Molecular epidemiology of paramyxoviruses in Zambian wild rodents and shrews

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33

Summary

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 RT-PCR assays were used to detect paramyxovirus RNA in 21% (96/462) of specimens analyzed. 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.

47 **Main Text**

48 Paramyxoviruses are well-known infectious agents of humans and animals. The
49 *Paramyxoviridae* family contain non-segmented negative-strand RNA viruses, and can be
50 divided into the subfamilies *Paramyxovirinae* and *Pneumovirinae*. The *Paramyxovirinae*
51 subfamily contains the genera *Avularirus*, *Rubulavirus*, *Respirovirus*, *Henipavirus*, and
52 *Morbillivirus*, along with some unclassified members. Rodents are classified in the order
53 *Rodentia* and are the most diverse and abundant mammals worldwide. They harbour a wide
54 range of viruses and can be reservoirs of zoonotic viruses such as Hantavirus, Lassa virus
55 and tick-borne encephalitis virus (Meerburg *et al.*, 2009). At least seven paramyxoviruses
56 (Sendai virus, Nariva virus, Mossman virus, J-virus, Beilong virus, Tailam virus and
57 Murine pneumonia virus) are thought to have originated in rodents.
58
59 Sendai virus, a member of the genus *Respirovirus*, is an etiological agent of pneumonia in
60 rodent species and is distributed worldwide (Faísca & Desmecht, 2007). Murine pneumonia
61 virus is classified within the *Pneumovirus* genus, in the *Pneumovirinae* subfamily. It was
62 isolated from the lung tissues of laboratory mice (Dyer *et al.*, 2012). Nariva virus was
63 isolated from *Zygodontomys b. brevicauda* in Trinidad. In Australia, Mossman virus was
64 isolated from *Rattus leucopus* and *Rattus fuscipes*, and J-virus was found in *Mus musculus*
65 (Jack *et al.*, 2005; Jun *et al.*, 1977; Lambeth *et al.*, 2009; Miller *et al.*, 2003). Beilong virus
66 was initially isolated from human kidney mesangial cell line in the laboratory, but the
67 origin of Beilong virus was expected to be rat kidney mesangial cell line (Li *et al.*, 2006).
68 Beilong virus variants have since been found in *Rattus norvegicus* and *Rattus rattus* from
69 China (Woo *et al.*, 2012). These findings would indicate that the natural host of Beilong
70 virus is a rodent. The complete genome sequence of another paramyxovirus identified in

71 *Rattus andamanensis* from China was designated Tailam virus (Woo *et al.*, 2011). With the
72 exception of Sendai virus and Murine pneumonia virus, these aforementioned
73 paramyxoviruses are phylogenetically distinct from other mammalian paramyxoviruses and
74 remain unclassified at the genus level.
75
76 Shrews are small mole-like mammals in the family *Soricidae*, order *Soricomorpha*.
77 Although they are similar in size and appearance to rodents, phylogenetic analyses based on
78 mitochondrial cytochrome *b* gene sequences has shown a clear genetic difference between
79 shrews and rodents (Guo *et al.*, 2013; Kang *et al.*, 2011). To date, there have been no
80 reports related to the presence and prevalence of paramyxoviruses in shrews. A single
81 paramyxovirus has been isolated from a treeshrew, however the animal is classified within
82 the different order, *Scandentia* (Tidona *et al.*, 1999).
83
84 The application of RT-PCR assays, using degenerate primers targeting the consensus region
85 of the paramyxovirus *L* gene, has revealed previously unrecognized paramyxoviruses,
86 particularly in bats (Baker *et al.*, 2012; Drexler *et al.*, 2012; Kurth *et al.*, 2012; Lau *et al.*,
87 2010; Sasaki *et al.*, 2012; Weiss *et al.*, 2012; Wilkinson *et al.*, 2012). However,
88 paramyxovirus diversity in rodents and shrews remains poorly understood. In this study we
89 aimed to investigate the presence and prevalence of paramyxoviruses in rodents and shrews
90 from Zambia.
91
92 Our study was conducted with permission from the Zambia Wildlife Authority. We
93 analyzed 431 wild rodents and 31 wild shrews collected across four locations in Zambia:
94 256 rodents and 5 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 2 shrews from Livingstone (17°50'8.72"S, 25°43'59.19"E); 48 rodents and 24 shrews from Mpulungu (08°45'5.45"S, 31°06'8.43"E); and 5 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 2010–2012. 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 analyzed 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 3 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 high prevalence rate and high viral load of paramyxoviruses in rodents, therefore it is considered to be the relevant organ 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 *Paramyxovirinae* subfamily 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 presence of the *L* gene. Among the 96 positive samples, 84 were from rodents and 12 were from shrews. Amino acid sequence similarities to *Paramyxovirinae* members ranged from 67–90%, with no previously known paramyxoviruses identified in this study. All sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB844333–AB844429 (Supplementary Table S1).

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 (Figure 1). All detected viruses could be grouped into four clades. Paramyxoviruses from 1 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 (Figure 1). Genotype 4 comprised paramyxoviruses from 6 rodents and 1 shrew, and a member of the *Morbillivirus* genus. In genotype 3, the virus sequences obtained from multiple *Mastomys 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

143 primers (RES-MOR-HEN-F1, RES-MOR-HEN-F2 and RES-MOR-HEN-R). Based on the
144 sequence of each obtained fragment, virus-specific primers were designed and used to
145 amplify a longer *L* gene fragment between regions targeted by the RES-MOR-HEN and
146 PAR primers. Conventional two-step RT-PCRs were performed with Superscript III reverse
147 transcriptase (Life Technologies, Invitrogen) and PrimeSTAR GXL DNA polymerase
148 (Takara Bio), according to the manufacturer's instructions. We obtained 31 *L* gene
149 fragments, ranging 1586–1793 bp, from 22 rodents and 9 shrews. Phylogenetic analyses
150 were conducted using representative nucleotide sequences with the maximum likelihood
151 method and the GTR+G+I substitution model (Figure 2). In parallel with the maximum
152 likelihood method, we also applied a Bayesian method to construct phylogenetic trees using
153 MrBayes software, version 3.2.2 (Ronquist *et al.*, 2012) (Supplementary Figure S1). Both
154 maximum likelihood and Bayesian trees resulted in the same topologies. The four clades
155 shown in Figure 1 also appeared in these trees, supporting the phylogenetic relationships in
156 Figure 1 and providing a deeper understanding of rodent and shrew paramyxovirus
157 phylogeny.
158
159 Virus isolation was conducted using Vero and BHK cells, which are used for propagation of
160 known rodent paramyxoviruses (Jack *et al.*, 2005; Lambeth *et al.*, 2009; Li *et al.*, 2006;
161 Miller *et al.*, 2003). Tissue homogenates [10% (w/v)] in Eagle's minimum essential
162 medium (MEM) were prepared from 18 kidney tissues that were positive for
163 paramyxovirus RNA, and used to infect Vero and BHK cells. Cells were maintained in
164 MEM containing 2% fetal bovine serum (FBS) and 2% antibiotic-antimycotic solution
165 (Life Technologies, Gibco). Supernatants were passaged onto fresh cells every 7 days; no
166 cytopathic effect was observed for 25 days post-inoculation. Paramyxovirus RNA was not

167 detected in culture supernatants when we used semi-nested RT-PCR assays. Consequently,
168 no paramyxoviruses were isolated from any of the tested tissues.
169
170 In this study, we detected various paramyxoviruses from different species of rodents in
171 Zambia. These viruses were related to the members of the *Morbillivirus* genus and
172 unclassified rodent paramyxoviruses (Mossman virus, Tailam virus and J-virus). Over the
173 last 50 years, six paramyxoviruses have been identified in rodents from Asian countries and
174 Australia (Faísca & Desmecht, 2007; Jun *et al.*, 1977; Lambeth *et al.*, 2009; Li *et al.*, 2006;
175 Miller *et al.*, 2003; Woo *et al.*, 2011). Drexler *et al*. detected paramyxoviruses that were
176 phylogenetically related to Morbilliviruses and Beilong virus in *Rhabdomys pumilio* from
177 South Africa using the different primer sets (Drexler *et al.*, 2012). These results give us an
178 indication of the distribution and genetic diversity of rodent paramyxoviruses in Africa.
179
180 Genome sequence analyses of rodent paramyxoviruses have revealed that they are not
181 phylogenetically grouped into any established genera (Jack *et al.*, 2005; Lambeth *et al.*,
182 2009; Li *et al.*, 2006; Miller *et al.*, 2003; Woo *et al.*, 2011). Our phylogenetic analysis
183 showed that many of paramyxoviruses detected from rodents in Zambia were distinct from
184 any established genera and related to previously identified rodent paramyxoviruses. These
185 paramyxoviruses could be classified into a new genus, or genera, of the *Paramyxovirinae*
186 subfamily.
187
188 We also identified unique paramyxovirus sequences from wild shrews. This is the first
189 report describing the prevalence of paramyxoviruses in shrews. The prevalence of
190 henipavirus-related paramyxoviruses in 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 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).

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283 variant of Beilong Paramyxovirus in rats, China. *Emerg Infect Dis* **18**, 1022-1024.
284
285
286

287 **Figure Legend**

288 Figure 1. Diversity of paramyxoviruses detected in wild rodents and shrews from Zambia.
289 A phylogenetic tree was generated based on a 176 amino acid sequence from a conserved
290 region of the paramyxovirus *L* gene corresponding to positions 13910-14439 in Nipah virus
291 genome (GenBank/EMBL/DDBJ accession number, NC_002728). We detected 84 rodent
292 paramyxoviruses (RodentPV) and 12 shrew paramyxoviruses (ShrewPV). These were
293 indicated by grey shading and analyzed alongside known paramyxoviruses. Genotype 1, 2,
294 3, and 4 represent Henipavirus-, Tailam virus-, Mossman virus- and Morbillivirus-related
295 paramyxoviruses, respectively. Previously identified rodent paramyxoviruses are marked
296 with an asterisk. Bootstrap values calculated from 1000 replicates are indicated at each tree
297 root. The horizontal scale bar represents a distance of 0.1 substitutions per site. Viruses and
298 their respective accession numbers are listed in Table S1. Species abbreviations are as
299 follows: *Acomys subspinosus* (Aco sub), *Aethomys chrysophilus* (Aet chr), *Arvicanthis* sp.
300 (Arv sp.), *Gerbilliscus leucogaster* (Ger leu), *Grammomys* sp (Gra sp.), *Hylomyscus* sp.
301 (Hyl sp.), *Mastomys natalensis* (Mas nat), *Mus minutoides* (Mus min), *Rattus rattus* (Rat
302 rat), *Saccostomus* sp. (Sac sp.), *Steatomys* sp. (Ste sp.), *Crocidura hirta* (Cro hir),
303 *Crocidura* sp. (Cro sp.).
304
305 Figure 2. Extended phylogenetic analysis of a portion of the *L* gene from paramyxoviruses.
306 Larger *L* gene fragments (1586–1793 bp) were detected in rodent and shrew specimens,
307 corresponding to nucleotides 12629–14439 in the Nipah virus genome (NC_002728). A
308 phylogenetic tree was generated using representative nucleotide sequences with the
309 maximum likelihood method and the GTR+I+G substitution model. Paramyxoviruses
310 detected in this study are indicated by shading (grey). Genotype 1, 2, 3, and 4 represent

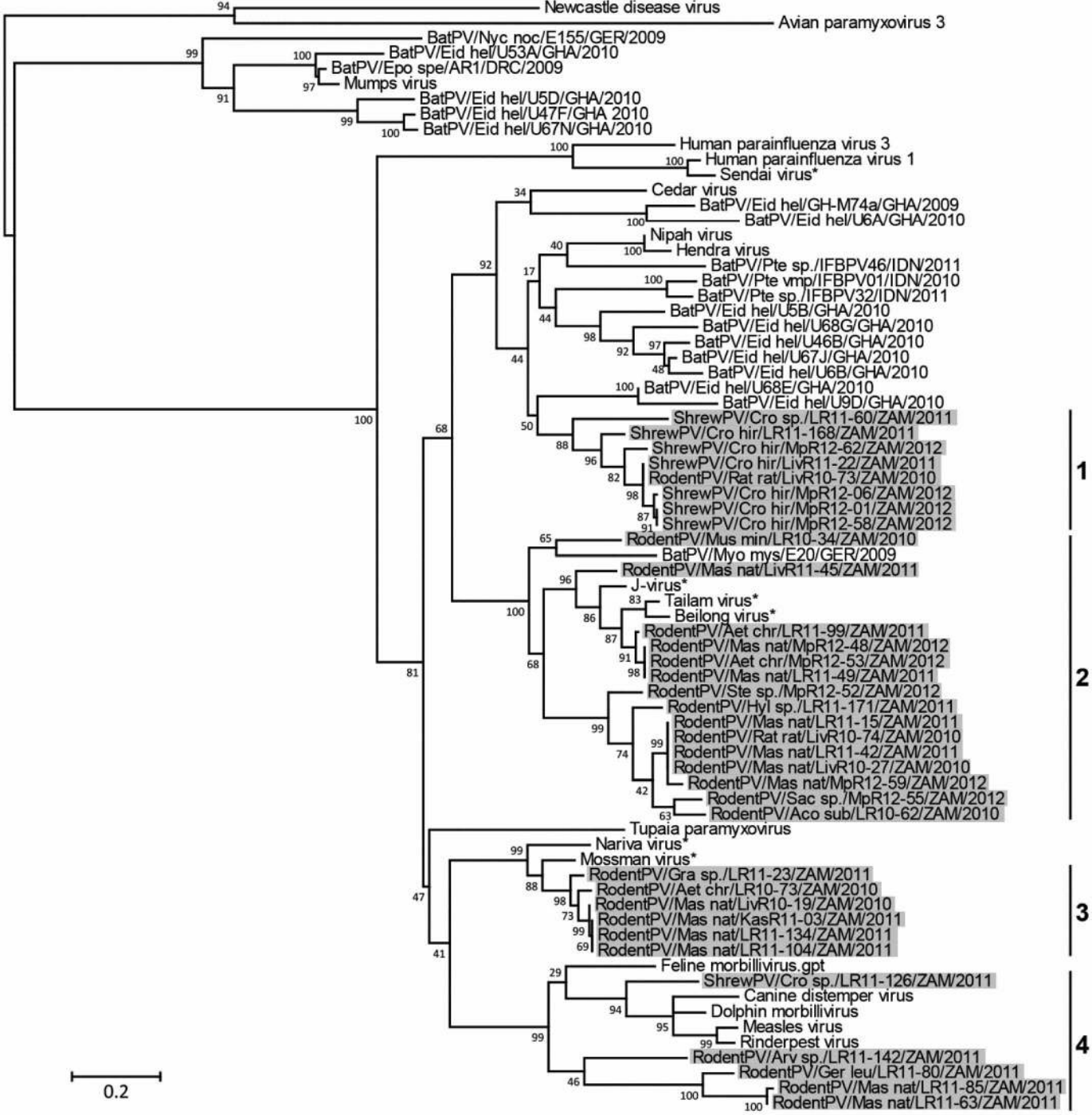
311 Henipavirus-, Tailam virus-, Mossman virus- and Morbillivirus-related paramyxoviruses,
312 respectively. Bootstrap values calculated from 1000 replicates are indicated at each tree
313 root. The scale bar represents a distance of 0.5 substitutions per site. Viruses and their
314 respective accession numbers are listed in Table S1.

Table 1. RT-PCR screening results

Species	Lusaka			Livingstone		Mpulungu	Kasanka	Total	Detected genotypes
	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 leucogaster	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	

*Number of RT-PCR-positive individuals per number of rodents or shrews captured

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


Newcastle disease virus
Avian paramyxovirus 3
BatPV/Nyc noc/E155/GER/2009
BatPV/Eid hel/U53A/GHA/2010
BatPV/Epo spe/AR1/DRC/2009
Mumps virus
BatPV/Eid hel/U5D/GHA/2010
BatPV/Eid hel/U47F/GHA 2010
BatPV/Eid hel/U67N/GHA/2010
Human parainfluenza virus 3
Human parainfluenza virus 1
Sendai virus*
Cedar virus
BatPV/Eid hel/GH-M74a/GHA/2009
BatPV/Eid hel/U6A/GHA/2010
Nipah virus
Hendra virus
BatPV/Pte sp./IFBPV46/IDN/2011
BatPV/Pte vmp/IFBPV01/IDN/2010
BatPV/Pte sp./IFBPV32/IDN/2011
BatPV/Eid hel/U5B/GHA/2010
BatPV/Eid hel/U68G/GHA/2010
BatPV/Eid hel/U46B/GHA/2010
BatPV/Eid hel/U67J/GHA/2010
BatPV/Eid hel/U6B/GHA/2010
BatPV/Eid hel/U68E/GHA/2010
BatPV/Eid hel/U9D/GHA/2010
ShrewPV/Cro sp./LR11-60/ZAM/2011
ShrewPV/Cro hir/LR11-168/ZAM/2011
ShrewPV/Cro hir/MpR12-62/ZAM/2012
ShrewPV/Cro hir/LivR11-22/ZAM/2011
RodentPV/Rat rat/LivR10-73/ZAM/2010
ShrewPV/Cro hir/MpR12-06/ZAM/2012
ShrewPV/Cro hir/MpR12-01/ZAM/2012
ShrewPV/Cro hir/MpR12-58/ZAM/2012
RodentPV/Mus min/LR10-34/ZAM/2010
BatPV/Myo mys/E20/GER/2009
RodentPV/Mas nat/LivR11-45/ZAM/2011
J-virus*
Tailam virus*
Beilong virus*
RodentPV/Aet chr/LR11-99/ZAM/2011
RodentPV/Mas nat/MpR12-48/ZAM/2012
RodentPV/Aet chr/MpR12-53/ZAM/2012
RodentPV/Mas nat/LR11-49/ZAM/2011
RodentPV/Ste sp./MpR12-52/ZAM/2012
RodentPV/Hyl sp./LR11-171/ZAM/2011
RodentPV/Mas nat/LR11-15/ZAM/2011
RodentPV/Rat rat/LivR10-74/ZAM/2010
RodentPV/Mas nat/LR11-42/ZAM/2011
RodentPV/Mas nat/LivR10-27/ZAM/2010
RodentPV/Mas nat/MpR12-59/ZAM/2012
RodentPV/Sac sp./MpR12-55/ZAM/2012
RodentPV/Aco sub/LR10-62/ZAM/2010
Tupaia paramyxovirus
Nariva virus*
Mossman virus*
RodentPV/Gra sp./LR11-23/ZAM/2011
RodentPV/Aet chr/LR10-73/ZAM/2010
RodentPV/Mas nat/LivR10-19/ZAM/2010
RodentPV/Mas nat/KasR11-03/ZAM/2011
RodentPV/Mas nat/LR11-134/ZAM/2011
RodentPV/Mas nat/LR11-104/ZAM/2011
Feline morbillivirus.gpt
ShrewPV/Cro sp./LR11-126/ZAM/2011
Canine distemper virus
Dolphin morbillivirus
Measles virus
Rinderpest virus
RodentPV/Arv sp./LR11-142/ZAM/2011
RodentPV/Ger leu/LR11-80/ZAM/2011
RodentPV/Mas nat/LR11-85/ZAM/2011
RodentPV/Mas nat/LR11-63/ZAM/2011
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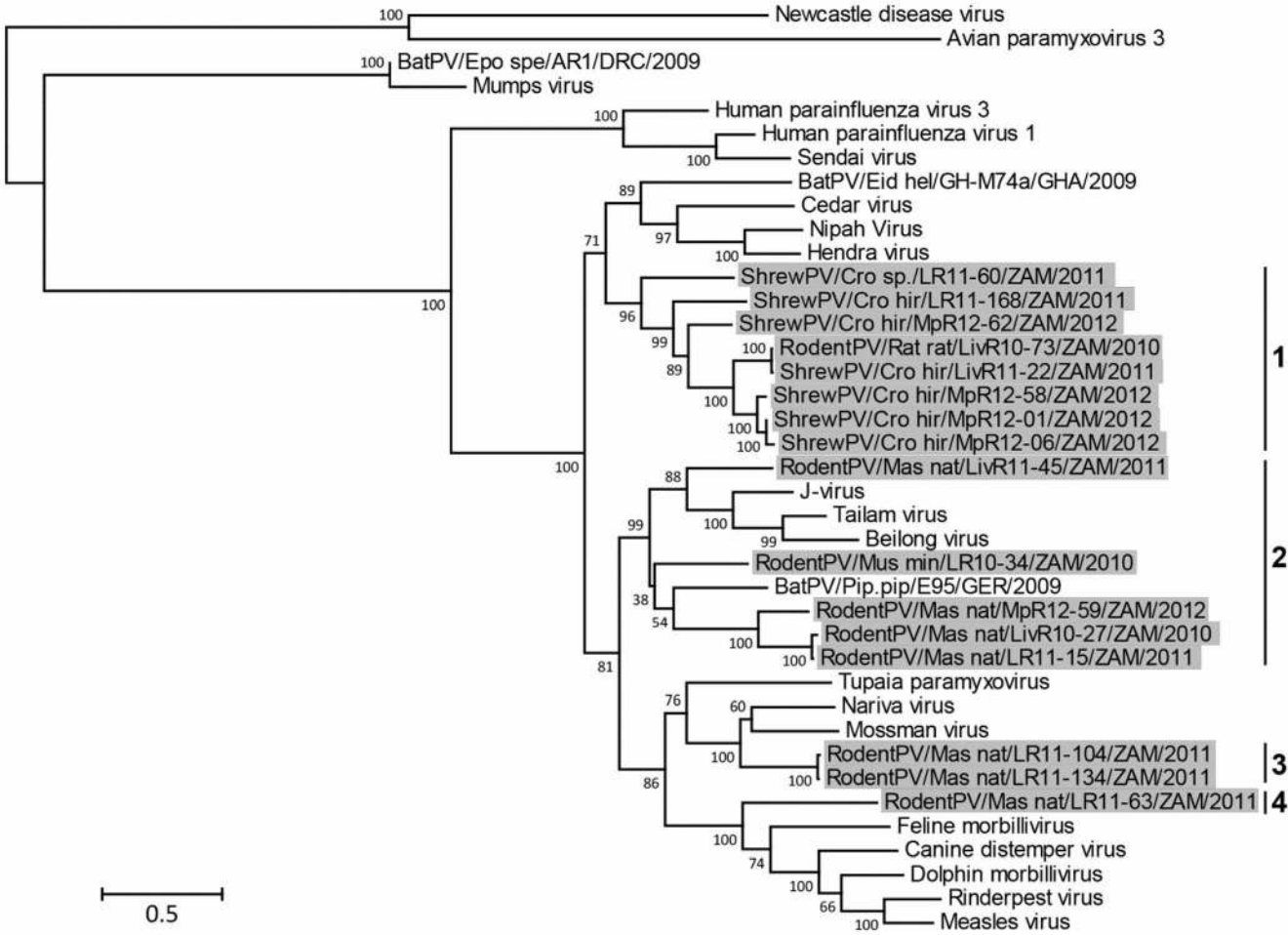
3
4
0.2


Newcastle disease virus
Avian paramyxovirus 3
BatPV/Epo spe/AR1/DRC/2009
Mumps virus
Human parainfluenza virus 3
Human parainfluenza virus 1
Sendai virus
BatPV/Eid hel/GH-M74a/GHA/2009
Cedar virus
Nipah Virus
Hendra virus
ShrewPV/Cro sp./LR11-60/ZAM/2011
ShrewPV/Cro hir/LR11-168/ZAM/2011
ShrewPV/Cro hir/MpR12-62/ZAM/2012
RodentPV/Rat rat/LivR10-73/ZAM/2010
ShrewPV/Cro hir/LivR11-22/ZAM/2011
ShrewPV/Cro hir/MpR12-58/ZAM/2012
ShrewPV/Cro hir/MpR12-01/ZAM/2012
ShrewPV/Cro hir/MpR12-06/ZAM/2012
RodentPV/Mas nat/LivR11-45/ZAM/2011
J-virus
Tailam virus
Beilong virus
RodentPV/Mus min/LR10-34/ZAM/2010
BatPV/Pip.pip/E95/GER/2009
RodentPV/Mas nat/MpR12-59/ZAM/2012
RodentPV/Mas nat/LivR10-27/ZAM/2010
RodentPV/Mas nat/LR11-15/ZAM/2011
Tupaia paramyxovirus
Nariva virus
Mossman virus
RodentPV/Mas nat/LR11-104/ZAM/2011
RodentPV/Mas nat/LR11-134/ZAM/2011
RodentPV/Mas nat/LR11-63/ZAM/2011
Feline morbillivirus
Canine distemper virus
Dolphin morbillivirus
Rinderpest virus
Measles virus
1
2
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4
0.5