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1 **A unique novel reptilian paramyxovirus, four adenovirus types and a reovirus**  
2 **identified in a concurrent infection of a corn snake (*Pantherophis guttatus*)**  
3 **collection in Germany**

4  
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15  
16 **Abstract**

17 In 2009, 26 clinical samples (organs and oral/cloacal swabs) from a total of 24 corn  
18 snakes (*Pantherophis guttatus*) from a single owner were sent to our laboratory to be  
19 tested for the presence of viruses. Paramyxoviruses (PMV), adenoviruses (AdV) and  
20 reoviruses were detected by RT-PCR, PCR and virus isolation methods. Three  
21 snakes were infected with all three viruses at the same time, while two other snakes  
22 had a double infection (PMV and reo, AdV and reo) and nine other snakes had a  
23 single infection with any of the three viruses. No viruses were detected in 10 animals.  
24 All isolated reoviruses were identical to one another and to the reptilian orthoreovirus  
25 isolate 55-02 in the partial RNA dependent RNA polymerase (RDRP) gene  
26 sequence. AdV partial polymerase sequences represented four different types, one

1 of which was first described here: most similar to SnAdV-1, while the other three  
2 were identical to known types: SnAV-1, -2 and -3. However, the detected single PMV  
3 differed distinctly from described reptile PMV and was a new type. According to  
4 partial L gene, HN gene and U gene sequences it may be the first described  
5 representative of a third squamamid PMV cluster: “group C” within the proposed  
6 reptilian PMV genus “*Ferlavirus*”. Nucleotide identity values for the L gene of the new  
7 PMV compared to group A viruses range between 76.5-80.3%, and between 80.5-  
8 81.2% compared to group B viruses. For the HN gene, these values were similar:  
9 78.2-80% (A) and 79.9-80.5% (B) and somewhat lower for the U gene: 72.7-75.4%  
10 (A) and 69.7-70% (B). No reports on the prevalence of concurrent viral infection in  
11 captive snake populations have been published so far. The possibility of concurrent  
12 infection with several different viruses and subsequent consequences for animal  
13 health should be kept in mind when testing reptile samples for viruses.

14

15

## 16 Introduction

17 Paramyxoviruses (PMV) have been isolated from several reptiles, mainly snakes,  
18 and have been characterized morphologically, immunologically and/or molecular  
19 biologically (Blahak, 1995; Richter et al., 1996; Ahne et al., 1999; Franke et al., 2001;  
20 Kindermann et al., 2001; Marschang et al., 2009).

21 The first recorded outbreak of PMV occurred in 1972 in a snake farm in Switzerland  
22 in common lancehead vipers (*Bothrops atrox*) (Fölsch and Leloup, 1976). The  
23 causative agent was named Fer de Lance virus (FDLV) and is regarded as the type  
24 species for reptilian PMV (Clark et al., 1979). Recently, the complete genome of  
25 FDLV has been fully sequenced (Kurath et al., 2004). This genome has a unique  
26 gene (U gene) that is absent in other PMVs, which encodes a short protein of

1 unkwon function. This feature together with the results of phylogenetic calculations  
2 supports the classification of FDLV in a new proposed genus "*Ferlavirus*" of the  
3 *Paramyxovirinae* subfamily. PMV have been detected in many different snake  
4 species from all major families including Boidae, Elapidae, Colubridae, and  
5 Viperidae. These viruses were first named ophidian (snake) paramyxoviruses  
6 (OPMV) (Essbauer and Ahne, 2001), but have since also been described in lizards  
7 and tortoises (Marschang et al., 2009; Papp et al., 2010b). Reptilian PMV are  
8 considered one of the most important pathogens of snakes and have been isolated  
9 from both private and zoological collections (Jacobson, 2007). They have also been  
10 described as emerging infectious diseases which may endanger wildlife (Daszak et  
11 al., 2000; Jacobson, 1993; Azevedo et al., 2001). Clinical signs observed in PMV  
12 infected snakes most commonly involve the respiratory tract, but central nervous  
13 system (CNS) disease is also regularly observed (Jacobson, 2007). Phylogenetic  
14 analysis of partial gene sequences of PMV isolated from snakes but also from lizards  
15 and a tortoise revealed that there are at least two squamatid lineages (groups) both  
16 containing snake and lizard isolates, whereas the sole tortoise PMV isolate from a  
17 Hermann's tortoise (*Testudo hermanni*) clusters as an ancient, separate lineage of  
18 the proposed new reptilian PMV genus "*Ferlavirus*" (Marschang et al., 2009). An  
19 additional study detected a squamatid PMV in a diseased leopard tortoise (Papp et  
20 al., 2010b), demonstrating the wide host range of some of these viruses.

21 Reovirus infections have been reported in several species of reptiles including  
22 snakes (Ahne et al., 1987; Blahak and Göbel, 1991; Blahak, et al., 1995; Jacobson,  
23 1986), lizards (Ahne at al., 1987; Drury et al., 2002; Marschang et al., 2002;  
24 Marschang and Papp, 2010), and tortoises (Drury et al., 2002; Marschang and Chitty,  
25 2004). Clinical signs associated with reovirus infections have been pneumonia and

1 neurologic problems that may appear similar to PMV infections (Wellehan et al.,  
2 2009).

3 The number of reports of adenovirus (AdV) infections in reptiles is increasing,  
4 apparently due to the growing popularity of reptiles as pets (Essbauer and Ahne,  
5 2001) and to sensitive diagnostic methods (Wellehan et al., 2004). AdV infections  
6 have been reported in several reptile species including crocodiles, snakes, and  
7 lizards (Frye et al., 1994; Jacobson and Gardiner, 1990; Kinsel et al., 1997; Ramis et  
8 al., 2000). In these species, adenovirus infection was considered the cause of  
9 gastroenteritis, hepatitis, nephritis, pneumonia and encephalitis (Frye et al., 1994;  
10 Helstab and Bestetti, 1984; Schumacher et al., 1994). The single fully sequenced  
11 reptilian isolate is from a corn snake (*Pantherophis guttatus*) (Farkas et al., 2002;  
12 2008). This snake adenovirus type 1 (SnAdV-1) represents the supposed reptilian  
13 lineage of the *Atadenovirus* genus. Further squamatid AdVs (including new snake  
14 types) have been partially sequenced and found to belong to the same genus  
15 (Wellehan et al., 2004; Garner et al., 2008; Papp et al., 2009a; Benkő et al., 2006;  
16 Péntzes et al., 2010). Interestingly, unlike PMVs, the recently described chelonian  
17 AdVs cluster separate from the squamatid AdVs, either to the supposed amphibian  
18 lineage *Siadenovirus* genus (Rivera et al., 2009) or outside of the five accepted  
19 genera of the family *Adenoviridae* (Farkas et al., 2009; Doszpoly and Harrach,  
20 personal communication; Wellehan et al., personal communication).

21 During routine diagnostic testing for reptilian viruses we found a concurrent ongoing  
22 infection with all three above described viruses in a collection of corn snakes  
23 including a snake that died suddenly showing dispnoe and vomitus prior to exitus.  
24 The survivors showed no specific symptoms. A follow-up study was performed in the  
25 animals from two consecutive collections for over 5 months, before they were finally  
26 dispersed. Detected viruses were partially sequence characterized, and most of the

1 viruses were also isolated on permanent cell lines. The PMV detected in these corn  
2 snakes is new to science, and seems to be the first described representative of a  
3 third major squamamid PMV group.

4

## 5 **Materials and Methods:**

### 6 *Samples*

7 In January 2009, a dead juvenile female corn snake (*Pantherophis guttatus*) (Lab.  
8 No. 6/3/09) was dissected at the Bird and Reptile Clinic, University of Leipzig,  
9 Leipzig, Germany. Its body weight was 4 g and the head-cloaca length 29 cm. Prior  
10 to death, the snake had dyspnoea and vomitus. Gross necropsy showed cachexia.  
11 The stomach was moderately filled with brown pasty content, the gut was empty and  
12 no macroscopic lesions were observed. Histopathologically the only finding was  
13 hyperplasia of melanophagocytes in the liver, while other organs showed advanced  
14 autolysis. According to the clinical symptoms, the hyperplasia of melanophagocytes,  
15 the juvenile age and the history of another death in the snake collection, a viral  
16 disease was suspected. Samples of lung, kidney, and intestine were sent to the  
17 University of Hohenheim, Stuttgart, Germany for virus detection. One month later, 13  
18 young corn snakes (body weight: 7-30 g) remaining in the possession of the same  
19 owners were swabbed (oral and cloacal) and tested (Lab. No. 14/1/09 to 14/13/09).  
20 Later, without further notice the owner dispersed the stock (some were reported to  
21 have died afterwards) and established a new snake collection. At this point - five  
22 months after the first tests - these 10 animals (Lab. No. 54/1/09 to 54/10/09) were  
23 also swabbed and tested. All together 26 clinical diagnostic samples including 3  
24 organs and 23 oral and cloacal swabs from a total of 24 corn snakes of two  
25 consecutive stocks kept at the same enclosure of one owner were sent to our  
26 laboratory and tested for the presence of PMV, AdV, and reoviruses (Table 1).

1 Tissue samples or swabs were immersed in 3 ml Dulbecco's modified Eagle's  
2 medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with antibiotics for  
3 further processing.

4

#### 5 *Virus isolation*

6 Isolation of viruses was attempted from all samples on Russell's viper heart cells  
7 (VH-2, ATCC: CCL-140) and iguana heart cells (IgH2, ATCC, CCL-108). Samples in  
8 DMEM were sonicated for destruction of cells and suspension of viruses. The  
9 samples were then centrifuged at 3000 x g for 10 min. for the removal of cell-debris  
10 and bacteria, then 200 µl of the homogenate was inoculated onto medium-free,  
11 approximately 70% confluent cell monolayers in 30 mm diameter Cellstar® tissue  
12 culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). After incubating  
13 for 2 h (at 28 °C, 5% CO<sub>2</sub>) 2 ml nutrient medium (DMEM supplemented with 2%  
14 foetal calf serum, 1% non-essential amino acids and antibiotics (200 U/ml penicillin-  
15 G, 380 U/ml streptomycin-sulfate, 6.4 U/ml gentamycinsulfate and 0.5 µg/ml  
16 amphotericin-B) were added to each dish. Cells were examined for the presence of  
17 cytopathic effects (CPE) approximately every 3 days with an inverted light  
18 microscope (Leitz, Weitzlar, Germany), dishes were frozen when extended CPE was  
19 seen. Dishes showing no CPE were frozen after 2 weeks of incubation for blind  
20 passaging. Two additional passages were performed from each dish after a freeze  
21 and thaw cycle and low speed centrifugation. RNA and DNA were prepared from the  
22 cell culture supernatant as described below for further identification and  
23 characterization of isolates.

24 In the case of mixed virus isolates, a 10 fold serial dilution of the supernatant was  
25 made. From each dilution step at least four wells of a 96-well FALCON MICROTEST



1 plate (Becton Dickinson Labware, Franklin Lakes, USA) were inoculated in parallel in  
2 order to separate the different viruses (end-point dilution, Reed and Muench, 1938).  
3 To determine whether virus isolates were enveloped, chloroform treatment was  
4 performed by mixing chloroform and virus suspension 1:10, followed by shaking on a  
5 vortex for 1h and centrifugation at 10.000 x g for 10 min. The supernatant was then  
6 diluted 1:10 in maintenance medium and inoculated into tissue culture dishes as  
7 described. Infectious cell culture supernatant without chloroform treatment was  
8 inoculated parallel into tissue culture dishes. Plague purification assay was also  
9 performed, as described by Bijlenga and Duclos (1989) with some modifications.  
10 Nutrient medium (see above) was prepared with an additional 1% of agar-agar  
11 (Merk, Darmstadt, Germany). Tissue culture dishes were infected with the mixed  
12 virus isolate, and after two hours of incubation 2 ml of this semi-solid medium was  
13 added. Dishes were incubated at 28 °C, 5% CO<sub>2</sub> for 3 days, microscopic agar-plaque  
14 samples were collected randomly using pipette tips and placed separately in 500 µl  
15 DMEM supplemented with a 2x concentration of antibiotics. Samples were then  
16 sonicated in an ice bath (Bandelin electronics, Berlin, Germany, 40 KHz) for 5  
17 minutes and re-inoculated onto one day-old tissue culture dishes and incubated for  
18 ten days.

19

20

#### 21 *RNA and DNA extraction.*

22 RNA was prepared from 300 µl of the sonicated clinical samples and virus isolate  
23 supernatants using the guanidinium isothiocyanate method described by Boom et al.  
24 (1990) and resuspended in 75 µl of RNase free water.

25 DNA extraction of the sonicated clinical samples and virus isolate supernatants (both  
26 from 200 µl) was carried out using the DNeasy® Blood and Tissue Kit (Quiagen

1 GmbH, Hilden, Germany) following the protocol of the manufacturer in a final elution  
2 volume of 100 µl.

3

#### 4 *PCR/RT-PCR and sequencing*

5 A nested RT-PCR targeting a portion of the L gene of ophidian PMV was carried out  
6 using the primers described by Ahne et al. (1999) in 25 µl reaction mixtures. The RT-  
7 PCR targeting portion of HN gene was performed as for L gene using primers F1 and  
8 R2 (Ahne et al., 1999) in the first round, and HN-cons Fwd-in and HN-cons Rev-in  
9 (Marschang et al., 2009) in the second round. The RT PCR targeting the complete  
10 ORFs of the U gene was carried out similarly using U-cons Fwd-out and U-cons Rev-  
11 out in the first round, and U-cons Fwd-in and U-cons Rev-in the second round  
12 (Marschang et al., 2009). The chemistry used for all PCRs is described in the latter  
13 paper.

14 For reovirus detection, a nested RT-PCR targeting a portion of the RNA-dependent  
15 RNA polymerase (RDRP) gene was carried out as described by Wellehan et al.,  
16 (2008). The primers 1607F and 2608R were used for the RT and first round PCR  
17 reactions, the second round of PCR amplification was carried out using the primers  
18 2090F and 2200R.

19 Nested AdV PCRs were carried out as described by Papp et al. (2009) using the  
20 primers originally described by Wellehan et al. (2004). The PCR protocol was  
21 optimized for some samples to remove unspecific bands by raising the annealing  
22 temperature to 50 °C and adding a total of 2 mM MgCl<sub>2</sub> in the second round.

23 Products of all PCR protocols were separated on 1.5% agarose gels (Bioenzym,  
24 Oldendorf, Germany) in TAE puffer containing 0.5 mg/ml ethidium-bromide and  
25 visualised under 320 nm UV light. Gel purified PCR amplicons (Invisorb Spin DNA  
26 Extraction Kit; Ivitek GmbH, Berlin, Germany) were sequenced directly using a Big-

1 Dye terminator kit v.1.1 and analysed on an ABI prism 310 automated DNA  
2 sequencer (both Applied Biosystems, Foster City, USA).

3

#### 4 *Analysis of sequences*

5 Raw sequences were processed by the ABI Sequence Analysis Programme 5.1.1  
6 (Applied Biosystems, Foster City, USA) then edited, assembled and compared using  
7 the STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield et  
8 al., 1995). The sequences were compared to data in GenBank (National Center for  
9 Biotechnology Information, Bethesda, USA) online ([www.ncbi.nih.gov](http://www.ncbi.nih.gov)) using  
10 BLASTN and BLASTX options. Multiple alignments of nucleotide sequences were  
11 performed with ClustalW algorithm of the BioEdit Sequence Alignment Editor  
12 programme (Hall, 1999). This alignment was further used for phylogenetic  
13 calculations in the PHYLIP program Package version 3.6. (Felsenstein, 1989)  
14 comparing parsimony, maximum-likelihood, and distance based methods to obtain  
15 an optimal tree. Bootstrap analysis of 100 replicates was carried out.

16

#### 17 **Results**

18 In this study we have investigated 26 organs and oral/cloacal swabs originating from  
19 24 corn snakes of two successive stock of a single owner and were able to detect  
20 PMV by PCR in the 7 snakes of the first stock, and AdVs from 3 snakes both in the  
21 first and second stocks. Reoviruses were successfully isolated from 7 & 2, PMV from  
22 2 & 0 and AdV from 2 & 0 snakes of the first & second stock respectively (Table 1).

23

#### 24 *(RT-)PCR results*

25 PMV L gene nested RT-PCR gave a positive signal (566 bp product) with the kidney  
26 and intestine of the dead animal and six swabs of the surviving snakes from the 1<sup>st</sup>

1 collection (50%) (Table 1), but none from the 2<sup>nd</sup> collection. From the L-gene positive  
2 samples each (50% of group one) were positive in the HN gene nested RT-PCR (399  
3 bp), and all but one (sample “14/8/09”, 42.8% positive of group one) using the U  
4 gene nested RT-PCR (640 bp). The homologous PMV partial gene sequences found  
5 in different samples were identical to one another, and represent a new reptilian PMV  
6 (assigned name: PanGut-GER09) (Fig. 1 and Fig S-1). The PanGut-GER09 partial  
7 gene sequences have been submitted to GenBank and assigned the accession  
8 numbers: HQ148084 to HQ148087.

9 AdVs were detected from the diagnostic samples using consensus nested PCR  
10 (product size: 320 bp) in 6 animals altogether (25%), 3 from the 1<sup>st</sup> group (21.4 %)  
11 (Table 1). The AdV from the kidney and intestine of the dead snake (6/3/09) had a  
12 partial polymerase sequence identical to the GenBank (FJ012163) snake adenovirus  
13 type 2 (SnAdV-2) sequence. Two surviving snakes of this 1<sup>st</sup> collection (14/2/09 &  
14 14/3/09) that were also identified with AdV infection by PCR revealed two different  
15 viruses based on their partial polymerase sequences with 100% and 93% identity  
16 respectively to the SnAdV-1 (DQ106414). This latter, new sequence is identical to  
17 that of a recently reported tiger python (*Python molurus*) AdV isolate (Papp et al.,  
18 2009b; Romanova et al., 2010), a supposed new serotype of the SnAdV-A virus  
19 species, and has been submitted to GenBank with an accession number: HQ148088.  
20 Three animals of the 2<sup>nd</sup> collection (30%) were AdV positive. The sequencing  
21 revealed three different viruses: beside the above mentioned SnAdV-1 (54/4/09) and  
22 SnAV-2 (54/6/09), a third type, identical in its partial polymerase sequence to the  
23 SnAV-3 (FJ012164) was also detected in a snake (54/1/09).

24 Reoviruses were not detected directly from the diagnostic samples using the nested  
25 RT-PCR targeting the RNA dependent RNA polymerase (RDRP).

26

## 1 *Isolation of viruses*

2 Adenoviruses were isolated in two cases (14.3%), once from the intestine of the dead  
3 snake (6/3/09) and from a swab taken from a survivor (14/2/09) in the first collection  
4 (Table 1). Observed cytopathic effects (CPE) with rounding and detachment of some  
5 cells (Fig S-1b) were typical of AdV. Paramyxoviruses were isolated from the kidney  
6 and intestine of the dead animal and from the swab of the same survivor as above.  
7 Typical CPE of giant fusion cells (syncytia) was observed. Reoviruses were isolated  
8 from a total of 9 snakes (37.5%), including all three organs of the dead snake and six  
9 survivors of the first collection, as well as two swabs from the second collection  
10 (Table 1). The cytopathic effect (CPE) caused by the PMV and reoviruses were both  
11 characterized by cytolysis and giant syncytial cell formation. Verification of the  
12 presence of reoviruses in cell culture supernatant by consensus nested RT-PCR  
13 (110 bp) was positive in all cases. All isolated reoviruses had identical partial RDRP  
14 genes sequences to one another and to the reptilian GenBank carpet python (*Morelia*  
15 *spilota*) orthoreovirus isolate 55-02 (GenBank accession No. E4309703).

16 In both cases of AdV isolation, PMV and reoviruses were also present in the isolates.  
17 The PMV isolates are easily overgrown with reovirus, yet the presence of PMV was  
18 verified by L, U, and HN gene RT-PCR amplification of PMV RNA from the cell  
19 culture supernatant. Using the end-point dilution method followed by chloroform  
20 treatment pure AdV was obtained from the 14/2/09 sample. Using an end-point  
21 dilution method followed by a plaque purification assay, a pure PMV isolate was  
22 obtained from the same sample, as verified by verified via (RT-)PCR. The PMV grew  
23 slowly in cell culture, with gradual development of a specific CPE with small syncytial  
24 cell formation (on day 10 post inoculation). In the case of 6/3/09, following chloroform  
25 treatment and end-point dilution of the isolate, the AdV specific CPE was still  
26 obscured by the syncytia of the fusogenic reovirus and it could not be isolated in a

1 pure culture using the methods above. Verification of the presence of AdV in cell  
2 culture was carried out by PCR amplification of AdV specific DNA in cell culture  
3 supernatant. Sequencing of the amplicons verified that there are two different AdV  
4 types (SnAdV-1 and -2) in the two isolates. All of the above listed isolates could be  
5 further passaged on homologous cells.

6

#### 7 *Infection rate*

8 Summarizing the PCR and virus isolation results, the following infection rates were  
9 observed. Three snakes (21.4%) of the 1<sup>st</sup> collection (6/3/09, 14/2/09 & 14/3/09) were  
10 identified with a triple viral infection with reoviruses, PMV, and AdV. One snake  
11 (7.1%) in this collection (14/6/09) had a double infection with a reovirus and PMV,  
12 three snakes (14/5/09, 14/10/09 & 14/13/09) had single infections with a reovirus and  
13 three further ones (14/8/09, 14/11/09 & 14/12/09) had a single infection with PMV.  
14 The remaining four (28.6%) of the 14 tested animals of the 1<sup>st</sup> collection were  
15 negative in all virus detection tests (Table 1). In the case of the 2<sup>nd</sup> collection one  
16 snake (54/4/09) (10%) had a double infection with a reovirus and AdV, two snakes  
17 (54/1/09 & 54/6/09) (20%) were infected with only AdV and one (54/3/09) with only  
18 reovirus. An additional six (60%) animals from this group were tested and found  
19 negative in the virus diagnostic tests.

20

#### 21 *Sequence analysis of new reptilian PMV*

22 L, HN, and U gene partial sequence data revealed a novel PMV type (PanGut-  
23 GER09) in seven animals of the first snake collection. This virus is nearly equally  
24 distant from the previously described "A" and "B" squamatid (snake and lizard) rPMV  
25 groups (Marshang et al., 2009). Nucleotide identity values for the new partial L gene  
26 sequence compared to group "A" member sequences are 76.5-80.3% and 80.5-

1 81.2% compared to group “B” ones. The identity value compared to the single  
2 tortoise isolate is 73.8%.

3 For the HN gene, these values are similar: 78.2-80% (“A”) and 79.9-80.5% (“B”) and  
4 somewhat lower for the U gene (including non-coding regions): 72.7-75.4% (“A”) and  
5 69.7-70% (“B”). Phylogenetic calculations (Fig. 1) based on the three partial gene  
6 sequences show that all rPMV form a single monophyletic cluster, representing the  
7 proposed new “*Ferlavirus*” genus. Within this genus the earlier described tortoise  
8 isolate and the “A” and “B” squamatid PMVs form separate groups. The novel  
9 PanGut-GER09 virus clusters with moderate bootstrap values either to the “B” group  
10 (L gene, Fig. 1a), or to group “A” (HN & U gene, Fig. 1bc), as a sister taxon.  
11 Alignments are shown in Figures S1-2.

12 In the transcriptional analysis of the squamatid (or reptile) PMV specific gene of  
13 unknown function (U gene) both earlier described consensus reading frames (ORF-1  
14 & -2) (Kurath et al., 2004; Marschang et al., 2009) were identified in the new isolate  
15 (Fig S1). The putative protein coded by ORF-1 has a predicted length of 82 aa  
16 (suppl.: Fig. S2c), whereas ORF-2 encodes a 144 aa protein (suppl: Fig. 2d), 1 or 2  
17 aa longer than those found in rPMV of group B or A respectively. Nucleotide identity  
18 values of the overlapping presumed coding regions of the U gene were comparable  
19 to those seen in the L and HN gene partial CDS. Yet unlike the latter two genes, the  
20 U gene putative ORFs of the new isolate had a distinctly higher similarity to the group  
21 “A” viruses than to group “B” ones: 85.9-86.7% (“A”) / 77.1-77.9% (“B”) for ORF1 or  
22 81.9-83.5% (“A”) / 78.4-78.6% (“B”) for ORF-2. Additionally, repeated sequencing  
23 verified that there is a single nucleotide variation at position 383 (Fig. S1/c) of the U  
24 gene sequence, resembling four (6/3/09, 14/2/09, 14/3/09, 14/11/09) and two  
25 (14/6/09, 14/12/09) samples respectively of the detected six cases, and coding a  
26 silent mutation of ORF-2.

1

2 **Discussion**

3 The purchase of new reptiles is associated with a particularly high risk of introducing  
4 disease into a collection. Viral infections such as inclusion body disease (IBD) and  
5 PMV often lead to devastating disease when they are transmitted to healthy snakes  
6 (Pasmans et al., 2008). Some studies demonstrate that there is a significant risk of  
7 hidden infectious diseases including PMV, inclusion body disease (IBD) and some  
8 bacterial infections in apparently healthy snake collections (Pees et al., 2010). Basic  
9 veterinary health checks and quarantine measures are of great importance to  
10 minimize the risk of infection among newly established snake groups.

11 In the current study, multiple viral infections were detected in captive corn snakes.  
12 The pathogenic role of the concurrent viral infection in the present case could not be  
13 fully clarified as only limited data were made available, and the owner dispersed the  
14 population without further notice soon after our second positive tests. We presume  
15 that mixing of previously untested animals of uncertain origins to form a new  
16 breeding population might be the source of infection in the 1<sup>st</sup> collection of this study,  
17 while in the case of the 2<sup>nd</sup> collection after dispersing the 1<sup>st</sup> snake group,  
18 inappropriate disinfection of the terrariums could also be a factor for transmitting viral  
19 infection into the newly introduced animals, as the same types of non-enveloped  
20 viruses (reo-, AdV) were detected in these animals as before, whereas the  
21 environmentally less stable PMV was no longer present. Detecting a further type of  
22 virus (SnAdV-3) in the 2<sup>nd</sup> collection might be due to previous infection in one of the  
23 new animals, leading to the introduction of this type into the newly formed collection  
24 as a result of a lack of screening and entry examinations. In addition to quarantine  
25 and disinfection procedures, screening and entry examinations are also of great



1 importance for detecting the prevalence of such infectious diseases (Pees et al.,  
2 2010).

3 Viral polymerases are often highly conserved, as they are essential for viral function.  
4 They are therefore often good targets for viral diagnostic assays. In the present  
5 paper we have used earlier described nested (RT-) PCRs targeting the polymerase  
6 genes to detect infection with reoviruses, PMV, and AdV (Wellehan et al., 2008;  
7 Ahne et al., 1999; Wellehan et al., 2004) from snake tissue samples and swabs. We  
8 have also used classical virus isolation method on permanent reptilian cell lines, and  
9 two further PCRs for the characterization of PMV (Marschang et al., 2009).  
10 Considering the results and in consensus with earlier papers (Marschang et al.,  
11 2009; Papp et al., 2010a) we can state that the nested RT-PCR targeting the L-gene  
12 (Ahne et al., 1999) is the most sensitive method currently available and the  
13 recommended procedure for basic diagnostic testing for PMV detection. A nested  
14 PCR targeting the polymerase gene (Wellehan et al., 2004) is also the most sensitive  
15 assay to date for the detection of AdV. However, in the case of reoviruses, the  
16 consensus nested RT-PCR targeting the RDRP gene (Wellehan et al., 2008) is less  
17 sensitive than the virus isolation method (Table 1, see also Marschang and Papp,  
18 2010), although it provides a useful tool for the initial characterization of these  
19 viruses. All reoviruses detected in the current study using the RT-PCR were from  
20 RNA extracts of cell culture isolates. No reoviruses were detected in original samples  
21 prior to isolation on cell culture. Optimization trials along with designing new primers  
22 might be necessary to increase the efficiency of this RT-PCR protocol.

23 Reoviruses are commonly described in reptiles, mostly in snakes (Jacobson, 2007).  
24 Previous studies have shown that reoviruses isolated from reptiles can differ from  
25 one another both serologically and molecularly (Blahak and others 1995, Wellehan et  
26 al., 2009; Pees et al., 2010), however, in the present study all isolated reoviruses

1 from nine snakes (including tissues of a dead one, seven survivors of the 1<sup>st</sup>  
2 collection and two snakes in the 2<sup>nd</sup> collection, Table 1) were identical to one another  
3 and 100% identical to the reptilian orthoreovirus isolate 55-02 (E4309703),  
4 suggesting a single source for the infection.

5 The AdV sequences detected in six snakes from the two collections, on the other  
6 hand, could be divided into four distinct types, representing the previously described  
7 SnAdV-1, -2 and -3 types (Farkas et al., 2002; 2008; Garner et al., 2008) and an  
8 additional SnAdV-1-like (93% id.) virus of the same species: SnAdV-A (Papp et al.,  
9 2009b). This novel virus type is to be better characterized in a separate publication  
10 together with similar novel types (Romanova et al., 2011). This finding is of great  
11 interest as it contributes new aspects to the postulated coevolution theory of  
12 adenoviruses (Harrach, 2000; Davison et al., 2003). The present paper shows that  
13 the same corn snake species (*Pantherophis guttatus*, Colubridae) can be infected  
14 with 4 different atadenovirus types (3 AdV species) that were earlier detected in other  
15 colubrid, boid, pythonid or viperid snake species.

16 Despite the numerous detections of reptilian AdV by electron microscopy and PCR,  
17 there are very few reported cases in which the virus was successfully isolated  
18 (Juhász and Ahne, 1992; Ogawa et al., 1992; Papp et al., 2009a). Partial sequence  
19 data of the different snake isolates suggest that these are the same type: SnAdV-1,  
20 which was also isolated in this study. However, this study is the first report on the  
21 isolation of the proposed type 2 snake AdV in cell culture (6/3/09 intestine, mixed  
22 with reo- & PMV). This snake AdV type 2 has been identified previously in the USA  
23 (Garner et al., 2008), Germany (Papp et al., 2009a) and Hungary (Farkas,  
24 unpublished data) by PCR. Continuous efforts are being made to obtain a pure  
25 culture of this AdV.

1 PMV infections have been involved in high mortality diseases in snake collections  
2 (Fölsch and Leloup, 1976; Jacobson, 1986). A recent publication reports concurrent  
3 infection with several distinct PMV in a single snake or group of snakes (Papp et al.,  
4 2010a) during one outbreak, yet all earlier papers (Ahne et al., 1999; Franke et al.,  
5 2001) identified the same PMV in animals of the same population or outbreak. In the  
6 present study, sequencing of three gene portions revealed a single novel unique  
7 rPMV in seven (one dead and six living) snakes from the 1<sup>st</sup> collection, which could  
8 be isolated first in a mixed, then in pure culture.

9 The first molecular based (L and HN gene) classification of reptilian PMV by Ahne  
10 and co-authors (1999) described two distinct “subgroups” with very low inner  
11 variance, called “a” and “b” and a set of more variable intermediate isolates. Later,  
12 Franke and co-authors (2001) described three clusters (A, B and C) on their L gene  
13 phylogenetic tree. Unfortunately, this latter publication did not include sequences  
14 from the earlier paper in the comparison, thus the denominations of their clusters do  
15 not refer to those of the subgroups (“cluster A” corresponded to the earlier “subgroup  
16 b”, “cluster B” to the “intermediate isolates”, and “cluster C” to the earlier “subgroup  
17 a”). This discrepancy in the names was resolved in recent publications by our  
18 research group (Marschang et al., 2009). Considering the seniority rule in  
19 nomenclature, and the high bootstrap monophyletic clusters of the L, HN and U gene  
20 trees, a revised grouping for the squamamid rPMV was suggested: A “sensu lato”  
21 group “A” contained the previous “subgroup a” and the old and new “intermediate  
22 isolates”, while the “sensu lato” group “B” those from “subgroup b” and similar  
23 squamamid PMVs. The use of “C” as a denomination for any group was omitted. A  
24 single tortoise isolate was suggested as the first representative of a separate  
25 chelonid PMV group, clustering with the two squamamid PMV groups into the  
26 proposed “*Ferlavirus*” genus (Kurath et al., 2004; Marschang et al., 2009).

1 The results of a later publication (Papp et al., 2010a) characterizing 38 samples from  
2 25 snakes, foreshadowed that all rPMV found in captive snake collections in  
3 Germany belong to one of the two squamatid PMV groups. The current study based  
4 on partial L, HN and U gene sequences first describes a representative of a novel  
5 third squamatid PMV cluster: “group C”, equally distant from the other two squamatid  
6 groups, within the proposed genus “*Ferlavirus*” genus (Fig. 1).  
7 These interesting new findings underline the significance of sequencing in routine  
8 reptilian virus diagnostics. It also shows the importance of testing for several different  
9 pathogens. Although each of the viruses detected in these snakes has been reported  
10 in connection with disease, the effect of combined infections and the prevalence of  
11 such combined infections is unknown. Concentrating on a single pathogen (e.g. PMV  
12 in snakes) can lead to underestimation of the importance of other infectious agents.  
13 The case report also emphasizes the importance of disinfection together with entry  
14 assessment prior to establishing new snake collections or introducing new animals  
15 into formerly established groups.

16

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1 **Table legend**

2

3 **Table 1. Virus positive corn snake samples.**

4 Abbreviations: AdV = adenovirus, o/cl = oral & cloacal, PMV = paramyxovirus, Sn =  
5 snake.

6 \*Virus isolation results summarized from VH2 and IgH2 cells, viruses were  
7 characterized based on the type of cytopathic effect (CPE), resistance to chloroform,  
8 and (RT-)PCR and sequence verification.

9 #For reoviruses, a 110 nt long portion of the RNA-dependent RNA polymerase  
10 (RDRP) gene was amplified and used for the sequence comparison (Wellehan et al.,  
11 2008). For AdV, a 318 nt long portion of the DNA dependent DNA polymerase gene  
12 was amplified and compared (Wellehan et al., 2004). For PMV, a 566 nt long portion  
13 of the the RDRP gene (L-gene) was amplified in the diagnostic PCR (Ahne et al.  
14 1999). That sequence, together with a 537 nt & a 648 nt long portion of two further  
15 genes (the hemagglutinin-neuraminidase (HN-gene) & the unique (U-gene)) were  
16 used for the seuquence comparison (Marschang et al., 2009). (All sizes are for the  
17 unedited PCR products.)

18 GenBank accession numbers: orthoreo isolate 55-02 (E4309703), snake AdV type 1  
19 (DQ106414), SnAdV-1 like new type from 14/3/09 swab (HQ148088), snake AdV  
20 type 2 (FJ 012163),snake AdV type 3 (FJ 012164). Paramyxovirus PanGut-GER09  
21 portions of L-, HN- & U-genes (HQ148084 to HQ148087).

22

23

24

1 **Figure legends**

2

3 **Fig. 1 (a, b & c). Phylogenetic distance trees of the reptilian PMV.**

4 Sequences were analyzed using the PHYLIP program package (DNA distance  
5 followed by FITCH). Bootstrap values over 60 from 100 resamplings of the FITCH  
6 are indicated beside the nodes. Branches with lower values are drawn with  
7 checkerboard lines.

8 Corresponding sequences (GenBank accession No.) from Newcastle disease virus  
9 /NDV/ (AF375823), Sendai (NC\_001552), and Human parainfluenza type 1 /HPIV-1/  
10 (AF457102) were used as outgroups. All other viruses on the trees are of reptilian  
11 origin, mainly from snakes. Lizard and tortoise isolates are printed in italics and  
12 marked with one or two crosses respectively. The novel PMV isolate '**Pangut Ger**  
13 **09**' is marked in bold.

14

15

16 **(a)** Phylogenetic distance tree, based on 443 bp of the large RNA-dependent RNA  
17 polymerase (L) gene. GenBank accession numbers for the reptilian PMV: Bush viper  
18 virus /ATCC-VR-1409/ (AF286043), CeraCe-98 (AF351137), Crot-GER03  
19 (GQ277611), CrotX1-96 (AF349405), Dasy-GER00 (GQ277613), ElaGut-91  
20 (AF349408), Fer de Lance virus (NC\_005084), GonoGER-85 (AF349404), *Igu-*  
21 *GER00*<sup>+</sup> (GQ277617), Neotropical rattlesnake virus /ATCC-VR-1408/ (AF286045),  
22 Orth-GER05 (GQ277616), Pyth-GER01 (GQ277612), *TORTOISE-GER99*<sup>++</sup>  
23 (GQ277615), *Xeno-USA99*<sup>+</sup> (GQ277614). For diagnostic non-isolated rPMV samples  
24 (Papp et al., 2010a): Pyt-2 kidney, Col-2c kidney, Pyt-6 intestine (GU393344 to  
25 GU393348). The following sequences (Ahne et al., 1999) were kindly provided by Dr.  
26 Gael Kurath and Dr. William N. Batts: Boa-CA98, Biti-CA98, Biti-GER 87, *Call-*

1 *GER88*<sup>+</sup>, *Crot1-VA95*, *Crot2-OH90*, *Crot3-CA98*, *Ela1-GER94*, *Ela2-GER93*, *Ela-*  
2 *FL93*, *Lamp-MD96*, *More-GER86*, *Pyth-GER88*, *Trim-MD97*.

3

4 **(b)** Phylogenetic distance tree based on 351 bp of the hemagglutinin-neuraminidase  
5 (HN) gene. The maximum likelihood analysis resulted in the same topology tree as  
6 the FITCH analysis, bootstrap values from both are indicated on the branches.

7 GenBank accession numbers: *Bush viper virus /ATCC-VR-1409/ (AF286044)*, *Dasy-*  
8 *GER00 (GQ277618)*, *Fer de Lance virus (NC\_005084)*, *Igu-GER00*<sup>+</sup> (*GQ277619*),  
9 *NTV /ATCC-VR-1408/ (AF286046)*, *Orth-GER05 (GQ277620)*, *Xeno-USA99*<sup>+</sup>  
10 (*GQ277621*). The other reptilian sequences (Ahne et al., 1999) were kindly provided  
11 by Dr. Gael Kurath and Dr. William N. Batts.

12

13 **(c)** Phylogenetic distance tree of the reptilian PMV based on 568 bp of the unique (U)  
14 gene. Please note that no homologous sequence from non-reptilian PMV could be  
15 applied as an outgroup. The maximum likelihood analysis resulted in the same  
16 topology tree as the FITCH analysis, bootstrap values are both indicated on the  
17 branches. "Variant 1 and 2" represent the single nucleotide sequence variation of  
18 "Pangut Ger09" U gene sequence found in samples: 6/3/09, 14/2/09, 14/3/09,  
19 14/11/09 and samples 14/6/09, 14/12/09 respectively.

20

21 GenBank accession numbers: *Biti-CA98 (AY534645)*, *Crot-GER03 (GQ277625)*,  
22 *Dasy-GER00 (GQ277627)*, *Fer de Lance virus (NC\_005084)*, *Gono-GER85*  
23 (*AY534644*), *Igu-GER00*<sup>+</sup> (*GQ277623*), *Orth-GER05 (GQ277622)*, *Pyth-GER01*  
24 (*GQ277624*), *Xeno-USA99*<sup>+</sup> (*GQ277626*)

25

26

Table 1.

	Lab. No.	Case history (& arrival date)	Samples tested	(RT-)PCR results of field samples <sup>#</sup>			Virus isolation*	Nucleotide identity of partial gene sequences <sup>#</sup>	Comment
				Reo	PMV	AdV			
G r o u p  O N E	6/3/09	A snake died in a new collection. (20-Jan-2009)	lung kidney intestine	- - -	- + +	- + +	Reo Reo, PMV Reo, PMV, AdV	Reo: 100% to <b>orthoreo 55-02</b> PMV: new type <b>PanGut-GER09</b> AdV: 100% to <b>SnAdV-2</b>	
	14/2/09	Swabs taken one month later from 13 survivors of the same group. (26-Feb-2009)	o/cl. swab	-	+	+	Reo, PMV, AdV	AdV is 100% ident. to <b>SnAdV-1</b> AdV is 93 % identical to <b>SnAdV-1</b>	The other four snakes: 14/1, 14/4, 14/7 & 14/9 were tested negative for the presence of viruses.
	14/3/09		o/cl. swab	-	+	+	Reo		
	14/5/09		o/cl. swab	-	-	-	Reo		
	14/6/09		o/cl. swab	-	+	-	Reo		
	14/8/09		o/cl. swab	-	+	-	-		
	14/10/09		o/cl. swab	-	-	-	Reo		
	14/11/09		o/cl. swab	-	+	-	-		
14/12/09	o/cl. swab		-	+	-	-			
14/13/09	o/cl. swab	-	-	-	Reo	All had the same reo, 100% identical to <b>orthoreo isol. 55-02</b>			
T W O	54/1/09	1 <sup>st</sup> group dispersed & new group established. 10 animals tested. (10-Jul-2009)	o/cl. swab	-	-	+	-	AdV: 100% to <b>SnAdV-3</b>	The other six snakes: 54/2, 54/5 & 54/7 to 54/10 were negative in virus detection tests.
	54/3/09		o/cl. swab	-	-	-	Reo	Reo: 100% to <b>orthoreo 55-02</b>	
	54/4/09		o/cl. swab	-	-	+	Reo	AdV: 100% to <b>SnAdV-1</b>	
	54/6/09		o/cl. swab	-	-	+	-	AdV: 100% to <b>SnAdV-2</b>	

Fig. 1/a

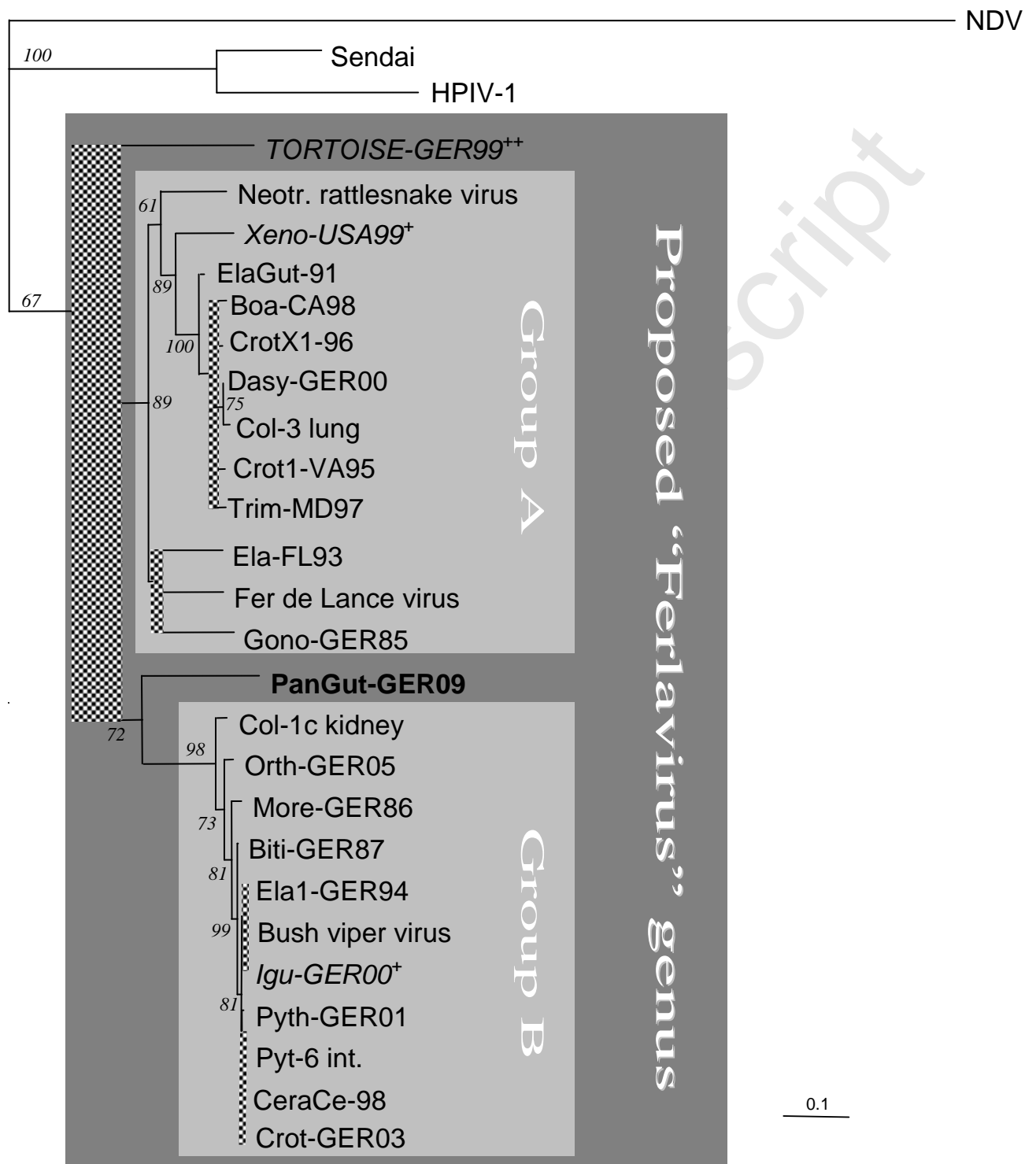


Fig. 1/b

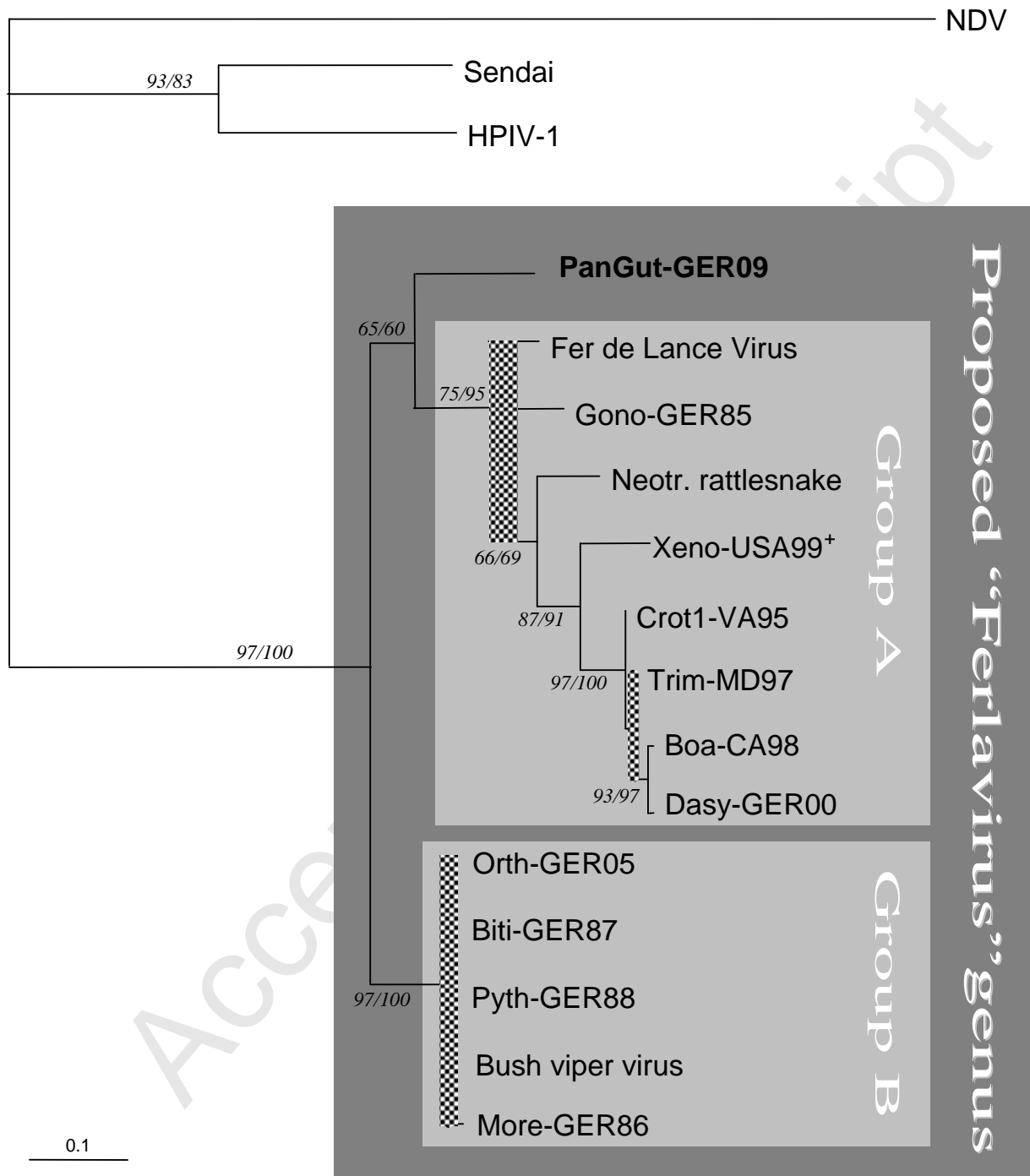


Fig. 1/c

