirus, Lassa virus and tick-borne encephalitis virus (Meerburg *et al.*, 2009). At least seven paramyxoviruses (Sendai virus, Nariva virus, Mossman virus, J virus, Beilong virus, Tailam virus and murine pneumonia virus) are thought to have originated in rodents.

Sendai virus, a member of the genus *Respirovirus*, is an aetiological agent of pneumonia in rodent species and is distributed worldwide (Faísca & Desmecht, 2007). Murine

pneumonia virus is classified within the genus *Pneumovirus*, in the subfamily *Pneumovirinae*. It was isolated from the lung tissues of laboratory mice (Dyer *et al.*, 2012). Nariva virus was isolated from *Zygodontomys brevicauda* in Trinidad. In Australia, Mossman virus was isolated from *Rattus leukopus* and *Rattus fuscipes*, and J virus was found in *Mus musculus* (Jack *et al.*, 2005; Jun *et al.*, 1977; Lambeth *et al.*, 2009; Miller *et al.*, 2003). Beilong virus was initially isolated from a human kidney mesangial cell line in the laboratory, but the origin of Beilong virus was expected to be a rat kidney mesangial cell line (Li *et al.*, 2006). Beilong virus variants have since been found in *Rattus norvegicus* and *Rattus rattus* from China (Woo *et al.*, 2012). These findings would indicate that the natural host of Beilong virus is a rodent. The complete genome sequence of another para-myxovirus identified in *Rattus andamanensis* from China was designated Tailam virus (Woo *et al.*, 2011). With the exception of Sendai virus and murine pneumonia virus, these aforementioned paramyxoviruses are phylogenetically distinct from other mammalian paramyxoviruses and remain unclassified at the genus level.

Shrews are small mole-like mammals in the family Soricidae, order Soricomorpha. Although they are similar in size and

appearance to rodents, phylogenetic analyses based on mitochondrial cytochrome *b* gene sequences have shown a clear genetic difference between shrews and rodents (Guo *et al.*, 2013; Kang *et al.*, 2011). To date, there have been no reports related to the presence and prevalence of paramyxoviruses in shrews. A single paramyxovirus has been isolated from a tree shrew; however, the animal is classified within the different order Scandentia (Tidona *et al.*, 1999).

The application of reverse transcription (RT)-PCR assays, using degenerate primers targeting the consensus region of the paramyxovirus L gene, has revealed previously unrecognized paramyxoviruses, particularly in bats (Baker *et al.*, 2012; Drexler *et al.*, 2012; Kurth *et al.*, 2012; Lau *et al.*, 2010; Sasaki *et al.*, 2012; Weiss *et al.*, 2012; Wilkinson *et al.*, 2012). However, paramyxovirus diversity in rodents and shrews remains poorly understood. In this study, we aimed to investigate the presence and prevalence of paramyxoviruses in rodents and shrews from Zambia.

Our study was conducted with permission from the Zambia Wildlife Authority. We analysed 431 wild rodents and 31 wild shrews collected across four locations in Zambia: 256 rodents and five shrews from Lusaka (eastern area: 15° 26′ 6.12″ S, 28° 26′ 9.93″ E; northern area: 14° 58′ 6.12″ S, 28° 14′ 8.33″ E; and southern area: 15° 34′ 6.88″ S, 28° 16′ 5.13″ E); 122 rodents and two shrews from Livingstone (17° 50′ 8.72″ S, 25° 43′ 59.19″ E); 48 rodents and 24 shrews from Mpulungu (8° 45′ 5.45″ S, 31° 06′ 8.43″ E); and five rodents from Kasanka (15° 34′ 6.88″ S, 28° 16′ 5.13″ E). Rodents and shrews were captured around houses and fields using Sherman traps and cage traps from 2010 to 2012. The captured animals were euthanized with diethyl ether and their kidneys removed. Rodent and shrew species were verified using nucleotide sequence analysis of the mitochondrial cytochrome *b* gene, as described previously (Ishii *et al.*, 2012). Around 61 % of the animals analysed were *Mastomys natalensis*, referred to as Natal multimammate mice. All captured shrews were members of the genus *Crocidura*. At least 19 species of rodent and three species of shrew were included in this study (Table 1).

Total RNA was extracted from the kidneys of all rodents and shrews using TRIzol reagent and a PureLink RNA Mini kit (Life Technologies, Invitrogen). Kidney has a high prevalence rate and a high viral load of paramyxoviruses in rodents; therefore, it is considered to be the relevant organ in which to detect rodent paramyxoviruses (Drexler *et al.*, 2012). We screened 462 RNA samples by semi-nested RT-PCR as described previously (Sasaki *et al.*, 2012). We used degenerate primers (PAR-F, PAR-F2 and PAR-R) that were specific for the L gene in subfamily *Paramyxovirinae* members (Tong *et al.*, 2008). Amplicons were electrophoresed on 1.6 % (w/v) agarose gels and purified with a QIAquick Gel Extraction kit (Qiagen). Sequences were determined by direct cycle sequence analysis in both directions using a BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Applied Biosystems). All obtained sequences were subjected to BLAST analysis.

Based on our RT-PCR results, approximately 21 % (96/462) of the RNA samples were positive for the presence of the L gene. Among the 96 positive samples, 84 were from rodents and 12 were from shrews. Amino acid sequence similarities to members of the subfamily *Paramyxovirinae* ranged from 67 to 90 %, with no previously known paramyxoviruses identified in this study. All sequences were deposited in the GenBank under the accession numbers AB844333–AB844336 and AB844338–AB844429 (Table S1, available in JGV Online).

A phylogenetic analysis of the deduced amino acid sequences for the L gene fragments amplified in this study was conducted. We generated a phylogenetic tree using MEGA5 software and the maximum-likelihood method with complete deletion option and the WAG + G + I substitution model (Tamura *et al.*, 2011). LR, LivR, MpR and KasR represent rodents or shrews from Lusaka, Livingstone, Mpulungu and Kasanka, respectively. The tree highlights the diversity of paramyxoviruses circulating in rodents and shrews from Zambia (Fig. 1). All detected viruses could be grouped into four clades. Paramyxoviruses from one rodent and 11 shrews branched from the lineage leading to the genus *Henipavirus* to form genotype 1. Tailam virus-related and J virus-related paramyxoviruses were detected in 30 rodents that formed genotype 2, while Mossman virus-related paramyxoviruses were detected in 47 rodents to form genotype 3 (Fig. 1). Genotype 4 comprised paramyxoviruses from six rodents and one shrew, and a member of the genus *Morbillivirus*. In genotype 3, the virus sequences obtained from multiple *M. natalensis* captured across different geographical locations were almost identical (99 %). Our results suggest that novel paramyxoviruses are endemic in Zambian rodents and shrews.

We amplified a different region of the L gene by semi-nested RT-PCR using degenerate primers (RES-MOR-HEN-F1, RES-MOR-HEN-F2 and RES-MOR-HEN-R) (Tong *et al.*, 2008). Based on the sequence of each obtained fragment, virus-specific primers were designed and used to amplify a longer L gene fragment between regions targeted by the RES-MOR-HEN and PAR primers. Conventional two-step RT-PCRs were performed with Superscript III reverse transcriptase (Life Technologies, Invitrogen) and PrimeSTAR GXL DNA polymerase (Takara Bio), according to the manufacturers' instructions. We obtained 31 L gene fragments, ranging from 1586 to 1793 bp, from 22 rodents and nine shrews. Phylogenetic analyses were conducted using representative nucleotide sequences with the maximum-likelihood method and the GTR + G + I substitution model (Fig. 2). In parallel with the maximum-likelihood method, we also applied a Bayesian method to construct phylogenetic trees using MrBayes software, version 3.2.2 (Ronquist *et al.*, 2012) (Fig. S1). Both maximum-likelihood and Bayesian trees resulted in the same topologies. The four clades shown in Fig. 1 also appeared in these trees, supporting the phylogenetic relationships in Fig. 1 and providing a deeper understanding of rodent and shrew paramyxovirus phylogeny.

Table 1. RT-PCR screening results

Results are shown as the number of RT-PCR-positive individuals per number of rodents or shrews captured.

Species	Lusaka			Livingstone		Mpulungu	Kasanka	Total	Detected genotype(s)*
	2010	2011	2012	2010	2011	2012	2011		
Rodent									
Acomys subspinosus	1/2	1/1	–	–	–	–	–	2/3	2
Aethomys chrysophilus	1/15	1/11	–	–	0/2	1/6	–	3/34	2, 3
Arvicanthis sp.	–	1/3	–	–	–	–	–	1/3	4
Cricetomys gambianus	–	–	0/2	–	–	0/3	–	0/5	
Cricetomys sp.	0/1	–	–	–	–	–	–	0/1	
Gerbilliscus leukogaster	0/12	1/12	–	–	–	0/1	–	1/25	4
Grammomys sp.	0/1	1/1	–	–	–	0/1	0/1	1/4	3
Graphiurus sp.	0/1	–	–	–	–	–	–	0/1	
Hylomyscus alleni	–	–	0/1	–	–	–	–	0/1	
Hylomyscus sp.	–	1/1	–	–	–	–	–	1/1	2
Lemniscomys rosalia	–	–	–	–	0/2	–	–	0/2	
Mastomys natalensis	6/44	41/131	0/2	7/42	7/35	7/28	1/2	69/284	2, 3, 4
Mus minutoides	1/2	1/1	–	–	–	–	0/2	2/5	2
Otomys sp.	–	0/1	–	–	–	–	–	0/1	
Paraxerus cepapi	–	–	–	–	–	0/2	–	0/2	
Rattus rattus	0/3	0/8	–	2/35	0/5	0/3	–	2/54	1, 2
Saccostomus campestris	–	–	–	0/1	–	–	–	0/1	
Saccostomus sp.	–	–	–	–	–	1/3	–	1/3	2
Steatomys sp.	–	–	–	–	–	1/1	–	1/1	2
Shrew									
Crocidura hirta	–	2/2	–	–	2/2	5/23	–	9/27	1
Crocidura luna	–	–	–	–	–	0/1	–	0/1	
Crocidura sp.	–	3/3	–	–	–	–	–	3/3	1, 4
Total	9/81	52/175	0/5	9/78	9/46	15/72	1/5	96/462	

*Genotypes were defined according to phylogeny results based on a portion of the paramyxovirus L gene.

Virus isolation was conducted using Vero and BHK cells, which are used for propagation of known rodent paramyxoviruses (Jack *et al.*, 2005; Lambeth *et al.*, 2009; Li *et al.*, 2006; Miller *et al.*, 2003). Tissue homogenates (10 %, w/v) in Eagle's minimum essential medium (MEM) were prepared from 18 kidney tissues that were positive for paramyxovirus RNA, and used to infect Vero and BHK cells. Cells were maintained in MEM containing 2 % FBS and 2 % antibiotic–antimycotic solution (Life Technologies, Gibco). Supernatants were passaged onto fresh cells every 7 days; no cytopathic effect was observed for 25 days post-inoculation. Paramyxovirus RNA was not detected in culture supernatants when we used semi-nested RT-PCR assays. Consequently, no paramyxoviruses were isolated from any of the tested tissues.

In this study, we detected various paramyxoviruses from different species of rodents in Zambia. These viruses were related to the members of the genus *Morbillivirus* and unclassified rodent paramyxoviruses (Mossman virus, Tailam virus and J virus). Over the last 50 years, six paramyxoviruses have been identified in rodents from Asian countries and Australia (Faísca & Desmecht, 2007;

Jun *et al.*, 1977; Lambeth *et al.*, 2009; Li *et al.*, 2006; Miller *et al.*, 2003; Woo *et al.*, 2011). Drexler *et al.* (2012) detected paramyxoviruses that were phylogenetically related to morbilliviruses and Beilong virus in *Rhabdomys pumilio* from South Africa using the primers (RES-MOR-HEN-F1, RES-MOR-HEN-F2 and RES-MOR-HEN-R). These results give us an indication of the distribution and genetic diversity of rodent paramyxoviruses in Africa.

Genome sequence analyses of rodent paramyxoviruses have revealed that they are not phylogenetically grouped into any established genera (Jack *et al.*, 2005; Lambeth *et al.*, 2009; Li *et al.*, 2006; Miller *et al.*, 2003; Woo *et al.*, 2011). Our phylogenetic analysis showed that many of the paramyxoviruses detected from rodents in Zambia were distinct from any established genera and related to previously identified rodent paramyxoviruses. These paramyxoviruses could be classified into a new genus, or genera, of the subfamily *Paramyxovirinae*.

We also identified unique paramyxovirus sequences from wild shrews. This is the first report to our knowledge describing the prevalence of paramyxoviruses in shrews. The prevalence of henipavirus-related paramyxoviruses in
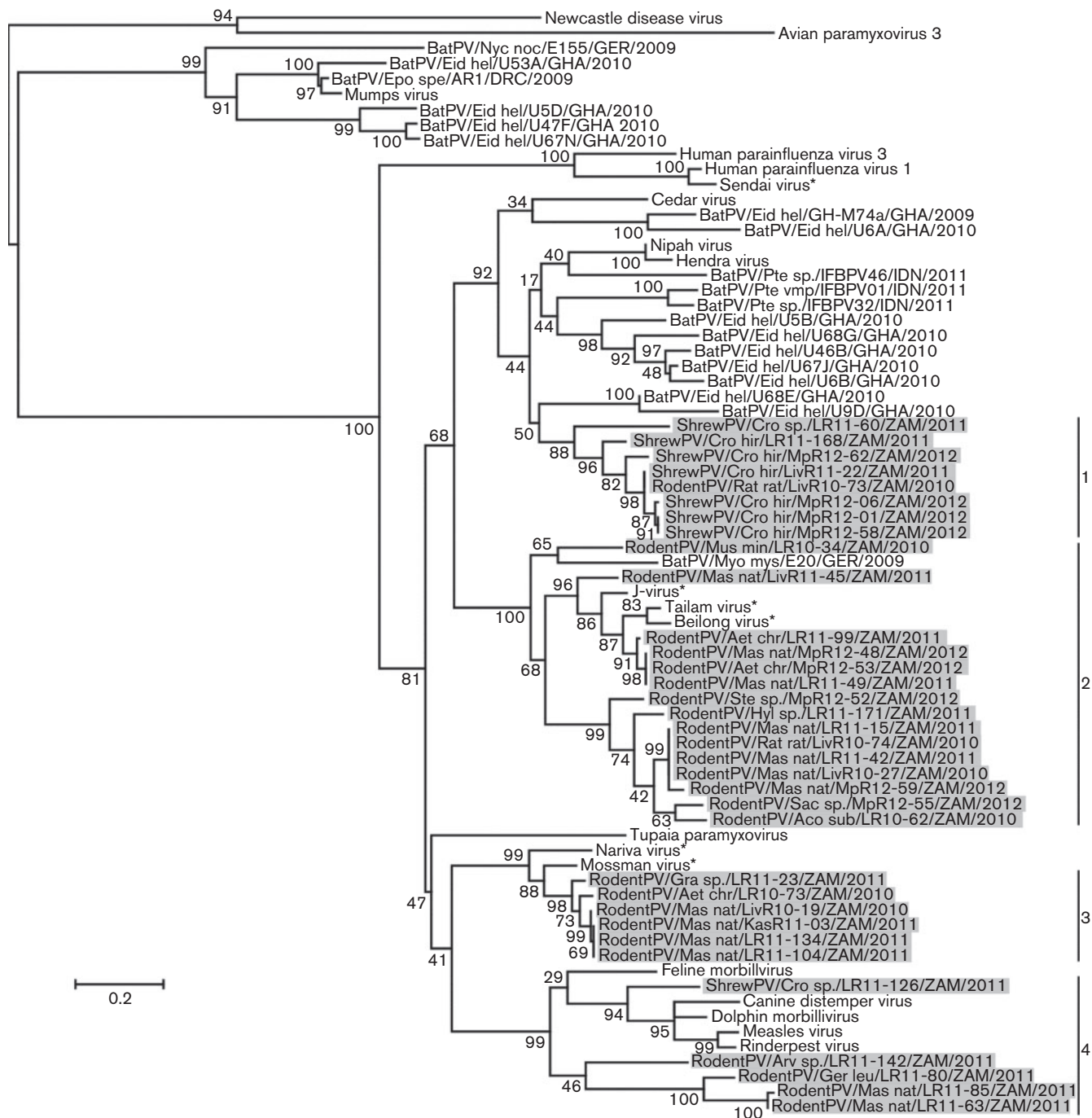
Fig. 1. Diversity of paramyxoviruses detected in wild rodents and shrews from Zambia. A phylogenetic tree was generated based on a 176 aa sequence from a conserved region of the paramyxovirus L gene corresponding to positions 13910–14439 in the Nipah virus genome (GenBank accession number NC_002728). We detected 84 rodent paramyxoviruses (RodentPV) and 12 shrew paramyxoviruses (ShrewPV). These are indicated by grey shading and were analysed alongside known paramyxoviruses. Genotypes 1, 2, 3 and 4 represent henipavirus-, Tailam virus-, Mossman virus- and morbillivirus-related paramyxoviruses, respectively. Previously identified rodent paramyxoviruses are marked with an asterisk. Bootstrap values (%) calculated from 1000 replicates are indicated at each tree root. Bar, 0.2 substitutions per site. Viruses and their respective GenBank accession numbers are listed in Table S1. Species abbreviations are as follows: Aco sub, *Acomys subspinosus*; Aet chr, *Aethomys chrysophilus*; Arv sp., *Arvicanthis* sp.; Eid hel, *Eidolon helvum*; Epo spe, *Epomophorus* sp.; Ger leu, *Gerbilliscus leukogaster*; Gra sp., *Grammomys* sp.; Hyl sp., *Hylomyscus* sp.; Mas nat, *Mastomys natalensis*; Mus min, *Mus minutoides*; Myo mys, *Myotis mystacinus*; Nyc noc, *Nyctalus noctula*; Pte sp., *Pteropus* sp.; Pte vmp, *Pteropus vampyrus*; Rat rat, *Rattus rattus*; Sac sp., *Saccostomus* sp.; Ste sp., *Steatomys* sp.; Cro hir, *Crocidura hirta*; Cro sp., *Crocidura* sp.
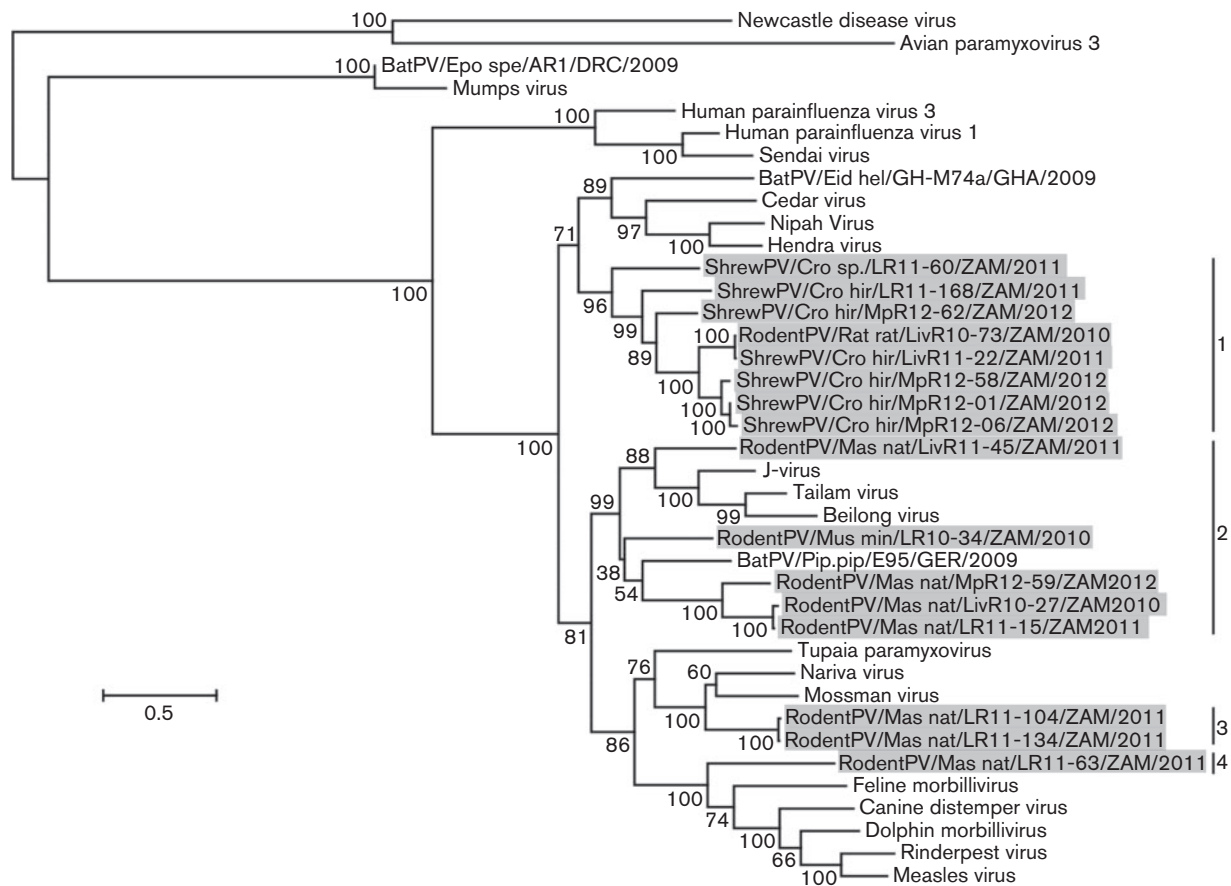
Fig. 2. Extended phylogenetic analysis of a portion of the L gene from paramyxoviruses. Larger L gene fragments (1586–1793 bp) were detected in rodent and shrew specimens, corresponding to nt 12629–14439 in the Nipah virus genome (GenBank accession no. NC_002728). A phylogenetic tree was generated using representative nucleotide sequences with the maximum-likelihood method and the GTR+I+G substitution model. Paramyxoviruses detected in this study are indicated by grey shading. Genotypes 1, 2, 3 and 4 represent henipavirus-, Tailam virus-, Mossman virus- and morbillivirus-related paramyxoviruses, respectively. Bootstrap values calculated from 1000 replicates are indicated at each tree root. Bar, 0.5 substitutions per site. Viruses and their respective GenBank accession numbers are listed in Table S1.

lesser red musk shrews (*Crocidura hirta*) was found to be high in this study. Shrew paramyxoviruses appear to be distinct from previously identified rodent paramyxoviruses, suggesting the presence of novel unique species within the subfamily *Paramyxovirinae*. It is possible that there are some differences in the genetic diversity of paramyxoviruses among rodents and shrews. Results from previous studies have described a clear phylogenetic division between hantaviruses from rodents and shrews (Guo *et al.*, 2013; Kang *et al.*, 2011).

Acknowledgements

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