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Diagnosis of ferlaviruses in snakes and characterization of isolates based on gene sequences

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- Papp, T., Farkas, L.S., **Abbas, M.D.**, Apfelbacher, N., Seybold, J., Erdélyi, K., Marschang, R.E. 2011: Paramyxovirus infections in reptiles, novel findings of the last years. Magy. Állatorvosok (in Hungarian), 133, 551-561. IF: 0.300

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- Marschang, R.E., **Abbas, M.D**., Pees, M., Schmidt, V., Seybold, J., Frost, J.W., Blahak, S., Papp, T. 2009: Detection and partial characterization of paramyxoviruses from reptiles. Proceedings ESVV 8th International Congress of Veterinary Virology, Budapest, Hungary, 23-26. August, 2009.

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Abbreviations

aa	amino acid(s)
AB	antibiotics
bp	bais pair(s)
CDS	coding regions
CPE	cytopathic effects
CS	cleavage site
C-terminus	carboxyl terminal end
DEPC	diethylpyrocarbonate
DMEM	dulbecco´s modified Eagle medium
dNTPs	deoxyribonucleotide triphosphate mix
EDTA	ethylenediamine-tetraaceticacid
ER	endoplasmic reticulum
F	fusion
FBS	fetal bovine serum
FDLV	fer de Lance Virus
FP	fusion peptide
G	glycoprotein
HA	haemagglutinin
HI	haemagglutination inhibition
HN	haemagglutin neuraminidase
HR	heptad repeat
ICTV	international Committee on Taxonomy of Viruses
IgH2	iguana heart cells
K	lysine
kDa	kilodalton
L	large protein
LB	lysis buffer
LZ	leucine zipper motif
M	matrix protein
ME	minimum evolution method

Milli-Q® water	millipore water
ML	maximum likelihood method
MP	maximum parsimony method
N	nucleocapsid protein
NC	negative control
NEA	non essential amino acids
N-G	glycosylation site
N-terminus	amino terminal end
nt	nucleotide(s)
NTV	neotropical virus
ORF	open reading fram
P	phosphorus protein
PC	positive control
PCR	polymerase chain reaction
R	arginine
RBCs	red blood cells
RdRP	RNA dependent RNA-Polymerase
RT-PCR	reverse-transcription followed by PCR
S	serine
SnAdV1	snake adeno virus type 1
SP	signal peptide
ss	single strand
SV5	simian virus type 5
TM	tansmembrane domain
U	unknown gene
UPGMA	unweighted pair group method with arithmetic mean
VH2	viper heart cells
WB	washing buffer

1 Introduction

The first paramyxovirus (PMV) outbreak in reptiles was documented in Switzerland in 1976, where a farm of Fer-de-lance snakes (*Bothrops atrox*) suffered from respiratory disease and death. The virus isolated during that outbreak was Fer de Lance Virus (FDLV), named after the species of snake affected. Clark et al. (1979) isolated this virus in cell culture and consequently several reports documented the occurrence of PMV infections in other snake families.

Like all other PMV, ferlaviruses are enveloped and form envelope glycolproteins fusion (F) and haemagglutin neuraminidase (HN) which have an essential role in the viral immunogenicity and pathogenecity. These glycoproteins play multiple roles in virus entry and the exit from its host.

Kurath et al. (2004) successfully sequenced the full genome of FDLV and detected a unique gene named unknown gene (U) which is absent in all other genera of *Paramyxovirinae*. Based on phylogenetic analyses and the unique genome characterization, Kurath (2009) proposed the classification of this new group of viruses into a novel genus *Ferlavirus* within the subfamily *Paramyxovirinae* with FDLV as the type species.

Ferlaviruses are important disease causing agents of reptiles including snakes, tortoises, and lizards. Earlier publications (Ahne et al., 1999; Franke et al., 2001) have characterized many PMV isolates and displayed inconsistent clustering of members of the genus *Ferlavirus*. In these publications, two to three distinct genetic variants were detected respectively, while several isolates clustered as “intermediate isolates”. A later study by Marschang et al. (2009) resolved this inconsistency by suggesting a revised grouping; sensu lato subgroups A and B. Recent studies by Abbas et al. (2011) and Papp et al. (2013) based on partial sequences of L, HN, and U genes, detected the first two representatives of a third subgroup C within the *Ferlavirus* genus.

The objectives of this study were to establish standard diagnostic methods for the detection of ferlaviruses in several snake families including Boidae, Pythonidae, Colubridae, and Viperidae, evaluate the nested RT-PCR method described by Ahne et al. (1999) targeting a partial sequence of the L gene and its efficiency as a standard diagnostic method, characterize novel types of viruses and increase our knowledge of their phylogenetic relationships and host specificity.

Several methods were used in order to optimize the sensitivity and specificity of the detection methods for ferlaviruses. Virus characterization and comparison were carried out based on partial sequences of L, HN, and U genes. In addition, the complete sequences of the surface glycoprotein encoding genes of nine ferlaviruses isolated from six snakes, two lizards, and a tortoise were amplified and compared. In addition, the complete set of the sequence motifs of F and HN genes were compared to detect the relation and the degree of similarity among genetically different ferlavirus isolates.

2 Literature review

2.1 Taxonomy of Paramyxoviruses (PMV)

The order *Mononegavirales* includes four families: *Bornaviridae, Filoviridae, Paramyxoviridae,* and *Rhabdoviridae* classified based on several criteria including genome size, virion morphology, replication site, host range, pathogenic potential and growth in cell culture (Easton and Ring, 2010) besides some other properties including genome organization and phylogenetic analyses (Wang et al., 2011). The common aspect which unites these enveloped viruses is the presence of a negative sense, single strand (ss) RNA genome in a helical nucleocapsid. This order of viruses comprises the agents of a wide range of diseases affecting humans, animals, and plants (Easton and Ring, 2010).

The family *Paramyxoviridae* has been classified into two subfamilies: the *Paramyxovirinae* which is currently divided into seven genera; *Ferlavirus*, *Aquaparamyxovirus*, *Rubulavirus*, *Avulavirus*, *Morbillivirus*, *Respirovirus*, and *Henipavirus* and the *Pneumovirinae* which is divided into *Pneumovirus* and *Metapneumovirus* (ICTV, 2011)[1] based on the biological activities of the proteins, the organization of the genome, morphologic criteria and the sequence relatedness of the encoded proteins (Lamb and Kolakofsky, 2001).

The *Paramyxoviridae* include a number of well-known disease-causing viruses that occur only in vertebrates. This family includes measles virus, respiratory syncytial virus, parainfluenza viruses, mumps virus, Newcastle disease virus, rinderpest virus, Hendra virus, and Nipah virus (Lamb and Kolakofsky, 2001; Wang et al., 2011) (Table 1.1).

[1] http://www.ictvonline.org/virusTaxonomy.asp?version=2011&bhcp=1

Table 1.1: list of genera of the family *Paramyxoviridae*

Genera of the family Paramyxoviridae, type species, total genome length, host range (Wang et al., 2011; Lamb & Jardetzky, 2007; Nylund et al., 2008). Abbreviations are as follows: HPIV-1: Human parainfluenza virus type 1, PIV-5: Parainfluenza virus type 5, SV-41: Simian virus 41, APMV-6: Avian paramyxovirus type 6, NDV: New castle disease virus, nts: Nucleotides.

	Genera	**Type species**	**Total length in (nts)**	**Haemagglutinin/ Neuraminidase**	**Host range**	**Examples**
Paramyxovirinae	*Rubulavirus*	*Mumps virus*	15,384	haemagglutinin /neuraminidase	humans, canines, monkeys, swine	PIV-5, SV-41
	Respirovirus	*Sendai virus*	15,348	haemagglutinin /neuraminidase	rodents, bovines, humans	HPIV-1
	Avulavirus	*NDV*	15,186	haemagglutinin/ neuraminidase	avians	APMV-6
	Henipavirus	*Hendra virus*	18,234	none	bats, swine, horses, humans	Nipah virus
	Morbillivirus	*Measles virus*	15,894	haemagglutinin	canines, bovines, humans, phocine, cetacean	Canine distemper virus
	Aquaparamyxovirus	*Atlantic salmon PMV*	16,965	haemagglutinin/ neuraminidase	fish	Pacific salmon PMV
	Ferlavirus	*FDLV*	15,378	haemagglutinin/ neuraminidase	reptiles	Gono GER95
Pneumovirinae	*Pneumovirus*	*Human respiratory syncytial virus*	15,222	hemagglutinin was reported in pneumonia virus of mice (PVM)	bovines, humans	Bovine respiratory syncytial virus
	Metapneumovirus	*Human metapneumovirus*	13,335	none	humans	Avian metapneumovirus

2.2 Structure of Paramyxoviruses

Paramyxoviridae are enveloped viruses 150 to 350 nm in diameter, spherical, although they can have pleiomorphic or filamentous forms (Lamb and Kolakofsky, 2001; Jacobson, 2007). Surface glycoproteins fusion (F) and attachment proteins: haemagglutinin neuraminidase (HN), haemagglutinin (HA) or glycoprotein (G) usually form spikes that are inserted into the lipid bilayer envelope and extend about 8 to 12 nm from the surface of the envelope. Underneath the envelope is another membrane associated matrix (M) protein. This protein is non-glycosylated and functions to stabilize the virion structure and associates with the inner face of the envelope (Fig 1). The viral nucleocapsid has helical symmetry and contains non infectious, negative sense single-stranded RNA genome and nucleocapsid associated proteins (Wang et al., 2011; Lamb and Kolakofsky, 2001).

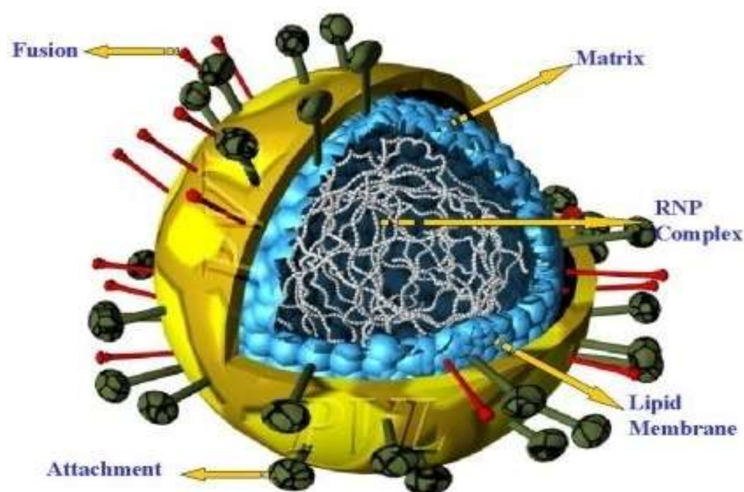


Figure 1: 3D image of of Pneumovirus reconstruction

Source: University of Warwick, Pneumovirus Laboratory, UK. (http://biowiki.org/view/Fall09/FinalProject *Paramyxoviridae*, 07.06.12)

The nucleocapsid protein (N) protects the RNA genome from digestion by nucleases. This protein together with the large polymerase protein (L) and phosphoproteins (P) form a complex that has RNA-dependent RNA transcriptase activity (Lamb and Kolakofsky, 2001).

The *Paramyxoviridae* genome varies in length among PMV genera from 13335 in *Metapneumovirus (Human Metapneumovirus*) to 18234 bp in *Nipahvirus (Hendra virus*) (Wang et al., 2011). Common in the genomes of members of *Paramyxovirinae* are six to ten transcriptional units listed in the order 3' N-P-M-F-HN-L 5'. N, P, M, F, and L are indicated as above. There is also diversity in genome length in PMV due to an unusual hallmark for some PMV (rule of six) where the N protein binds six nucleotides of RNA, therefore the genome length of these viruses are multiples of six nucleotides (nts). Nevertheless, most viruses in the order *Mononigavirales* do not fit to this rule (Easton and Ring, 2010).

Common in members of *Paramyxovirinae* are “accessory” proteins, which are encoded generally by alternative transcriptional units, Open Reading Frames (ORFs) that overlap within the P gene (Lamb and Kolakofsky, 2001). During mRNA synthesis, glycine residue(s) are added into its editing site in a planned pattern which will in turn shift the translational reading frame to an alternative reading frame(s). This will in turn lead to the translation of new proteins as (P, V, W, Y1, Y2) (Lamb and Kolakofsky, 2001). These proteins play a role in the inhibition of interferon induction and signaling which in turn activate transcription (Easton and Ring 2010). Moreover, Lamb and Kolakofsky (2001) addressed the role of these proteins in organizing viral RNA synthesis.

2.3 Replication strategy of paramyxoviruses

Members of the *Paramyxoviridae* typically attach to receptors on the plasma membrane of cells and fuse directly with the plasma membrane. These two actions seem to be mediated by HN, HA or G for attachment and the F protein for fusion (Easton and Ring, 2010).

In the case of PMVs, e.g New castle disease virus (NDV), attachment of HN protein to the sialic acid binding sites on the plasma membrane of susceptible cells catalyze

the fusion protein into its fusogenic state through an association between HN and F proteins (Morrison, 2003).

Subsequent to this reorganization, the amino terminal end (N-terminus) of the F protein is able to interact with the cytoplasmic membrane of the target cell, while the carboxyl terminal end (C-terminus) of the same protein is anchored in the viral envelope. Further conformational changes of the F protein bring its two terminal sides in to closer proximity and that in turn brings both the target and the viral membrane closer and leads to membrane fusion (Lamb & Jardetzky, 2007; Morrison, 2003).

The viral envelope of PMV generally merges with the plasma membrane at neutral pH. Infected cells expressing viral glycoproteins can merge with neighboring cells causing syncytia formation, a feature of PMV infection (Klenk and Garten, 1995). Fusion of the viral envelope with cellular membranes results in the discharge of viral nucleocapsids into the cytoplasm where replication occurs. The positive-sense antigenome RNA is subsequently used as the template for the replication of the viral RNA.

Transcription of mRNA starts at the 3' end of the template and is catalyzed by the RNA polymerase at the first transcription initiation signal sequence located next to the genome promoter and requires the N, P, and L proteins in addition to the genome as a template (Easton and Ring, 2010).

Upon transcribing the gene, the polymerase adds a series of uridine residues at the 5' end of the gene subsequent to the transcription termination codon and ceases transcription. Due to the presence of the termination signal and the short non-transcribed intergenic region between gene start and gene stop, transcription produces mRNA in a chronological mode. The reinitiation of this procedure occasionally fails several times. Therefore, the transcription is arranged in a way to produce a gradient of transcription proteins with those genes closer to the promoter at the 3' end being expressed at higher levels than those located further away. Thus, L protein, coded at the 5' end of the genome, reaches the lowest level concentration, whereas the N protein, coded at the 3' end of the genome reaches the highest concentration. The new positive sense strand created is used further as a template

for new negative-sense genomes which can be assembled into new virus particles (Easton and Ring, 2010).

Post translation, surface glycoproteins of viruses traverse a secretory pathway through the rough endoplasmic reticulum (ER) in a process called translocation. This process is directed by a short sequence known as signal peptide (SP) located at the N- terminus of the proteins (Hunter, 2001). These proteins undergo post translation modifications in the ER and Golgi apparatus and are subsequently transported to the cytoplasmic membrane of the infected cell, where assembly takes place (Easton and Ring, 2010).

Several steps of virus assembly are mediated by matrix proteins starting from adherence of these proteins via their hydrophobic domains to regions of the cytoplasmic membrane, recruitment of nucleocapsids to these sites and reorganization of the nucleocapsids into structures with helical symmetry. Progeny virions are then set free by budding at the plasma membrane (Easton and Ring, 2010).

2.4 Viral proteins

Members of the *Paramyxovirinae* encode 7-10 structural proteins (5-250 kilodalton (kDa)) in addition to 2-4 proteins encoded by additional genes which are encoded by overlapping ORFs of the P gene (Wang et al., 2011; Lamb and Kolakofsky, 2001). In general, viral proteins of all *Paramyxovirinae* genera are nucleocapsid associated proteins including: L, N, and P proteins and membrane associated proteins including: F, Attachment and M proteins. Among the genera of the subfamily *Paramyxovirinae* L, N, and M showed the highest sequence relation between the related proteins, while surface proteins are less conserved (Wang et al., 2011).

The N protein (43-58 kDa) coats the genomic RNAs forming the helical structure and is essential for transcription and replication. The P protein (27-72 kDa) is phosphorylated and required for transcription and replication. The M protein, which is the major structural component of the PMV virion is important for particle morphogenesis and is considered the key determinant of virus assembly and budding in viruses of the family *Paramyxoviridae.* The L protein is the largest viral

protein measuring 200 to 260 kDa. It is highly conserved within the family *Paramyxoviridae* and is presumed to contain the major RNA dependent RNA-Polymerase (RdRP) enzymatic activities in the nucleocapsid complex. This protein is responsible for transcription including capping and polyadenylation and replication of the viral RNA (Easton and Ring, 2010).

Surface glycoproteins: an attachment protein and a fusion (F) protein are encoded in all *Paramyxoviridae* members and responsible for the steps starting from the first contact with the host cell membrane to the release of the viral genome into the cytoplasm. These proteins will be discusses in detail in the following sections (2.4.1 and 2.4.2).

Normally, the surface proteins are glycosylated during post-translation modification in the ER (Lamb and Kolakofsky, 2001), where an N-linked carbohydrate attaches covalently to asparagines based on the sequence motif NXT (Aspargine-X-Threonine) or NXS (Aspargine-X-Serine), where X is any amino acid (aa) except proline or aspartic acid. In Simian Virus 5 (SV5), removal of oligosaccharides from the F1 subunit of the F protein of SV5 resulted in delayed intracellular transport and decreased stability of the protein (Panda et al., 2004).

2.4.1 F protein

F proteins of PMVs are glycosylated, heterodimer, pH independent and classified as type I glycoproteins (Lamb and Kolakofsky, 2001). Klenk and Garten (1994) have described the role the F protein of avian and mammalian PMV play in viral virulence which is mediated by the interaction between this protein and cellular proteases which regulate viral reproduction, host range and viral pathogenecity.

The F proteins of PMV contain at their N-terminus a cleavable signal peptide that targets via their hydrophobic intermediate sequence the freshly translated polypeptide to the ER (Lamb and Kolakofsky, 2001). The signal peptide contains three different regions. The first is the positively charged N- terminus, the second is the highly hydrophobic intermediate motif and the third is the polar C- terminus which determines the signal peptide (SP) cleavage site (Von Heijne, 1985). Following translocation of these proteins into the lumen of the ER, the SP is cleaved from the

rest of the protein by a complex of enzymes known as signal peptidase to mature the protein (Hunter, 2001).

Proteolytic cleavage is a common post translational modification of F protein (inactive precursor F0) to produce the active form of the glycoprotein (F1 and F2) (Klenk and Garten, 1994). Cleavage is carried out by intracellular enzymes (furin) which are provided by the infected cells. Interestingly, the cleavage efficiency and the cellular site where the cleavage is supposed to occur are strongly influenced by the sequences of the furin recognition site as has been shown in some NDV strains. For instance, if only a single basic aa is present at the furin recognition site, the inactive precursor F0 cannot be cleaved as usual by the protease enzymes provided in the Golgi apparatus. This protein can then alternatively be cleaved by extra enzymes (tryptase Clara) in the respiratory tract (Klenk and Garten, 1994). Therefore, such infections are restricted to the respiratory tract and cannot induce systemic infections. In comparison, PMV encoded F proteins with the furin recognition site R-X-K-R/R (R: arginine; K: lysine, X: any aa) can be cleaved in the trans Golgi apparatus and thus induce systemic infection and death (Klenk and Garten, 1995). Post cleavage, disulfide linked F1 and F2 fusogenically active subunits are formed (Scheid and Choppin, 1974). The newly formed fusion peptide (FP) positioned at the amino terminus of the F1 inserts into the target membrane initiating its fusion (Hernandez et al., 1996; Morrison, 2003). The hydrophobic domain FP is a distinctive feature for most fusion glycoproteins (Hernandez et al., 1996). The sequence of FP can regulate syncytia formation (Gething et al., 1986).

F proteins of PMVs consist of repeat patterns of seven aa, positioned from a~g and called heptad repeats (HR). Amino acids of each HR are positioned to form helical wheels in the α helices coiled coil structure. Residues at positions a and d of each wheel are usually hydrophobic and form the interior hydrophobic core of the coiled coil. When leucine residues are predominant at the a positions of the HRs, this motive is recognized as a leucine zipper motif (LZ) where leucine residues form the teeth of the zipper. This motif provides a bonding force in the analogous α helices with a hydrophobic interior allowing the motif to fasten together (Franke et al., 2006; Joshi et al., 1998; and[2]). Two HR were predicted for the F protein of SV5; HRA, which is located at the C- terminus of the FP and HRB, which is located at the N-

[2] http://encyclopedia.thefreedictionary.com/Leucine+zipper

terminus of the tansmembrane domain (TM). Both are mapped to the protein anchored subunit F1 (Joshi et al., 1998). The hydrophobic TM domain (around 20 residues) is located near the C- terminus of the protein. Once the TM domain is synthesized, the translocation machinery of the polypeptide chain into the ER stops. TM domain anchors the protein in the membrane, leaving a short cytoplasmic tail (20 – 40 aa) at the C- terminus of the protein in the cytoplasm and the rest of the protein extracellular (equivalent to ER lumen/ virion surface/ cell surface) which is typical for type I orientation proteins (Lamb and Kolakofsky, 2001).

Residue substitutions and mutations within several domains of the F protein including cleavage site (CS), FP, HRs, LZ, and TM can influence activation of this protein (Watanabe et al., 1992; Reitter et al., 1995; Sergel-Germano et al., 1994; Morrison et al., 2003; Bosch et al., 1989).

The crystal structure of NDV uncleaved F protein was resolved by Morrison 2003, showing homotrimers which are intertwined with each other, each consisting of head, neck, and long stalk. The stalk in that 3 dimensional (3D) diagram is made up of coiled coil trimers that consist of HRI and HRII.

Current studies characterizing ferlavirus glycoproteins are limited. Franke et al. (2006) characterized and compared portions of the F gene from fourteen PMV of reptilian origin and predicted conserved domains along this gene. Based on the entire sequence of F gene of both FDLV and Gono GER85, a universal model of F protein was predicted by Franke et al. (2006).

2.4.2 Attachment protein (HN, H or G)

HN glycoproteins are the main antigenic factor of PMV (Lamb and Kolakovfsky, 2001) The attachment protein has different names based on its activities; for instance haemagglutinin (HA) when it lacks neuraminidase, but agglutinates red blood cells (RBCs), as for morbilliviruses, or glycoprotein (G) when it has neither function like in henipaviruses, or haemagglutinin-neuraminidase (HN) when it performs both actions as in avula-, respiro- and rubulaviruses (Chua et al., 2000; Morrison, 1988; Lamb and Kolakovfsky, 2001). Merz et al. (1981) documented that the two different functions may occur at one sialic acid site, while Morrison and Portner (1991) have documented that both activities may occur at two distinct sialic acid sites. Regarding the HN protein of NDV, Lamb and Kolakovfsky (2001) have suggested that both

functions may happen at one site. The attachment protein is pH dependent; the acidic atmosphere is favorable for hemagglutination, and neuraminidase activity (Lamb & Kolakofsky, 2001). Beside these two functions, HN protein functions to trigger the fusion process (Zaitsev et al., 2004; Yuan et al., 2011).

Morrison (1988) has compared the protein sequences of attachment proteins of several paramyxoviruses (sendai virus, simianvirus type 5, NDV, and parainfluenzavirus type 3) and found two short but very conserved regions. The first contained the sequence NRKSCS (N: aspargine; S: serine; C: cysteine) and the second contained the sequence GAEGR (G: glycine; A: alanine; E: glutamic acid). The conservation of these regions has directed Morrison to suggest the potential importance of these sites to the HN protein functionality and structure. Mirza et al. (1994) have studied the effect of several mutations introduced by site directed mutagenesis for the motif NRKSCS, on the several functions assigned to this protein and found that any mutation for each of the four aa residues (N-R-K-S) of this motif reduced the neuraminidase dramatically.

Several reports have demonstrated the structures of many PMV attachment proteins which consist of a transmembrane domain, stem and head (Lamb and Kolakofsky, 2001; Yuan et al., 2011). Some reports demonstrated the presence of a cytoplasmic tail at the N- terminus of the proitein in addition to the previous components in the HN protein structure (Chanock et al., 2001).

The transmembrane domain is the single hydrophobic domain located close to the N-terminus that is non cleavable and works as both signal and anchorage motif (Lamb and Kolakofsky, 2001). This motif functions to target the freshly translated poly peptide chain to the ER and ensuring their translocation. This process will in turn bring the TM domain in the lipid bilayer, leaving the N- terminus of the protein including the cytoplasmic tail (as for the low pathogenic parainfluenzavirus type-3 PIV-3) in the cytoplasm and the C- terminus of the protein extracellular which is typical for type II protein orientation (Chanock et al., 2001).

The head was proposed to contain neuraminidase domains where the sialic acid binding sites were located while the stalk was predicted to have specific sites that were important for triggering the F protein. Therefore, mutations in this region, depending on their exact localization, might either affect fusion activation only or

might in addition have a double affect on fusion and neuraminidase activity (Yuan et al., 2011).

2.5 Paramyxoviruses in reptiles

Reptile PMV are important disease etiological factors of snakes and have been isolated from both private and zoological collections (Jacobson, 2007). The first documentation of a PMV outbreak in reptiles was from Zurich in Switzerland in 1976, with an explosive respiratory disease in farmed Fer-de-lance snakes (*Bothrops atrox*) (Fölsch and Leloup, 1976). The causative agent of that outbreak was FDLV, named after the infected snakes and isolated by Clark et al. (1979). In that study, Clark suggested that FDLV is of ectothermic vertebrate origin based on several observations, including low temperature requirement for replication (the optimum range of 25-30 C^o), efficient replication in all reptilian cell types and lack of antigenic relation to all known mammalian and avian Myxoviruses. Consequent cases were reported in several snake families including Elaphidae, Colubridae, Boidae and Pythonidae (Ahne et al., 1987; Essbauer and Ahne, 2001; Marschang et al., 2009; Abbas et al., 2011). Kurath et al. (2004) sequenced the full genome of FDLV and found the presence of the unique unknown gene (U) between the N and P genes. Kurath et al. (2004) also found other criteria when comparing the sequences of the complete genome of FDLV with other established PMVs including low levels of sequence identity (ICTV, 2011). All previously mentioned characteristics supported grouping of described PMV of reptilian origin in the new genus *Ferlavirus* within the subfamily *Paramyxovirinae* with FDLV as the type species (Kurath et al., 2004, 2009; Marschang, 2011).

In snakes, the target organ for ferlaviruses seems to be the lung. In general, PMV infections should be considered in snakes with respiratory distress, anorexia, emaciation, lethargy and sudden death (Jacobson et al., 2007). However, ill snakes can display neural symptoms like head tremor, weak muscles, opisthotonus (stargazing), and flaccid paralysis (Fölsch and Leloup, 1976; Jacobson et al., 1981;).

Gross post mortem examination often reveals exudative pneumonia with pulmonary lesions, congestive and haemorrhagic lungs, enlarged pancreas/ liver and fibrinonecrotic exudate in the coelomic cavity which often form aggregates or clumps (Stacy and Pessier, 2007).

Microscopically, histologic changes are often observed in affected lungs including hyperplasia of respiratory epithelial cells and variable diffuse interstitial infiltrates of lymphocytes, heterophiles, plasma cells and macrophages (Jacobson, 1992, 1997; Stacy and Pessier, 2007). In some cases, epithelial syncytial cell formation or intraepithelial eosinophilic intracytoplasmic inclusions are also formed (Homer et al., 1995). While proliferative pneumonia is suggestive of PMV infection, other chronic non viral pneumonias including bacterial and parasitic pneumonias should be taken into acount (Homer et al., 1995).

PMV infections have also been doccumented in lizards, though less frequently than in snakes (Jacobson, 2007). Antibody surveys of captive and wild-caught lizards often demonstrate high level antibody titres against PMV (Marschang et al., 2002; Gravendyck et al., 1998; Marschang et al., 2009). A paramyxo-like virus was first isolated from a false tegu (*Callapistes maculatus*), however, no clinical signs associated with the infection were reported (Ahne and Neubert, 1991). The first report documenting PMV infection associated with clinical signs was in an epidemic of proliferative pneumonia caused by PMV infection in a group of caiman lizards (Jacobson et al., 2001). A PMV was isolated from a clinically healthy wild caught mexican lizard (Marschang et al., 2002). In 2012, a case study documented the first detection of PMV in apparently healthy bearded dragons (*Pogona vitticeps*) from a rescue centre in Munich (Abbas et al., 2012).

In chelonians, the information available on ferlavirus infections is even rarer (Essbauer and Ahne, 2001; Jacobson, 2007; Marschang et al., 2009). In one report, PMV were described in a group of Hermann's (*Testudo hermanni*) and spur-thighed (*T. graeca*) tortoises in Switzerland associated with dermatitis (Zangger et al., 1991). In another case, a double infection of PMV and herpesvirus was detected in South Africa within a mixed group of tortoises during an epidemic (Oettle et al., 1990). The sole PMV isolate (Ther GER99) reported to date was obtained from a Hermann's tortoise in Germany. The infection was associated with pneumonia. This isolate was identified as a ferlavirus based on morphological, biochemical and molecular characteristics (Marschang et al., 2009). In 2010(a), Papp et al. documented a ferlavirus infection in a leopard tortoise (*Geochelone pardalis babcocki*) with lethargy and respiratory distress. The viruses found in that animal differed on a genomic level

from that previously found in a Hermann's tortoise highlighting the lack of species specificity within these viruses (Papp et al., 2010a).

Concurrent infections have been documented along with ferlavirus infections in reptiles, mainly with at least two ferlavirus strains (Papp et al., 2010a&b) and infection with other viruses like reoviruses (Gravendyck et al., 1998; Marschang et al., 2002; Abbas et al., 2011), or with both reo- and adenoviruses (Abbas et al., 2011).

Hyndman et al. (2012) described a new PMV (Sunshine) from Australian pythons with neuroresperatory disease. Phylogenetic calculations of the complete genome has revealed clustering within the family *Paramyxoviridae* but could not be assigned to any of the subfamilies *Paramyxovirinae* or *Pneumovirinae*. This virus is quite distinct from the previously described ferlaviruses.

Serological relatedness was documented via Haemagglutination inhibition (HI) by Blahak (1995) between snake PMVs and avian PMV serotypes 1 and 7 (aPMV). However no antigenic relatedness was observed between PMVs of reptile origin and members of mammalian and avian PMV serotypes (1,2,3,4,6,7,8,9) (Richter et al., 1996). Cross reaction was noticed within PMVs of reptile origin when rabbit anti sera originally directed against neotropical virus (NTV), reacted as well against other PMV of reptile origin including NTV (Clark et al., 1979). A recent study has described cross reactivity within ferlaviruses (Rösler et al., 2013), where an antiserum originally directed against ferlavirus isolate Crot GER03 reacted similarly against all of the squamate and chelonid ferlaviruses.

2.6 Taxonomy of ferlaviruses

Previous studies compared ferlaviruses based on partial genome sequences: L and HN genes (Ahne et al., 1999) and L and F genes (Franke et al., 2001). While Ahne et al. (1999) proposed classification of these viruses into two subgroups designated A and B with intermediate isolates, Franke et al. (2001) suggested the classification into three clusters designated A, B and C. Unfortunately, Franke did not integrate isolates from the previous study, therefore, members of the three clusters did not correspond to those from Ahne et al. (1999). This inconsistency was resolved by Marschang et al. (2009) based on partial genome sequences (L, HN and U genes)

when comparing several PMV isolates from snakes, lizards, and a tortoise. All PMV of squamate origin (snakes and lizards) included in that study clustered into two subgroups designated A and B. The only chelonian PMV (Ther GER99) available, isolated from a Hermann's tortoise, was found to cluster very close to the squamate PMVs based on partial L gene sequence. However, this virus could not be assigned to any of the known subgroups (A, B) within the proposed *Ferlavirus* genus (Marschang et al., 2009).

A recent study by Abbas et al. (2011) based on partial genome sequences of the L, HN, and U genes of PMVs from a corn snake (*Patherophis guttatus*) revealed a new PMV type. This study incorperated information on almost all of the PMV included in the previous study by Marschang et al. (2009). The novel PMV showed moderate distances to all members of subgroups A and B suggesting a new classification; the first representatives of subgroup C within the newly accepted genus *Ferlavirus*. A new study from Papp et al. (2013), based on partial genome sequence of the L, HN, and U genes of a PMV from a masked water snake (*Homalopsis buccata*) has revealed the second representative of subgroup C. The exact taxonomic positions of these different clusters and whether they represent different species within the genus remains to be determined. The single chelonian isolate (Ther GER99) has consistently been shown to cluster within the genus *Ferlavirus* but as a sister group to the A, B, and C subgroups (Marschang et al., 2009; Abbas et al., 2011).

Several studies (Ahne et al., 1999; Marschang et al., 2009; Franke et al., 2001) have shown that ferlaviruses are not species specific. In one study, PMVs from lizards were shown to be closely related to various snake isolates and did not form a separate cluster (Marschang et al., 2009); in another, two PMVs detected in a leopard tortoise clustered with those of squamate origin (Papp et al., 2010a), indicating no species specificity.

2.7 Detection of Ferlaviruses

Ferlaviruses have been isolated from both living animals (oral and cloacal swabs) (Pees et al., 2010) and from different tissues including kidney, small intestine, lung, trachea, liver, and heart (Blahak, 1994; Papp et al., 2010b; Abbas et al., 2011). The

most commonly used methods to detect ferlaviruses in reptiles are described in the following.

Virus isolation in cell culture is the first method that was used for the detection of ferlaviruses. It has often been used for screening snakes, lizards, and tortoises for ferlaviruses (Marschang et al., 2009; Papp et al., 2010a&b; Abbas et al., 2011).

Clark et al. (1979) isolated the first FDLV by inoculating lung suspension from a *Bothrops* that suffered a fatal infection into the embryonated eggs of the snake *Cyclagras gigas.* The homogenate of the infected embryo was then inoculated into a wide range of reptilian and mammalian cell lines and caused cytopathic effects (CPE) when incubated at 30 ºC.

Reptile PMV can grow readily on several reptilian cell lines including iguana heart cells (IgH2) (Ahne et al., 1999), viper heart cells (VH2) (Clark et al., 1979; Richter et al., 1996), and rattlesnake fibroma cells (Jacobson et al., 1980), or on mammalian cell lines like vero cells (Mayr et al., 2000) and BHK/21 from Syrian hamster (Clark et al., 1979). They have also been propagated in embryonated gecko and chicken eggs (Clark et al., 1979). Depending on the cell type used, the CPE, which includes all morphological and pathological changes, varies from spindling and rounding of cells to giant cell formation followed by cell detachment and cell lysis (Clark et al., 1979; Franke et al., 2001). In some cases, CPE formation may take a long time; for that reason blind passages are of great importance for virus recovery (Origgi and Paré, 2007).

Nowadays and most commonly, diagnosis of PMV infection in reptiles is confirmed using reverse-transcription followed by PCR (RT-PCR). This molecular based technique is very sensitive. In order to obtain molecular information from PMVs of reptile origin, Ahne et al. (1999) described a nested RT-PCR protocol using synthetic oligonucleotide primers targeting portions of the L and HN genes.

Subsequently, additional primer design was accomplished targeting portions of several genes in addition to the L gene. Franke et al. (2001) described a semi nested RT-PCR protocol targeting partial F gene sequence. Marschang et al. (2009) described new degenerate primers targeting partial sequances of the HN gene along with several primers targeting partial sequence of the U gene. Those primers

described in Ahne et al. (2009) have been further used for the detection and characterization of new ferlaviruses (Abbas et al., 2011; Papp et al., 2013).

A comparison of sequences of the L, HN, and U genes (Marschang et al., 2009) showed that the L gene was the most conserved gene, followed by the HN gene, while the U gene was the least conserved. Therefore, the protocol described by Ahne et al. (1999) targeting a partial sequence of the L gene has been established as a standard molecular diagnostic technique for the detection and characterization of ferlaviruses in reptiles (Franke et al., 2001; Kindemann et al., 2001; Marschang et al., 2009; Abbas et al., 2011).

HI testing is one of the serological tests that has been used to reveal the antigenic relationship between ferlaviruses and between these viruses and others including avian and mammalian PMV (Richter et al., 1996; Blahak, 1995; Rösler et al., 2013).

3 Materials

3.1 Laboratory equipment

Table 3.1: Equipment used for cell culture and virus isolation

Name of article	Resource	Application
Biosafety cabinet Heraeus	Kendro Laboratory Products, Hanau.	Sterile cabinet
Fireboy S1000	Tecnorama, Fernwald	Bunsen burner
CO_2-Incubator	Binder Tuttlingen	Incubator
Sterile workbench	Prettl, Bempflingen	Laminar flow cabinet
Inverted light microscope	Leitz, Wetzlar	Microscope

Table 3.2: Equipment used for processing diagnostic samples

Name of article	Resource	Application
Varifuge 3.2 RS,	Heraeus Osterode	Centrifuge
Branson-250 Sonifier	Ausrüstung 707 Schwäbisch Gmünd	Disruption of cell membranes

Table 3.3: Equipment used for the extraction of viral RNA/ RT-PCR

Name of article	Resource	Application
Centrifuge 5415 R	Eppendorf, Wesseling Berzdorf	Table centrifuge
Centrifuge 5415 D	Eppendorf, Wesseling Berzdorf	Table centrifuge
Vortex Genie 2™	Bender & Hohlbein AG, Zurich (CH)	Vortexer
Dry Bath Incubator	Kisker, Steinfurt	Heating block
Peqlab Primus 35 Adv.	Peqlab, Erlangen	Thermal cycler
Doppio Thermocycler	VWR, Darmstadt	Thermal cycler
Mastercycler Gradient	Eppendorf, Wesseling Berzdorf	Thermal cycler

Table 3.4: Equipment used for gel electrophoresis/ Gel extraction

Name of article	**Resource**	**Application**
Sterile Surgical Blades,	(Aesculap Ag, Tuttlingen)	Gel extraction
Consort 443, Electrophoresis	Keutz, Reiskirchen	Power supply
EC-105, Electrophoresis	Biometra, Gottingen	Power supply
Infinity	1000 Vilber Lourmat,Eberhardzell	UV-transilluminator
Agagel Mini G45/2- Gel chamber	Biometra, Gottingen	Gel electrophoresis

3.2. Cell lines

Table 3.5: Description of cell lines used in experimental trials

Cell type	**Reference No.**	**Source**	**Description**
Viper heart cells (VH2)	ATCC, CCL-140	American Type Culture Collection USA	Established from the heart of a female Russells Viper (*Vipera russelli*)
Iguana heart cells (IgH2)	ATCC, CCL-108	American Type Culture Collection USA	Established from the heart of a green iguana (*Iguana iguana*)

Table 3.6: Other materials used for different applications

Name of article	**Source**	**application**
BD Falcon BD Falcon™ with vented cap (75 cm^3),	Becton Dickinson GmbH, Heidelberg	Tissue Culture Flasks
30mm diameter Cellstar®	Greiner Bio-One GmbH, Frickenhausen	Tissue culture dishes
BD Falcon™ 15 ml polypropylene conical tubes	BD Biosciences Discovery Labware, Heidelberg	Sample preparation
1000. 200, 10 µl Pipettes 0.5 ml PCR Tubes	Biozyme scientific GmbH, Oldendorf	RNA preparation

3.3 Chemicals, reagents and solutions

3.3.1 Cell culture and virus isolation

Water, deionised and filtered

Milli-Q® water (Millipore GmbH, Eschborn)

Dulbecco´s modified Eagle medium (DMEM)

13.4 g/l DMEM medium, 4.5 g/l D-glucose, with L-glutamine, were all dissolved in 1 l Milli-Q® water. 2.2 g/l $NaHCO_3$ (Merck KGaA, Darmstadt) was added and the solution was stirred thoroughly until the ingredients were dissolved completely. After that, the medium was sterile filtered and kept at 4°C for future use.

Amphotericin B

The lyophelized Amphotericin B (Biochrom AG, Berlin, Germany) was suspended in 5 ml Milli-Q® water. The final concentration of the stock solution was about 250 µg/ml.

Gentamicin sulfate solution

640 U/mg Gentamicin sulfate (Biochrom AG, Berlin, Germany) dissolved in Milli-Q® water to achieve a concentration of approx. 3200 U/ml in the stock solution.

Penicillin-G solution

Penicillin-G (1664 U/mg) (Biochrom AG, Berlin, Germany) was dissolved in Milli-Q® water to achieve a concentration of about 100000 U/ml in the stock solution.

Streptomycin sulfate solution

Streptomycin sulfate (758 U/mg) (Biochrom AG, Berlin, Germany) was dissolved in Milli-Q® water to achieve a concentration of about 190000U/ml in the stock solution.

FBS

Fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany)

Non essential amino acids (NEA)

(Biochrom AG, Berlin, Germany)

Table 3.7: Maintenance medium Dulbecco´s modified Eagle medium (DMEM) for cell culture and virus isolation.

Concentrations used for virus isolation are written in Italics

Constituent	**Volume in ml /500 ml DMEM**	**Final Concentration**
FBS	50 *10*	10 % (v/v) *2% (v/v)*
NEA	5 *5*	1% (v/v) *1% (v/v)*
Penicillin-G-solution	2 *2*	200 U/ml *200 U/ml*
Gentamycin sulfate-solution	2 *2*	6.4 U/ml *6.4 U/ml*
Streptomycin sulfate-solution	2 2	380 U/ml *380 U/ml*
Amphotericin B-solution	4 *4*	0.5 µg/ml *0.5 µg/ml*

Table 3.8: Trypsin-versen (TV) solution 0.05 %

Ingredient / Company	**Volume g/l**	**Concentration**
KCl (Merck KgaA, Darmstadt)	0.20	3 mM)
KH_2PO_4 (Merck KgaA, Darmstadt)	0.20	1 mM
Na_2HPO_4x12 H_2O (Merck KgaA, Darmstadt)	2.31	6 mM
$CaCl_2$ x 2 H_2O (Merck KgaA, Darmstadt)	0.132	0.9 mM
NaCl (Merck KGaA, Darmstad)	8.00	136 mM
Trypsin-dry substance (Biochrom AG, Berlin)	0.5	1:250 U/mg
Versen (Titriplex III) (Merck KgaA, Darmstadt)	1.25	3 mM
Streptomycin sulfate (Biochrom AG, Berlin)	0.05	37900 U/l
Penicillin-G (Biochrom AG, Berlin)	0.06	100000 U/l

The ingredients were dissolved in Milli-Q® water, adjusted with 1 M NaOH to pH 7.00, and sterile filtered and kept at -20 °C for future use.

3.3.2 Other materials used for processing clinical samples

Table 3.9: DMEM supplemented with 2x antibiotic concentration (2x AB DMEM) used for processing diagnostic samples

Constituent	Volume in ml /500 ml DMEM	Final Concentration
Penicillin-G-solution	4	400 U/ml
Gentamycin sulfate-solution	4	12.8 U/ml
Streptomycin sulfate solution	4	760 U/ml
Amphotericin B-solution	8	1 µg/ml

3.3.3 Extraction of viral RNA

Nuclease-free water

(QIAGEN Hilden, Germany)

Diethylpyrocarbonate (DEPC) as nuclease free water

10 ml DEPC (Fluka, Buchs SG, Switzerland) was dissolved in 90 ml of absolute ethanol to reach a concentration of 10% of the DEPC stock solution. This solution was placed in brown bottles and kept in the dark. A 1% working solution was made by mixing 1ml of the stock solution in 99 ml sterile Milli-Q® water. The mixture was then autoclaved at 120 °C for 30 min and stored at 4 °C until use.

Silica-matrix

60 g of Silicon dioxide (SiO_2) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added to a measuring cylinder and filled with DEPC water up to 500 ml and mixed thoroughly. The cylinder was placed at room temperature for 24 hours to allow good sedimentation. Using a pipette, 430 ml of the supernatant were removed and discarded. The cylinder was again filled with DEPC water up to 500 ml to re-suspend the sedimented Silica particles. Following an additional sedimentation of 5 hours, 440 ml of the supernatant were discarded. The pH of the solution was adjusted to 2.00 by the addition of 600 µl of 25 % HCl (6.85 M). The solution was shaken to re-suspend

the silica, and then aliquoted into 1.5 ml nuclease free tubes in 1 ml portions, autoclaved at 121 °C for 15 min and kept at room temperature in the dark.

Tris-HCl

12.1 g Tris-HCl (Carl Roth GmbH & Co, Karlsruhe, Germany) was dissolved in 1L Milli-Q® water and the pH of the solution was adjusted to 6.40 with 0.01 M HCl. The solution was stored at 4 °C. The final concentration of Tris- HCl solution was 0.1 M

Ethylenediamine-tetraaceticacid (EDTA)

7.44 g EDTA (Carl Roth GmbH & Co, Karlsruhe, Germany) was dissolved in 100 ml DEPC water to reach a final concentration of about 0.2 M. With 5N NaOH, the pH of the solution was adjusted to 8.00. The solution was then autoclaved at 120 °C for 30 min and stored at 4 °C until use.

Lysis buffer (LB)

1 ml Triton X-100 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 48 g Guanidinethiocyanate (Carl Roth GmbH & Co, Karlsruhe, Germany), 8.8 ml of 0.2 M EDTA (pH 8.0) and 40 ml 0.1 M Tris HCl (pH 6.40) were mixed together in a 250 ml-glass beaker. The beaker was wrapped in aluminum foil and placed in a water bath (56 °C, 15 min) to dissolve the ingredients. The solution was then stored in the dark at room temperature for future use.

Washing buffer (WB)

48 g Guanidinethiocyanate and 40 ml 0.1 Tris HCl (pH 6.40) were mixed in a 250 ml-glass beaker. The beaker was wrapped with aluminum foil and placed in a water bath (56 °C, 15 min) to dissolve the contents. The solution was stored in the dark at room temperature until use.

70 % Ethanol

35 ml of absolute ethanol Rotipuran® 99.8 % (Carl Roth GmbH & Co, Karlsruhe, Germany) and 15 ml Milli-Q® water were mixed in a nuclease free 50 ml tube. The tubes were then kept at room temperature.

RNase inhibitor

RiboLock™ RNase Inhibitor 40 U/µl (Fermentas GmbH, St. Leon-Rot, Germany) was kept frozen at – 20 ºC until use.

Acetone

Acetone Rotipuran® 99.8 % (Carl Roth GmbH & Co, Karlsruhe, Germany) was kept in nuclease free 50 ml plastic tube and stored at room temperature.

3.3.4: Reverse transcription polymerase chain reaction (RT-PCR)

Table 3.10: Chemicals and reagents used for RT-PCR (Fermentas, St. Leon-Roth) and long-RT-PCR (Analytik Jena AG, Jena).

Those used for long RT-PCR are written in Italics.

Reagent	**Volume/ 25µl**	**Final concentration**
10X Taq buffer (with KCl)	2.5 µl	1x
10x PCR buffer long PCR, (with KCl and $(NH_4)_2SO_4$)	*2.5 µl*	1x
25 mM $MgCl_2$	2.5 µl	2.5 mM
25 mM $MgCl_2$ solution long PCR	*2.5 µl*	*2.5 mM*
primer	2.5 µl	1 µM
dNTPs	2.5µl	0.2 mM
dNTPs	*3µl*	*0.24 mM*
40 U/µl RiboLock™ RNase Inhibitor	0.15 µl	0.24 U/µl
200 U/µl RevertAid™ Reverse Transcriptase	0.35 µl	2.8 U/µl
5 U/µl Taq DNA Polymerase	0.25 µl	0.05 U/µl
5 u/µl Innu Taq Long Range Polymerase	*0.25 µl*	*0.05 U/µl*

Primers

Primers were ordered from biomers.net (Ulm, Germany). The stock solution (100 pmol/µl) was then prepared by dissolving the lyophilized primers with the appropriate amount of Milli-Q^R water (calculated by the provider) into 1.5 ml RNase-free centrifuge tubes. The tubes were then vortexed for five seconds and allowed to stand on ice for 15 min. Subsequently, the tubes were again shortly vortexed and centrifuged for five seconds to ensure the proper mixing of the primer components. The stock solution was tenfold diluted in Milli-Q^R water to achieve working solutions of 10 pmol/µl. The final concentration of the primers in the master mix was 1 µM.

dNTPs 2´- Desoxynucleosid-5´-Triphosphat dATP, dCTP, dGTP, and dTTP (100 mM, Fermentas,St. Leon-Rot)

A working solution (2 mM) was prepared from the stock solution (100 mM) by adding 40 µl of each nucleotide of the stock solution to 1840 µl Milli-Q^R water. After mixing well, the solution was aliquoted and stored at -20° C until use. The final concentration of each dNTP in the master mix was 0.2 mM in the normal RT-PCR and 0.24 mM in the long RT-PCR.

3.3.5 Gel electrophoresis

1x TAE-buffer (Roth, Karlsruhe,Germany)

The stock solution of 50x TAE buffer consisted of 242 g Tris, 57.1 ml acetic acid and 100 ml 0.5 M EDTA, maintained at pH 8.00. To prepare the working solution (1x), 40 ml 50x TAE buffer were diluted with 1960 ml Milli-Q^R water.

Ethidium bromide solution (Roth, Karlsruhe,Germany)

The working solution was prepared by dissolving 10 mg ethidium bromide in 10 ml aqua dest under the laminar flow cabinet. This solution was stored in the dark and handled carefully using nitrile gloves.

Table 3.11: Other chemicals used for gel electrophoresis

Chemical/ Reagent	Source
Agarose	Roth (Karlsruhe)/Germany
6x Loading Dye	Fermentas (St. Leon-Roth),Germany
GeneRuler™ 100 bp DNA Ladder	Fermentas (St. Leon-Roth),Germany
GeneRuler™ DNA Ladder Mix	Fermentas (St. Leon-Roth),Germany

3.3.6 Gel extraction

Chemicals, regents and materials

PeqGOLD Gel-Extraction Kit (PEQLAB, Erlangen). Detailed protocol: see methods section.

3.4 Software programs for sequencing and phylogenetic analyses

Table 3.12: Software programs for processing DNA sequences

Program description	Producer	Application
Staden Package version:1.7.0 (2003.0) Pregap4 and Gap4	Andrew Withwham, (Bonfield et al., 1995)	Genome assembly program: assembling, editing, correcting and aligning DNA sequence
BioEdit 7.0.5.3 (2005)	Ibis therapeutics Carlsbad CA92008, (Hall, 1999)	Alignment editor
NCBI BLAST, BlastX and BlastN	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Sequence analysis and source for additional sequences

Table 3.13: Software programs for phylogenetic analyses

Program description	Producer	Application
PHYLIP Package, Versions 3.64	Felsenstein, University of Washington (Felsenstein, 1989)	Phylogenetic analysis
Mega 5.05 (2011)	Tamura et al., 2011	Phylogenetic analysis

3.5 Processing of clinical samples

3.5.1 Samples

During the period from 2009 until the end of 2011, a total of 495 clinical samples from 251 snakes were tested for the occurrence of PMV by RT-PCR as described in the methods section. The snakes originated from private collections, mostly from Germany under cooperation with the Clinic for Birds and Reptiles, Leipzig University, and in a few cases from Hungary, Great Britain, Italy, and Denmark. All clinical samples (different organs, oral/ cloacal swabs, and tracheal washes) were collected by veterinary practitioners. List of samples: see supplementary data, Table 1.

3.5.2 Primers

Table 3.14: Description of oligonucleotide primers for amplification of partial sequences of L, U, and HN genes of ferlaviruses

Name	Sequence	Position 5' in FDLV	References
L 5F	GCAGAGATTTTCTCTTTCTT	9768	Ahne et al., 1999
L 6R	AGCTCTCATTTTGTATGTCAT	10394	Ahne et al., 1999
L 7F	TAGAGGCTGTTACTGCTGC	9811	Ahne et al., 1999
L 8R	CATCTTGGCAAATAATCTGCC	10376	Ahne et al., 1999
U-cons Fwd-out	ACCARYYATRRRCHTGYVRARATG	1571	Marschang et al., 2009
U-cons Rev-out	GTTWGCCATTMTTACTGGATCTC	2254	Marschang et al., 2009
U-cons Rev-in	ATCTCAGATACCTTTGATCCTAAG	2236	Marschang et al., 2009
U-cons Fwd-in	RARATGATTAAAGAAAACCTAGG	1589	Marschang et al., 2009
HN 1F	AAATCCTGCAGTACCGTGGCA	7532	Ahne et al., 1999
HN 2R	AGATATCTGTGTAAACTCCTG	8210	Ahne et al., 1999
HN cons F1	AATGTYRTRGARGATGARAG	7583	Marschang et al., 2009
HN cons R2	CARGGYYYGTTMCCWGGYCTYG	8154	Marschang et al., 2009

3.5.3 RT-PCR Positive controls

09/05/03 Crot GER03 (Marschang et al., 2009)

06/03/09 Pangut GER09 (Abbas et al., 2011)

3.6 Materials used for optimization trials

The established RT-PCR protocol from Ahne et al. (1999) targeting partial sequence of the L gene has been used as a standard diagnostic detection tool for PMV in reptiles. In order to increase the specificity of this protocol, multiple trials were performed targeting this L gene.

3.6.1 Optimization trials of RT-PCR targeting a portion of the L gene

3.6.1.1 Chemicals and reagents used for optimization trials of the RT-PCR targeting a partial sequence of the L gene (Ahne et al., 1999).

Table 3.15: RT-PCR matrix with different $MgCl_2$ concentrations

Reagent	1st round RT-PCR	2nd round RT-PCR				Final concentration			
	volume in 25 µl total amount	volume in 25 µl total amount							
Millipore water	9.25 µl	12.75	12.25	11.75	11.25 µl				
Taq-Buffer+KCl 10x	2.5 µl	2.5 µl				1x			
25 mM $MgCl_2$	2.5 µl	1 µl	1.5 µl	2 µl	2.5 µl	1 mM	1.5 mM	2 mM	2.5 mM
2 mM DNTPs	2.5 µl	2.5 µl				0.2 mM			
10 pmol/µl Primer Farward	2.5 µl	2.5 µl				1 pmole/µl			
10 pmol/µl Primer reverse	2.5 µl	2.5 µl				1 pmole/µl			
5 u/µl Taq DNA-Polymerase	0. 25 µl	0. 25 µl				0.05 U/µl			
40 u/µl Ribolock inhibitor	0.15 µl					0.24 U/µl			
200 u/µl RT (ReverseTranscriptase)	0. 35 µl					2.8 U/µl			
Template	2.5 µl of extracted viral RNA sample	1µl of the 1st round RT-PCR products							

3.6.1.2 Isolates

All ferlavirus isolates were identified as PMV by syncytia formation on VH2, sensitivity to chloroform and by detection of the partial L gene sequence (RT-PCR-Ahne et al., 1999) followed by sequencing.

Table 3.16: PMV isolates used for optimization trials.

Isolate No/ year	Isolate name	Sub group	Host	Species common name	Literature
3319/95	Var GER95	A	*Varanus prasinus*	Emerald tree monitor	Gravendycket al., 1998 & Marschang et al., 2002
28xpc/99	Xeno USA99	A	*Xenosaurus platyceps*	Flathead knob-scaled lizard	Marschang et al., 2009
9/5/03	Crot GER03	B	*Crotalus horridus*	Timber rattlesnake	Marschang et al., 2009
936/00	Dasy GER00	A	*Dasypeltis atra*	Montane egg-eating snake	Marschang et al., 2009
324/00	Igu GER00	B	*Iguana iguana*	Green iguana	Marschang et al., 2009
5688/91	Pangut GER91	A	*Pantherophis guttatus*	Kornnatter	Blahak, 1994
1356/90	Crot GER90	B	*Crotalus catalinensis*	Santa Catalina Island Rattlesnake	Blahak, 1994
2766/90	Vip GER90	B	*Vipera palaestinae*	Palestine viper	Blahak, 1994
20/3/05	Orth GER05	B	*Orthriophis taeniurus*	Beauty snake	Marschang et al., 2009
tPMV/99	Ther GER99	-	*Testudo hermanni*	Hermann’s tortoise	Marschang et al., 2009
6/3/09	Pangut GER09	C	*Pantherophis guttatus*	Corn snakes	Abbas et al., 2011

3.6.1.3 Samples and positive control

Optimization trials were carried out with samples (Table 3.17) that gave unspecific reactions in the original PCR and with samples that were positive for snake adenovirus (SnAdV-1).

Table 3.17: Diagnostic Samples used for the optimization trials.

Some of the samples represent false positive/ unspecific products including bacteria[π], adenovirus[Ω], and some are PMV which were used as positive controls[PC].* products obtained after applying the RT-PCR protocol from Ahne et al. (1999) targeting partial L gene sequence. SnAdV1= Snake Adeno Virus Type 1.

Samples	**Product length after sequencing***	**Sequencing result**
123/10	500 bp	*Acinetobacter baumanni*
104/4/11[π]	550 bp	*Enterobacter cloaca*
104/5/11[π]	550 bp	*Enterobacter cloaca*
101/1/11	550 bp	*Acinetobacter baumanni*
17/2/10	500 bp	*Pseudomonas putida*
61/11[π]	500 bp	*Pseudomonas aeroginosa*
AdV[Ω]	350 bp	SnAdV1
6/11 [pc]	550	PMV
86/11 [PC]	550	PMV
Pangut GER 09 [PC]	550	PMV

3.6.2 Designing new primers targeting partial sequence of the L gene of ferlavirus isolates

3.6.2.1 Primers

Table 3.18: Description of oligonucleotide primers designed for RT-PCR amplification of L gene and used for the optimization trials.

Name	Sequence	Position 5' in FDLV
PMV F1 out	GAYATHYTHTAYCCDGARTG	34
PMV F2 in	CAYYTNRAYTCHCCDATHG	55
PMV F3 in	CRTTYHTRACHTGGTT	524
PMV R1 out	CKCATHTCHYWYTTRAT	544
PMV R3	GTYADRAAYGRBYKRTDCCA	514
PMV R2	AACCADGTYADRAAYG	524
PMV R4 out	CTNCCYTCBACHACRTCRC	729
PMV R5 out	CCNCCRTGYCTYTCYC	1238
PMV R6 out	GGRTANACDGWRTCCCA	1462

3.6.2.1: Isolates

Nine ferlavirus isolates (Dasy GER00, Pangut GER91, Crot GER03, Igu GER00, Vip GER90, Crot GER90, Orth GER05, Pangut GER09, Ther GER99) as indicated in Table 3.16 were used for testing the new primers.

3.7 Materials used for amplification of full CDS of F and HN genes

3.7.1 Virus isolates

Table 3.19: Description of ferlavirus isolates analyzed in this study

Isolate No/ year	Isolate name	Sub group	Host	Species common name	Literature
3319/95	Var GER95	A	*Varanus prasinus*	Emerald tree monitor	Gravendycket al., 1998 & Marschang et al., 2002
368/01	Pyth GER01	B	*Python regius*	Ball python	Marschang et al., 2009
9/5/03	Crot GER03	B	*Crotalus horridus*	Timber rattlesnake	Marschang et al., 2009
324/00	Igu GER00	B	*Iguana iguana*	Green iguana	Marschang et al., 2009
1356/90	Crot GER90	B	*Crotalus catalinensis*	Santa Catalina Island Rattlesnake	Blahak, 1994
2766/90	Vip GER90	B	*Vipera palaestinae*	Palestine viper	Blahak, 1994
20/3/05	Orth GER05	B	*Orthriophis taeniurus*	Beauty snake	Marschang et al., 2009
tPMV/99	Ther GER99	-	*Testudo hermanni*	Hermann’s tortoise	Marschang et al., 2009
6/3/09	Pangut GER09	C	*Pantherophis guttatus*	Corn snakes	Abbas et al., 2011

3.7.2 Primers

Table 3.20: Description of oligonucleotide primers designed for RT-PCR amplification of full CDS region of HN and F genes of nine ferlavirus isolates.

[π]Primers were kindly provided by Tibor Papp, PhD, DVM.

Name	Sequence	Position 5’ in FDLV
F-gen cons 68 F	TTGATMAHCTWGAACARRTAGG	5089
F gene 130 F	CCRGTCAGYAMWGCRACAATGAT	5157
F gene 257 F	CCATWRAYAARHTVARBACTG	5284
F gene 1631 R	AKAAARCCKAGRTTRTTGTAD	6658
OPMV F gene 274 F	ACTGAYATYASTGTVVTNGARGG	5301
OPMV F gene 1502 R	GCRATAGAGRYHGRAGMDKTYA	6518
F-gene cons 5409F	GCMCARATMACRGCDGGRATTGC	5406
F-gene cons 6324R	GGYTTRATYTGYAGVCCRTC	6316
F-HN cons 6305F	GAYGGBCTRCARATYAARCC	6296
F-HN cons 7837R	GRGCTTGRCACATRGCYTG	7829
F-HN cons 7837R	GRGCTTGRCACATRGCYTG	7829
HN 7562 F tPMV	GCTTATCTCTTATGTACTCATG	7565
HN 8257 R tPMV	CCAGATAACAATGTGACAGTGG	8262
HN 7671 F tPMV	GCTATAGGTACTCAGACAGG	7674
HN 8119 R tPMV	GGTCTCTATCCAACTGATCC	8123
HN middle 7984F	TCHCARGTRTGGCTHGGTGCAG	7974
HN middle 8335R	TGACTATCWGCMACBCGYCC	8325
HN gene 1303 F[π]	GAYCCHATAAGAATAAGYTGG	8093
L gene 1217 R[π]	CCRTGTCTCTCYCTRAAYCC	9943
HN cons. 8421FWD	GAGYTHAGYCCTGCYACYYTRGG	8415
HN cons. 9574 REV	TCATASACYTTATTACCDAG	9562
HN gene 1351 F[π]	GGKAACRRBCCYTGYYCWGC	8141
L gene 1184 R[π]	GARCARAAHACNGCATGYCC	9910
HN 8060 F1 tort	GGTTGGCATGCCTTGGCACAG	8051
HN 10280 R1 tort	GGATCGAAATCCATATCTTC	10258
HN 8199 F2 tort	TGTATCACTGGTGTTTATAATG	8183
HN 9970 R2 tort	GATCCTCCGTGTCTCTCTC	9950
F gene 5755 REV	GCATTDATRTCRCCATCC	5735
F gene 5881 REV	GGRAARGACATCTCRAT	5831

Table 3.21: Software programs for surface protein analyses

Program description	Producer/ Reference	Application
GENtle	Magnus Manske at the University of Cologne, Germany/ Kyte & Doolittle, 1982	Prediction of hydrophobicity plots, leusine zipper (LZ)
NetNGlyc 1.0 (2002)	Center for Biological Sequence Analysis (Denmark)[3]	Prediction of glycolysation sites
TMHMM Server 2.0 (2009)	Center for Biological Sequence Analysis (Denmark)/[4]	Prediction of transmembrane domains
SignalP V 2.0	Center for Biological Sequence Analysis (Denmark)/[5] Nielsen et al., 1997	Prediction of signal peptides

4. Methods

4.1 Processing of diagnostic samples

Swabs or tissue samples of examined snakes were placed in 15 ml tubes, immersed in 3 ml growth medium DMEM supplemented with 2x concentration of antibiotics (Table 3.9) and sonicated at an output level of 30 for 3 impulses for the destruction of cell membranes. For removal of bacteria and cell debris, samples were subsequently centrifuged at 3000xg for 15 min and kept at -4 °C.

4.2 Subculture and preparation of tissue culture dishes for virus isolation

After discarding the DMEM from a complete cell monolayer flask (75 cm^3) and washing with 5 ml trypsin, the cells were incubated with 1 ml trypsin at room

[3] http://www.cbs.dtu.dk/services/NetNGlyc/
[4] http://www.cbs.dtu.dk/services/TMHMM/
[5] http://www.cbs.dtu.dk/services/SignalP/

temperature for 15 minutes. Subsequently, the flask was shaken by hand until the cells were detached. Afterward, 4 ml of growth medium was added to the subcultured cells. A glass pipette was used to separate the cells by pipetting them up and down several times. The cells were diluted at a ratio of 1:10, 1:5, or 1:2 during subcultivation (depending on the density of the cells) by transferring 0.5, 1, or 2.5 ml of the cell suspensionrinto 17 ml DMEM. To prepare tissue culture dishes the cell suspension was mixed 1:10 with DMEM in a cell reservoir to prepare 30 mm tissue culture dishes. 2 ml of this diluted suspension was added to all dishes. 1 day old dishes were used for virus isolation. All flasks and dishes were incubated at 28 °C with 5 % CO_2.

4.3 Virus isolation

Virus isolation trials were carried out from all clinical samples on VH2 and IgH2. 200 ml of the sample supernatant were inoculated onto one day old, medium-free tissue culture dishes, with about 70% confluent cell monolayers. Dishes were then incubated for 2 hours at 28 ºC with 5% CO_2.. After incubation, 2 ml DMEM supplemented with 2% FBS, 1% NEA and antibiotics (Table 3.7) were added to each dish. One dish was always left uninoculated as a cell control. Inoculated cells were checked for the occurrence of CPE using a light microscope about every 2-3 days. Virus isolates were identified by the form of the CPE and resistance to chloroform. When CPE was seen, dishes were frozen at -80 ºC and thawed for another passage. When no CPE was seen, dishes were incubated for two weeks and then frozen for blind passaging. Subsequent to a freeze and thaw cycle, two further passages were carried out from each dish.

Chloroform treatment

Chloroform treatment was performed by mixing chloroform and virus suspension at a dilution of 1:10, followed by shaking vigorously for 1h and centrifugation at 10.000 rpm for 10 min. The supernatant was then diluted 1:10 and inoculated into tissue culture dishes.

4.4 Extraction of viral RNA

RNA – Preparation modified from Boom et al. (1990)

1- 300 µl of the cell culture supernatant (see 4.3) or of the homogenous supernatant after processing the diagnostic samples (see 4.1), was mixed with 900 µl lysis buffer and 40 µl silica matrix in 2 ml tubes. The suspension was then incubated (lying on a table) at room temperature for 10 minutes and vortexed every 3 minutes.
2- The suspension was centrifuged (1 minute, 14000 rpm, 4°C) and the supernatant was discarded.
3- The pellet was washed twice with 1000 µl washing buffer and 1000 µl 70% ethanol respectively. After each washing step, the pellet was vortexed, centrifuged (0.5 minute, 14000 rpm, 4°C) and the supernatant was discarded.
4- The washing process was repeated once with aceton, followed by centrifugation for 3 minutes. The aceton was then decanted and the pellet dried (10 min. 56 °C) on a heating block.
5- Following complete drying, 60 µl of nuclease-free water and 1 µl RNase-inhibitor were added to the pellet, vortexed and incubated at 56°C for 15 minutes. The tubes were then vortexed every 5 minutes, followed by centrifugation (3 min. 14000 rpm, 4°C).
6- The supernatant was carefully removed with a micropipette (without any contact to the silica matrix) from each tube and transferred to a new 0.5 ml tube. This process was repeated once again and the supernatant stored immediately at -80 °C.

4.5 Methods used for RT-PCR

4.5.1 RT-PCR protocol used for processing diagnostic samples

Table 4.1: Nested RT-PCR protocol targeting partial sequence of the L (Ahne et al., 1999), HN, and U genes (Marschang et al., 2009)

Reagent	1st round RT-PCR	2nd round RT-PCR	Final concentration
	volume in 25 µl total amount	**volume in 25 µl total amount**	
Millipore water	9.25 µl	11.25 µl	
10x Taq-buffer+KCl	2.5 µl	2.5 µl	1x
25 mM MgCl2	2.5 µl	2.5 µl	2.5 mM
2 mM dNTPs	2.5 µl	2.5 µl	0.2 mM
10 pmol/µl primer forward	2.5 µl	2.5 µl	1 pmole/µl
10 pmol/µl primer reverse	2.5 µl	2.5 µl	1 pmole/µl
5 u/µl Taq DNA-polymerase	0. 25 µl	0. 25 µl	0.05 U/µl
40 u/µl Ribolock inhibitor	0.15 µl		0.24 U/µl
200 u/µl RT (reverse transcriptase)	0. 35 µl		2.8 U/µl
Template	2.5 µl of extracted viral RNA sample	1µl of the 1st round RT-PCR products	

Table 4.2: Description of primer combinations, thermocycler programs and expected product lengths used for RT-PCR amplification of partial sequences of L, HN and U genes of ferlaviruses from diagnostic samples.

PCR Name	*Primer PCR round I*		*Thermocycler program 1st round*	*Primer PCR round II*		*Thermocycler program 2nd round*	*Expected Product length (bp)*
	Fwd	*Rev*		*Fwd*	*Rev*		
L gene	L5F	L6R	42°C for 1 h 95°C for 5 min. 95°C for 30 sec. 45°C for 30 sec. 72°C for 2 min. 72°C for 7 min.	L7F	L8R	95°C for 2 min. 95°C for 30 sec. 45°C for 30 sec. 72°C for 1 min. 72°C for 5 min.	626, 566
HN gene	HN 1F	HN 2R		HN cons F1	HN cons R2		678, 399
U gene	U-cons Fwd-out	U-cons Rev-out		U-cons Rev-in	U-cons Fwd-in		684, 648

4.5.2 RT-PCRs used for the optimization trials

4.5.2.1. Optimization trials for the detection of ferlavirus L gene

In order to increase the specificity of the RT-PCR (Ahne et al., 1999), multiple trials were performed, both to lower the number of products with unexpected sizes as well as to increase the number of virus specific products of the expected size. RT-PCRs carried out in these trials targeted the L gene, as this gene is highly conserved and is therefore considered a good target for a group of genetically diverse viruses.

Optimization trials were carried out with samples that gave unspecific reactions in the original PCR (3.5.1.3) as well as with positive controls (PC) (a ferlavirus isolate and two diagnostic samples verified by sequencing as PMV positive) and samples that were positive for other viruses frequently found in snakes, particularly a snake adenovirus (SnAdV-1). Optimization procedures included the use of multiple $MgCl_2$ concentrations. Three different annealing temperatures were used for each master mix. Differences were applied for the 2nd round of the RT-PCR only. The first round

was carried out as described by Papp et al. (2010). All primer combinations and thermo cycler programs are listed in Table 4.3.

Primer combination/ thermocycler program

The primer combination targeting partial L gene sequence described by Ahne et al., 1999 (Table 2.4) and the corresponding thermo cycler programs were used for the optimization trials. In addition, $MgCl_2$ concentrations were varied and each concentration was used with three different annealing temperatures, as listed in Table 3.15

4.5.2.2 Designing new primers targeting partial L gene sequence

As a second attempt to achieve a more specific assay for ferlaviruses, degenerate primers targeting a portion of the L gene were designed based on several members of each ferlavirus subgroup including; Var GER95 (subgroup A), Pyth GER01, Crot GER03, Igu GER00 (subgroup B), Pangut GER09 (subgroup C) and the Ther GER99. All information regarding these isolates is listed in Table 3.16. Extended portions of partial L gene sequences were obtained from these isolates and used for the alignments. Deduced amino acid sequences of L gene (514 aa) are indicated in supplementary data, Figure 1.

RT-PCR protocol

The primer combinations (Table 4.3) targeting partial L gene sequences were tested for six ferlaviruses as follows: Xeno USA99, Var GER95, Pangut GER 09, Ther GER99, Igu GER00, Dasy GER00. All information regarding these isolates is listed in Table 3.16.

Table 4.3: Description of primer combinations, thermo cycler programs and expected product lengths used for RT-PCR amplification of partial L gene.

Abbreviations: *Composition of chemicals and thermo cycler programs used in the RT-PCR protocol were the same as those used by Ahne et al. (1999) and Marschang et al. (2009) (Table 4.1, 4.2). $^{\pi}$ The sequences of all primers are listed in Table 3.18.

PCR Name	***Primer PCR round I$^{\pi}$***		***Thermocycler program 1st round****	***Primer PCR round II$^{\pi}$***		***Thermocycler program 2nd round****	***Expected Product length (bp)***
	Fwd	***Rev***		***Fwd***	***Rev***		
A	F2 in	R5 out	42°C for 1 h 95°C for 5 min. 95°C for 30 sec. 45°C for 30 sec. 72°C for 2 min. 72°C for 7 min.	F3 in	R4 out	95°C for 2 min. 95°C for 30 sec. 45°C for 30 sec. 72°C for 1 min. 72°C for 5 min.	190
B	F2 in	R6 out		F3 in	R5 out		929
C	F1 out	R6 out		F3 in	R5		929
D	F1 out	R1 out		F2 in	R2		451
E	F1 out	R1 out		F2 in	R3		441
F	F1 out	R2		F2 in	R3		441
G	F1 out	R5 out		F2 in	R4 out		656
H	F1 out	R5 out		F3 in	R4 out		190
I	F1 out	R6 out		F2 in	R4 out		656

4.5.3 Methods used for the amplification of the full CDS of F and HN genes

Table 4.4: RT-PCR protocol utilized for the amplification of full CDS of surface proteins Those written in Italics were used for long RT-PCR.

	1st round RT-PCR	**2nd round RT-PCR**	
Reagent	**volume in 25 µl total amount**	**volume in 25 µl total amount**	**Final concentration**
Millipore water	9.25 µl *8.75 µl*	11.25 µl *10.75 µl*	
10x Taq-Puffer+KCl *10x PCR Buffer long PCR*	2.5 µl *2.5 µl*	2.5 µl *2.5 µl*	1x 1x
25 mM MgCl2 *25mM MgCl2 long PCR*	2.5 µl *2.5 µl*	2.5 µl *2.5 µl*	2.5 mM 2.5 mM
2 mM dNTPs	2.5 µl *3 µl*	2.5 µl *3 µl*	0.2 mM o.24 mM
10 pmol/µl primer forward	2.5 µl	2.5 µl	1 µM
10 pmol/µl primer reverse	2.5 µl	2.5 µl	1 µM
5 u/µl Taq DNA-polymerase *5 u/µl Innu Taq long range polymerase*	0. 25 µl *0. 25 µl*	0. 25 µl *0. 25 µl*	0.05 U/µl 0.05 U/µl
40 u/µl Ribolock inhibitor	0.15 µl		0.24 U/µl
200 u/µl RT (reverse transcriptase)	0. 35 µl		2.8 U/µl
Template	2.5 µl of extracted viral RNA sample	1µl of the 1st round RT-PCR products	

Table 4.5: Description of Synthetic oligonucleotide primer combinations, thermal cycler programs and expected product lengths used for RT-PCR amplification of full CDS region of HN and F genes of nine ferlavirus isolates.

Abbreviations: ł gap of about 400bp at the end of the HN gene. ¡ gap of about 10 bp. ± a genomic region lying at the end of the F gene and at the beginning of the HN genes.. Ĭ PCR targets the beginning of the F gene. Primers have been described in earlier publications; ǂAhne et al. (1999), ∞ Marschang et al. (1999)

PCR Name	***Primer PCR round I***		***Primer PCR round II***		***Modification applied to the RT-PCR (Ahne et al., 1999)***	***Expected product length (bp)***
	Fwd	***Rev***	***Fwd***	***Rev***		
F gene I	130F	1631 R	274F	1502R	PCR; 95C⁰/45 sec. Annealing;45C⁰/45 sec. Elongation 72C⁰/2 min. for both rounds.	1501,1228
F gene II	257 F	1631 R	274F	1502R		1374,1228
F gene III	130F	1631 R	257F	1502R		1501,1245
F gene VI	257 F	1631 R	274F	1631 R		1374,1357
F subgroup \`C\`	257 F	1631 R	5409F	6324R		1501, 915
F-HN[±]	257 F	HN cons R2∞	F-HN 6305	F 7837R	Annealing; 44 C⁰/ 45 sec. Elongation; 72C⁰/ 3 min. for both 1st and 2nd rounds.	2870, 1532
HN gene	HN 1F[ǂ]	HN R2[ǂ]	HN cons F1∞	HN cons R2∞	RT-PCR has been applied without modifications.	678, 571
HN 10 bp[¡]	7984 F	8335 R	1 round PCR with 45 cycles		PCR; 95 C°/ 1 min. Annealing; 45C⁰/ 45 sec.Elongation; 72 C⁰/ 3 min.	351
HN gap[ł] 400 bp	HN 1351 F	L 1184 R	8421F	9574R	PCR; 95 C°/ 1 min. Elongation;72 C⁰/ 3 min. for both rounds	1769, 1147
HN tPMV gap	HN 7562 F	HN 8257 R	HN 7671 F t.	HN 8119 R t.	RT-PCR has been applied without modifications.	697, 448
HN-L	HN 1303F	L 1217R	HN 1351F	L 1184R	Annealing; 45C⁰/ 45 sec. Elongation; 72C⁰/ 3 min. for both rounds.	1847, 1769

PCR Name	Primer PCR round I Fwd	Primer PCR round I Rev	Primer PCR round II Fwd	Primer PCR round II Rev	Modification applied to the RT-PCR (Ahne et al., 1999)	Expected product length (bp)
HN-L tortoise	HN 8060 F1 tort	L 10280 R1 tort	HN 8199 F2 tort	L 9970 R2 tort	Annealing; 45C^{o}/ 45 sec. Elongation; 72C^{o}/ 3 min. for both rounds.	2207, 1767
F gene left side[i]	U cons. F in∞	F 5755R F 5881R	1 round PCR with 45 cycles		Annealing; 45C^{o}/45 sec. Elongation; 72C^{o}/4 min.	750

4.6 Gel electrophoresis

1.5% agarose solution was prepared by dissolving 1.5 g agarose in 100 ml 1x TAE-buffer followed by heating in a microwave for about 2 min. After heating, the solution was supplemented with 5 µl of the ethidium bromide solution and mixed by gentle shaking. The mixture was then poured into the gel chamber provided with a comb and left to solidify for about 20 minutes at room temperature. After drying, the comb was removed and the gel was placed in the gel chamber and immersed in 1x TAE-buffer. 2 µl Loading dye (6x) was mixed with 8 µl of each of the DNA samples and pipetted into the gel slots. 3 µl of DNA size ladder (100 bp DNA ladder or DNA ladder mix, based on the expected length of the products) was added to at least two side slots of each row. Gel electrophoresis was run for about 20-30 min at about 110 V, the gel was then visualized with a 320 nm UV-transilluminator.

4.7 Gel extraction

With the use of surgical blades, the bands of the fourfold volume of PCR II amplicons were incised accurately under visualization on the UV-transilluminator (the whole process should not exceed more than 30 seconds). Each band was put in a 2 ml microcentrifuge tube (previously weighed and numbered).

Gel extraction was carried out according to the protocol provided by the manufacturer for the peqGOLD Gel-Extraction Kit (PEQLAB Biotechnology GmbH, Erlangen, Germany) as follows:

1. After supplying each gel slice with an equal amount of Binding Buffer , the tubes were incubated on a heating block at 65°C, (vortexing every 3 min) until the gel was totally dissolved. The pH of the solution was controlled based on its color; if it was orange or red instead of yellow, about 5 µl of 5 M Na-Acetate were added and vortexed immediately.

2. 700 µl of the solution was then pipetted onto PerfectBind DNA columns with a membrane for adsorption of DNA, which were plugged into collection tubes. The tubes were centrifuged (always for 1 min at 10,000 rpm) and the flow-through was discarded. If the solution was more than 700 µl, the process was repeated using the same collection tubes and columns.

3. Columns were washed once with 300 µl Binding Buffer and twice with 300 µl Wash Buffer supplemented with EtOH. Prior to centrifuging (1 min at 10,000 rpm), the columns were incubated for about 3 min at room temperature.

4. For drying the columns, a further centrifugation step (same conditions as above) was applied. The collection tubes were then discarded and the columns were placed into fresh 1.5 ml microcentrifuge tubes.

5. For elution of the DNA from the membrane, 15 µl Elution Buffer was added (exactly on the membrane), the tubes were then incubated for about 3 min at room temperature. After that, the tubes and the membrane were centrifuged for 1 min at 5,000 rpm. This elution step was repeated once to get a final volume of about 30 µl of the extracted DNA which was stored at -4ºC until sequencing.

All steps used for gel electrophoresis were carried out with all extracted DNA samples to determine the efficiency of the gel purification, by mixing 2 µl of each purified sample with 8 µl 1x concentration loading dye.

4.8 Sequencing

Purified DNA samples were diluted (1:2-1:20), based on the strength of the band visualized on the UV-transilluminator. For sequencing, 15 µl of the diluted samples and 20 µl of the corresponding primer were sent to a commercial lab (Eurofins, Ebersberg).

Analyses of sequences

The raw sequences were first downloaded from the website of the company http://www.eurofinsdna.com/de/home.html and processed using the ABI Sequence Analysis Programme 5.1.1 (Applied Biosystems, Foster City, USA). Sequences were further edited, joined and aligned using several software programs (all programs involved are mentioned in Table 3.12). Identity matrices were generated by the BioEdit program (Hall, 1999)

Phylogenetic calculations were carried out using the unweighted pair group method with the arithmetic mean (UPGMA), maximum likelihood (ML), maximum parsimony (MP) and minimum evolution (ME) trees calculated using Mega 5.05 (Tamura et al., 2011) model; ML and distance based Fitch trees using the PHYLIP Package (Felsenstein, 1989) model (Table 3.13). Protein analysis was carried out using several programs (Table 3.21), under the supervision of Prof. Pfitzner from the Institute for Genetics, Subinstitute for General Virology of the University of Hohenheim.

5 Results

5.1 Prevalence of ferlaviruses in diagnostic samples

5.1.1 RT-PCR/ sequencing analyses

Molecular diagnostics based on L gene RT-PCR verified by sequencing revealed ferlavirus positive results (566 bp products) from 19 samples (3.83% of the 495 screened) originating from 15 snakes (5.97% of the 251 snakes tested). All ferlavirus positive snakes were from the families *Colubridae* and *Pythonidae* (Table 5.1.1*)*, while no ferlaviruses were detected from other snake families (supplementary data, Table 1 in Appendix). Ferlavirus was detected in 8 dead snakes (Col-1,-2, Pyt-1a,b,c, Pyt-3,4,5) and from 6 live animals (Pyt-2, Col-1a,b,c,d,e,f). Col-1 and the live animals tested (except Pyt-2) came from one enclosure (Abbas et al., 2011).

In the dead snakes, the following tissues tested positive by RT-PCR (number positive/number tested): intestine (3/7), lung (5/6), kidney (2/7), pancreas (1/4), liver (1/1). Heart (0/1), and head (0/1) were negative (Table 5.1.1). Among the ferlavirus positive snakes intestine, kidney and lung were all examined 5 times and all three were positive in one case (Col-1) (20%). Only lung was positive in three cases (Pyt1-a,b,c) (60%) while the other two organs were negative in these cases. In our study, PMV was only detected in the pancreas and not in any other tissues once (7.14% of the 14 snakes that tested positive) in a dead royal python (Pyt-3).

The RT-PCRs targeting the HN and U genes were carried out on all L gene positive samples which were confirmed by sequencing. Positive products (399bp) were obtained from 14 samples (73.68% of the 19 ferlavirus positive samples) for the HN gene and from 10 samples (648bp) (52.63%) for the U gene.

All specific size RT-PCR amplicons obtained for all three genes (L, HN, and U) were sent for sequencing by a commercial company (MWG Operon, Ebersberg) and then processed as described in the Methods section (4.8). Ten RT-PCR partial L gene, nine partial HN gene and eight partial U gene sequences were identical to each other representing subgroup C (Pangut-GER09) (HQ148084–HQ148087). Three amplicons from Pyt-1a~c were identical to the previously described Crot-GER03 (Subgroup B), (Acc No: GQ277611). Another three products from Pyt-5 were also identical to one another and to Igu GER00 (Subgroup B), (Acc No: GQ277617). The

three amplicons representing partial sequences of L, HN and U genes obtained from Col-2 belonged to the new subgroup C (Hobuc HUN09) (Acc No JX186193-JX186195) (Papp et al., 2013). The partial RT-PCR L gene obtained from Pyt-4 was 99% similar to Crot GER03 (GQ277611) (Subgroup B) (Table 5.1.1).

An RT-PCR targeting partial L gene sequence revealed a positive signal from the throat and cloacal swab of Pyt-2 (an Indian python). Sequencing resulted in a product of 234 bp as sequence data from the rest of RT-PCR product was not reliable. The partial L gene RT-PCR obtained from this sample was 97% identical to FDLV (AY141760.2) (Subgroup A).

5.1.2 Virus isolation

Virus isolation attempts were carried out for all samples. Ferlaviruses were isolated on VH2 from three snakes (Col-1, -1a, Pyt-4) (20%), all of which were also positive in the RT-PCR from original samples and from RNA prepared from the virus isolates. Infection rates with ferlaviruses obtained from isolation and RT-PCR results of all tested snakes were 5.97% (15 of the 251 tested snakes) (Table 5.1.2).

Of those animals that tested ferlavirus positive by RT-PCR, reoviruses were isolated in four cases (26.66%) (Col-1, -1a,b,c) while AdVs were isolated twice (13.33%) (Col-1&1a). Of those found negative for ferlaviruses by RT-PCR, reoviruses were isolated from five animals that originated from two groups of snakes owned consecutively by the same owner (14/5, 10, 13/09 and 54/3, 4/09) (Supplementary data, Table 1). Multiple viral infections with three viruses (PMV, AdV, and reoviruses) were detected in two animals (Col-1, 1a) which belonged to a group of captive corn snakes of the same enclosure. These viruses were isolated in the first case from the intestine of a dead animal and in the second case from a swab of a live snake which belong to a group of 13 survivals which were swabbed one month later. Unknown viruses were isolated on VH2 in two cases (Lab No; 3/2/09 and 106/09) that tested negative for ferlavirsues by RT-PCR (L gene) and in three cases (Lab No; 56/10, 60/10 and 157/10) that tested positive for ferlaviruses by RT-PCR and revealed no significant results after sequencing (supplementary data, Table 1 in Appendix).

Applying an end-point dilution technique followed by plaque purification, a pure ferlavirus isolate was obtained from Col-1a, as confirmed by RT PCR. While applying the same techniques in conjunction with chloroform treatment, a pure AdV isolate was obtained from the same sample (Abbas et al., 2011). Syncytial cell formation and cytolysis were the hallmark for the CPE caused by the ferla- and the reoviruses. As compared to ferlaviruses in cell culture, which grew slowly forming small syncytial cells (Figure 2), the reoviruses grew quickly, forming giant syncytial cells (Figure 3). Virus isolates forming fusogenic CPE and sensitive to chloroform were further identified as PMV by RT-PCR (Ahne et al., 2009), whereas isolates forming fusogenic CPE and resistant to chloroform were further identified as reoviruses using a reovirus specific RT-PCR (Wellehan et al., 2009). The AdV caused a CPE consisting of fast detachment of cells with developement of small round cells and was identified by PCR (Wellehan et al., 2004) (Figure 4).

All orthoreoviruses isolated from *Pantherophis guttatus* were identical to one another and to orthoreovirus 55/02 (E4309703) based on nested RT-PCR targeting partial sequence of the RDRP gene. The adenovirus isolated from the dead *Pantherophis guttatus* (Col-1) was 100% similar to Snake AdV type 2 (FJ 012163) based on partial DNA dependent DNA polymerase gene sequences while the AdV isolated from the live snake Col-1a of the same species revealed 100% similarity to Snake AdV type 1 (DQ106414).

Figure 2. Cytopathic effects (CPE) of a ferlavirus isolate from corn snakes.

(A): Negative control VH2. (B): 2nd passage of ferlavirus isolate from the intestine of Col-1 on VH2 cells 4 days post inoculation (Magnification: 400x). The cytopathic effect (CPE) consisted of the development of syncytial cells and cytolysis.

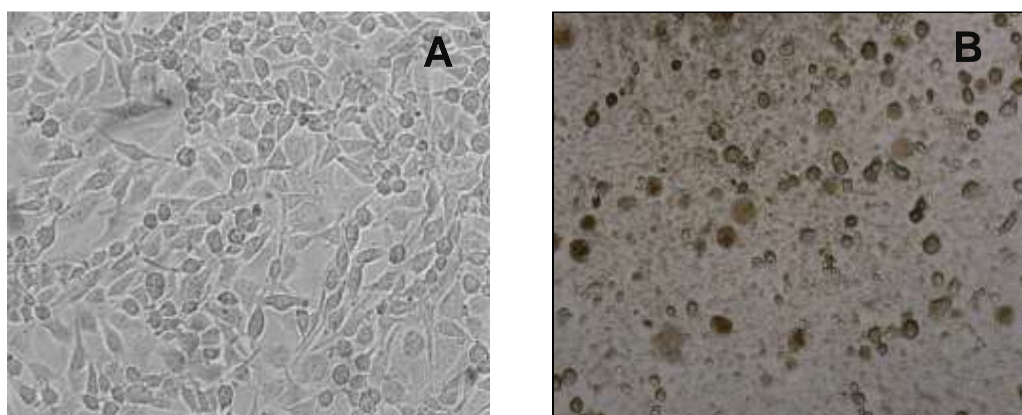


Figure 3. Cytopathic effects (CPE) of a reovirus isolate from corn snakes.

(A): Negative control VH2. (B): 2nd passage of reovirus isolate from oral/ cloacal swab from (Col-1a) on VH2 cells 7 days post inoculation (Magnification: 400x). The cytopathic effect (CPE) consisted of the development of giant multinuclear syncytial cells and cytolysis.

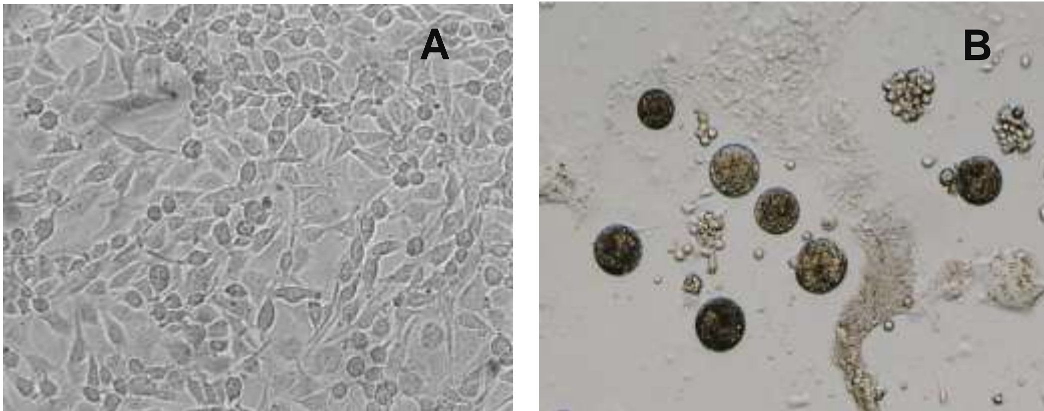


Figure 4. Cytopathic effects (CPE) of an AdV isolate from corn snakes.

(A): Negative control IgH2. (B): 4th passage of an AdV isolate from oral/ cloacal swab from corn snake Col-1a on IgH2 3 days post inoculation (Magnification: 400x). (CPE) consisted of rounding and detachment of some cells.

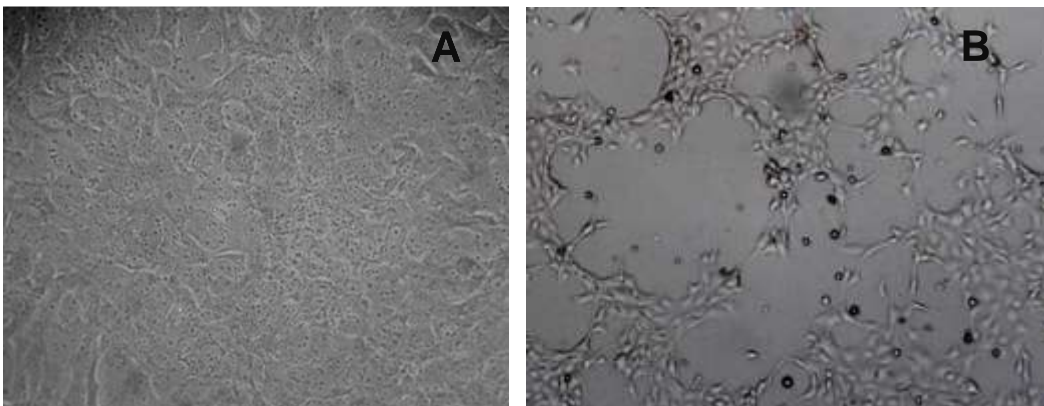


Table 5.1.1: PMV positive diagnostic samples.

Abbreviations: *, **: indicate animals are from the same owner and enclosure; o/cl. swab = oral and cloacal swabs, +: positive, –: negative, AdV= adenovirus, Ref1.= case reported in: Abbas et al. (2011), Ref2 =case reported in Papp et al. (2013). [R] Sequencing of PCR products from all reovirus isolates revealed identical partial RDRP gene sequences to the orthoreovirus isolate 55/02 (Wellehan et al., 2009).

Lab. No.	Species	Case history	Tissue tested	PMV RT-PCR			Nucleotide identity of partial L gene sequences	Virus isolation	Comments/ further findings (other PCRs)
				L gene	HNGene	U gene			
Col-1* 6/3/09	Corn snake (*Pantherophis guttatis*)	A snake died in a new collection. Ref1 dyspnoea and vomitus noticed prior to death	Lung	+	+	-	Pangut GER09	Orthoreovirus[R]	Accompanying two animals were negative for PMV.
			Kidney	+	+	+		PMV, orthoreovirus	
			Intestine	+	+	+		PMV, orthoreovirus, SnAdV-2	

Lab. No.	Species	Case history	Tissue tested	PMV RT-PCR			Nucleotide identity of partial L gene	Virus isolation	Comments/ further findings (other PCRs)
Col-1a* 14/2/09		Swabs taken one month later from 13 survivors of the same group. Ref1	O/cl. swab	+	+	+	All had the same PMV, a new type designated Pangut-GER09	PMV, orthoreovirus, SnAdV-1	Tested positive for AdV by PCR
Col-1b* 14/3/09			O/cl. swab	+	+	+		orthoreovirus	Tested positive for AdV by PCR
Col-1c* 14/6/09			O/cl. swab	+	+	+		orthoreovirus	Seven snakes from this group tested negative for the presence of PMV (14/1, 14/4, 14/5, 14/7, 14/9, 14/10, 14/13). Reovirus was isolated from three of those tested negative (14/5, 14/10, 14/13).
Col-1d* 14/8/09			O/cl. swab	+	+	+		-	
Col-1e* 14/11/09			O/cl. swab	+	+	+		-	
Col-1f* 14/12/09			O/cl. swab	+	+	+		-	

Lab. No.	Species	Case history	Tissue tested	PMV RT-PCR			Nucleotide identity of partial L gene	Virus isolation	Comments/ further findings (other PCRs)
Col-2 80/09	masked water snake *Homalopsis buccata*	35 cm long young snake showed anorexia prior to death. Pneumonia with secondary bacterial infection [Ref1]	Lung	+	+	+	New type designated Hobuc HUN09 (identity value 83% to Pangut GER09)	-	
Pyt-1a** 45/13/09	Indian python *Python molurus*		Lung Intestine Kidney Pancreas	+ - - -	+	-	100% similar to Crot GER 03	-	RT-PCRs for the L gene were negative from intestine, kidney and pancreas of these three snakes. Accompanying animal found negative for PMV and positive for AdV.
Pyt-1b** 45/14/09	Indian python *Python molurus*		Lung Intestine Kidney Pancreas	+ - - -	+	-	100% to Crot GER 03	-	
Pyt-1c** 45/15/09	Indian python *Python molurus*		Lung Intestine Kidney Pancreas	+ - - -	+	-	100% to Crot GER 03	-	

Lab. No.	Species	Case history	Tissue tested	PMV RT-PCR			Nucleotide identity of partial L gene	Virus isolation	Comments/ further findings (other PCRs)
Pyt-2 9/2/10	Indian python *Python molurus*		Throat, tracheal and cloakal swab	+	-	-	97% similarity to FDLV based on 200 bp	-	
Pyt-3 86/1/10	Royal python *Python regius*		Pancreas Lung Kidney Intestine	+ - - -	-	-	100% to Pangut GER 09	-	One animal died, seven survivors tested negative in the group
Pyt-4 25/11	Ball python	Animal died, enteritis noticed	Intestine Kidney	+ -	-	-	99% to Crot GER 03	PMV	
Pyt-5 6/1/11	Python		Liver Kidney Intestine Heart Head	+ + + - -	- + -	- + -	100% to Igu GER00	-	Group of about 40 pythons died of fatal pneumonia. The group was treated with gentamycin that had no effect. Septisemia was found after dissection. Tested positive in liver and head for AdV by PCR

Table 5.1.2: Infection rates in respect to all tested viruses (ferla-, reo- and adenoviruses) obtained from isolation and/ or (RT-) PCR results of all tested snakes.

Abbreviations: *: case reported in Abbas et al., (2011), **: case repoted in Papp et al., 2013

Number of snakes infected with the following viruses				**Infection rate**
	Ferlavirus	**Reovirus**	**SnAdV**	
Three snakes with triple infection*	Col-1, 1a, 1b	Col-1, 1a, 1b	Col-1, 1a, 1b	1.19%
Three snakes with double infection*	Col-1c, Pyt-5	Col-1c, 54/4/09 (Abbas et al., 2011)	Pyt-5, 54/4/09 (Abbas et al., 2011)	1.19%
Fourteen snakes with single infection	Ten snakes: Col-1d, e, f, Col-2**, Pyt-1a~c, Pyt-3&4 and Pyt2	Four snakes: 14/ 5,10&13/09 and 54/3/09		5.57%

5.1.3 Phylogenetic analyses

Six different partial L gene sequences were obtained from 19 RT-PCR products. The RT-PCR product obtained from Pyt-2 (234 bp) was not included in the phylogenetic analyses. Three of these produtcs (from Pyt-1a~Pyt-1c, Pyt-4, and Pyt-5) cluster to subgroup B while the rest (from Col -1; Col -1a~col-1e; Pyt-3 and Col-2) cluster into one branch forming the new subgroup C within the *Ferlavirus* genus (Figure 5A). This figure shows the same topology as in earlier publications (Abbas et al., 2011; Papp et al., 2013) where the ferlaviruses clustered into three closely related subgroups forming a seperate genus (Marschang et al., 2009; Kurath et al., 2004). Within this genus, the first two representatives of the novel subgroup C clustered together.

Phylogenetic calculations based on partial L gene sequence were carried out using several methods within the Mega program 5.05 as mentioned previously (Table 3.13). In all methods used, the two isolates Pangut GER09 and Hobuc HUN09 clustered together within the *Ferlavirus* genus supported with significant bootstrap values (61-83%). Depending on the method applied, this subgroup sometimes clustered close to subgroup B isolates, supported with low bootstrap values (39-53%). In one method (ML) this subgroup clustered as its own branch, the proposed subgroup C supported with high bootstrap values (95%) (Figure 5B).

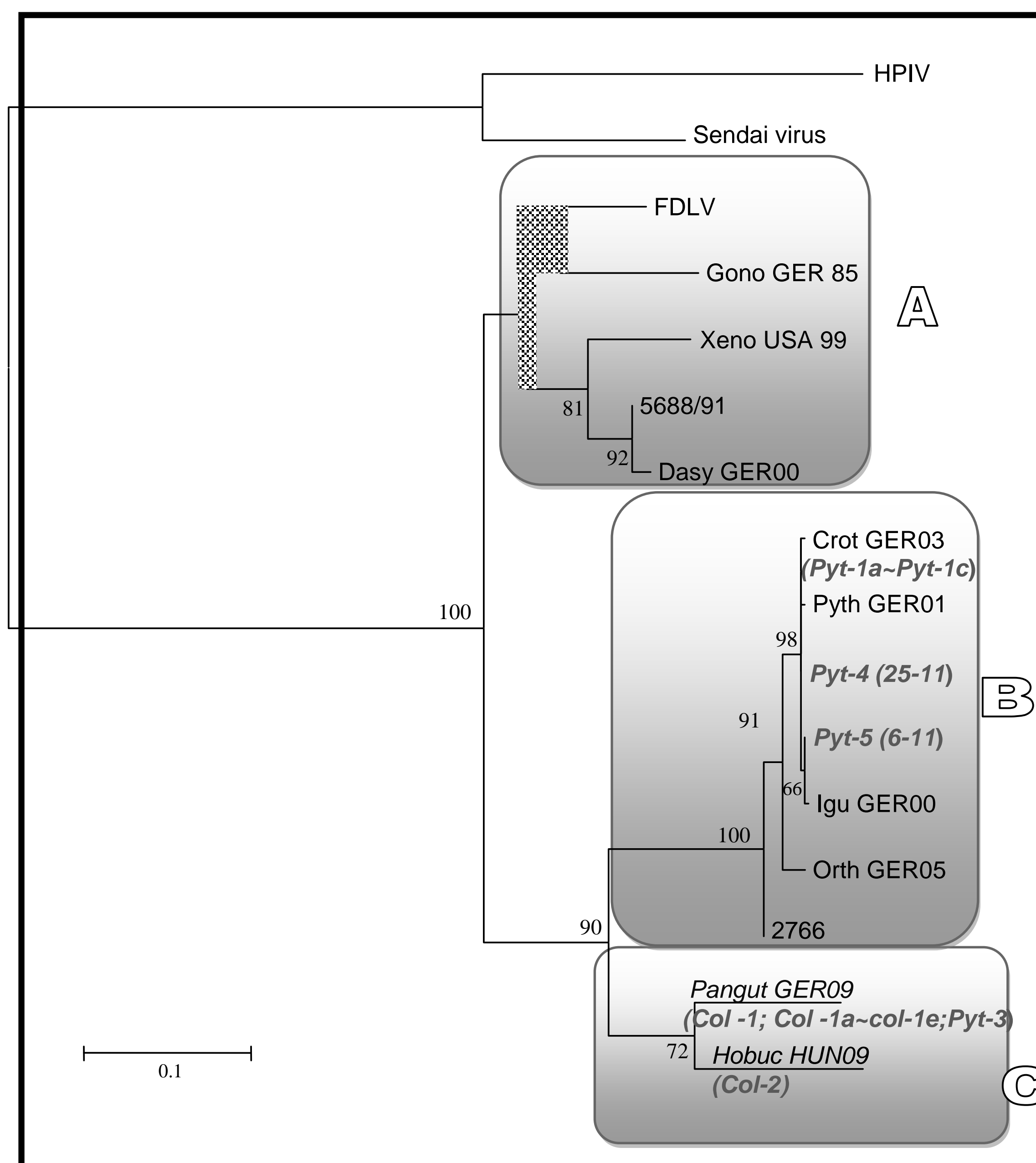


Figure 5A: Phylogenetic tree (Maximum likelihood analysis) based on partial L gene sequences 456 nucleotides (nt).

Bootstrap values were calculated for 500 replicates. Bootstrap values 60% and higher are indicated beside the nodes, while the branches with lower values are shown with checkerboard lines. Clinical samples from this study are printed in italics. GenBank accession numbers: FDLV (NC_005084), Crot GER03 (GQ277611), Dasy GER00 (GQ277613), GonoGER 85 (AF349404), Igu GER00 (GQ277617), NTV (AF286045), Orth GER05 (GQ277616), Xeno USA99 (GQ277614), Pyth GER01 (GQ277612), Pangut GER09 (HQ148084), Hobuc HUN09 (JX186195), Ther GER99 (GQ277615). Avian PMV-6 (AY029299), NDV = Newcastle disease virus (AF375823), Hendra virus (AF017149), Nipah virus (NC_002728), HPIV-1 (AF457102), Sendai (NC_001552), Canine distemper virus (AF378705), Measles virus (AF266289), Mumps virus (NC_002200), SV-41 = Simian virus 41 (NC_006428) and Tupaia paramyxovirus (AF079780).

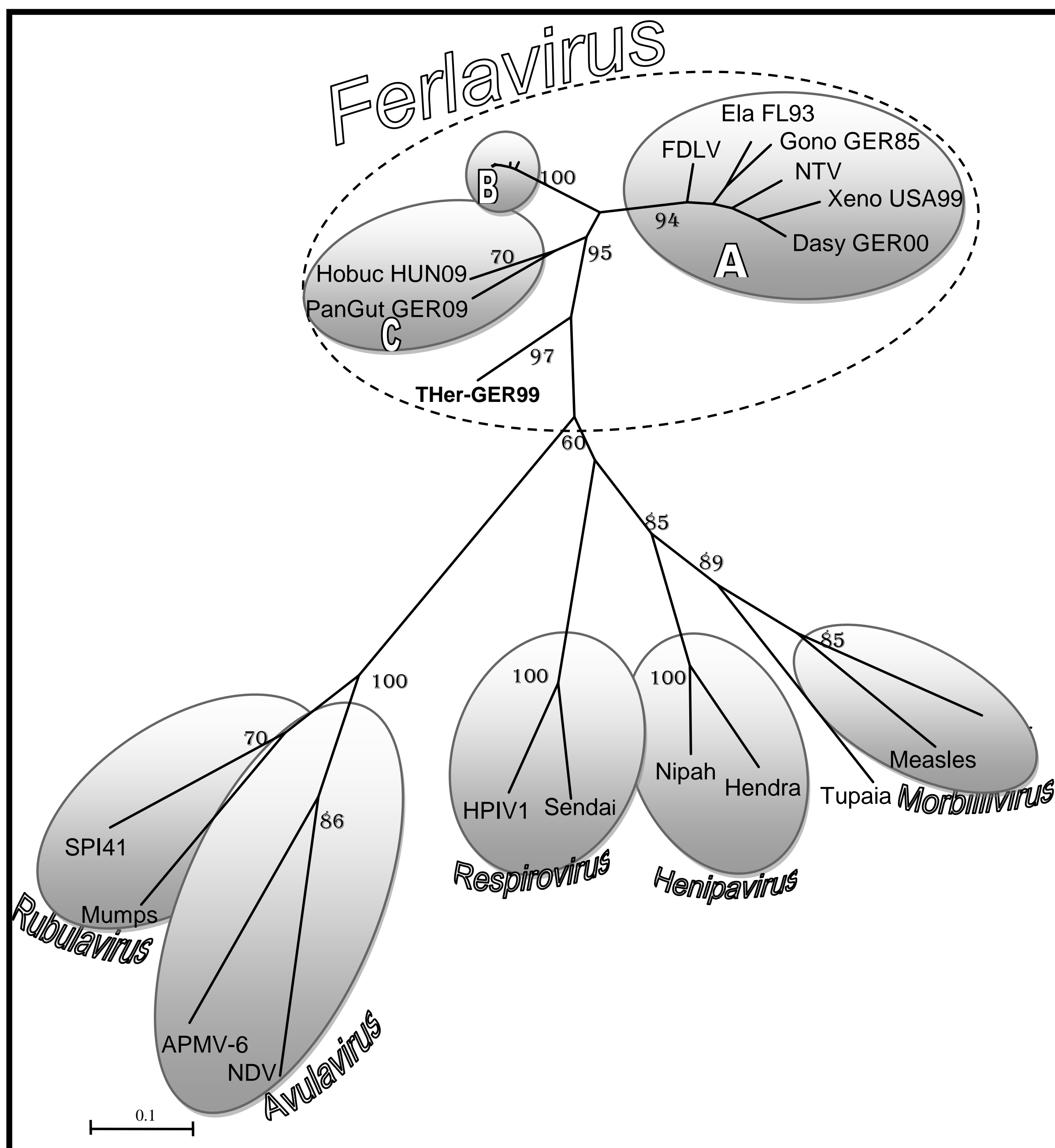


Figure 5B: Phylogenetic tree (Maximum likelihood analysis) based on partial L gene 463 nucleotides (nt).

Bootstrap values were calculated for 500 replicates. Values of 60% and higher are indicated beside the nodes, while the branches with lower values are shown with checkerboard lines. Clinical samples from this study are printed in italics. GenBank accession numbers: FDLV (NC_005084), Crot GER03 (GQ277611), Dasy GER00 (GQ277613), GonoGER 85 (AF349404), Igu GER00 (GQ277617), NTV (AF286045), Orth GER05 (GQ277616), Xeno USA99 (GQ277614), Pyth GER01 (GQ277612), Pangut GER09 (HQ148084), Hobuc HUN09 (JX186195), Ther GER99 (GQ277615). Avian PMV-6 (AY029299), NDV = Newcastle disease virus (AF375823), Hendra virus (AF017149), Nipah virus (NC_002728), HPIV-1 (AF457102), Sendai (NC_001552), Canine distemper virus (AF378705), Measles virus (AF266289), Mumps virus (NC_002200), SV-41 = Simian virus 41 (NC_006428) and Tupaia paramyxovirus (AF079780).

5.1.4 Evaluation of the nested RT-PCR method (Ahne et al., 1999) targeting the ferlavirus partial L gene and its efficiency as a standard diagnostic method

A product of approximately 566bp was obtained from a total of 61 samples from 50 animals. (For more details, see supplementary data, Table (1) in Appendix). The size range of these products was between 500 and 600 bp. All products within this size range were submitted for sequencing. No sequence was obtained from 49.18% (30 samples of the 61 reactive samples). The sequence obtained from 7 samples (11.47%) was bacterial in origin. Amplicons from an additional five samples (8.19%) were non specific, while 19 samples (31.14%) had ferlavirus specific sequences. Unspecific products of the expected size but not specific sequence made up 29.23% of the apparently positive samples (8 samples of the 27 that resulted in expected size products).

In order to increase the specificity of the RT-PCR multiple trials were performed targeting the highly conserved L gene.

5.2 Optimization trials

5.2.1 Optimization trials for the nested RT-PCR (Ahne et al., 1999) targeting a portion of the L gene.

Despite the changes in the annealing temperature (45, 48 and 51) and in the $MgCl_2$ concentrations (1, 1.5, 2, and 2.5) mM, all of the samples that had resulted in unspecific products in the original RT-PCR (listed in Table 3.17) also resulted in unspecific products in the adjusted RT-PCRs. The AdV positive sample gave false positive specific size products (~566 bp) with all three annealing temperatures used when concentrations of 2 and 2.5 mM $MgCl_2$ were used. However, when lower $MgCl_2$ concentrations were used with all three annealing temperatures, this sample gave unspecific size amplicons (350 bp of the DNA polymerase gene) which is the predicted size length when applying the AdV PCR (Wellehan et al., 2004). No signals were detected for negative controls (NC) in any of the trials. All three samples used as PC showed the specific size amplicon (566 bp) for all applications except for sample No 10 which showed either too faint or no amplicons when using 2.5 mM $MgCl_2$ at 45 and 51 ºC annealing temperatures and 2 mM $MgCl_2$ at 48 ºC annealing

temperature. All RT-PCR results of these trials are shown in the following figures (Figure 6.1- 6.3).

Sensitivity testing using ferlavirus isolates from cell culture showed that the RT-PCR using the new primers had a lower sensitivity than the original PCR. This PCR was therefore discarded as a possible alternative diagnostic method.

Figure 6.1: RT PCR targeting a portion of the L gene modified from Ahne et al. (1999) using an annealing temperature of 45 ^{0}C with four different concentrations of $MgCl_2$; (A) 1 mM, (B) 1.5 mM, (C) 2 mM, (D) 2.5 mM.

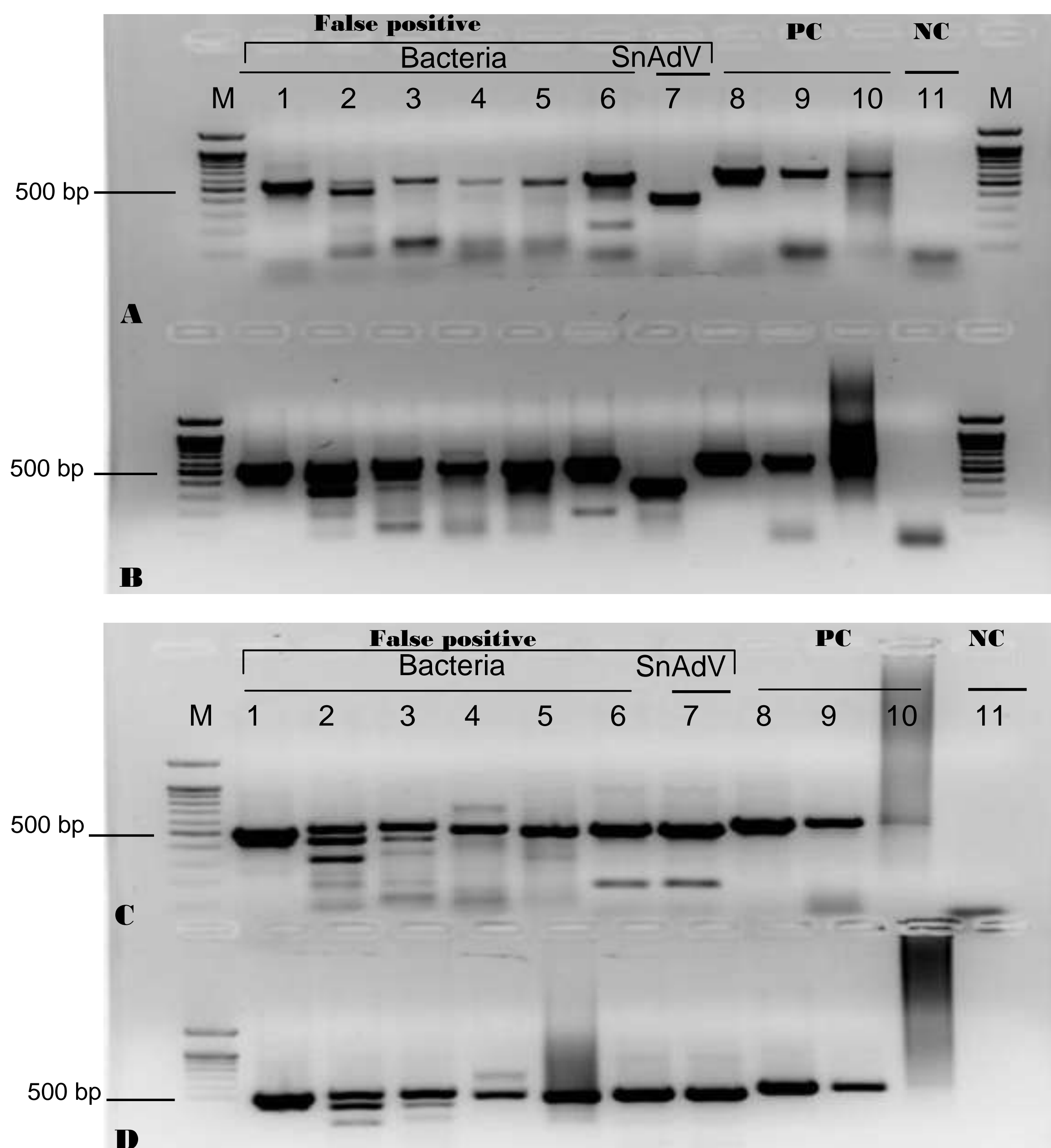


Figure 6.2: RT PCR targeting a portion of the L gene modified from Ahne et al. (1999) using an annealing temperature of 48 ⁰C with four different concentrations of $MgCl_2$; (A) 1 mM, (B) 1.5 mM, (C) 2 mM, (D) 2.5 mM.

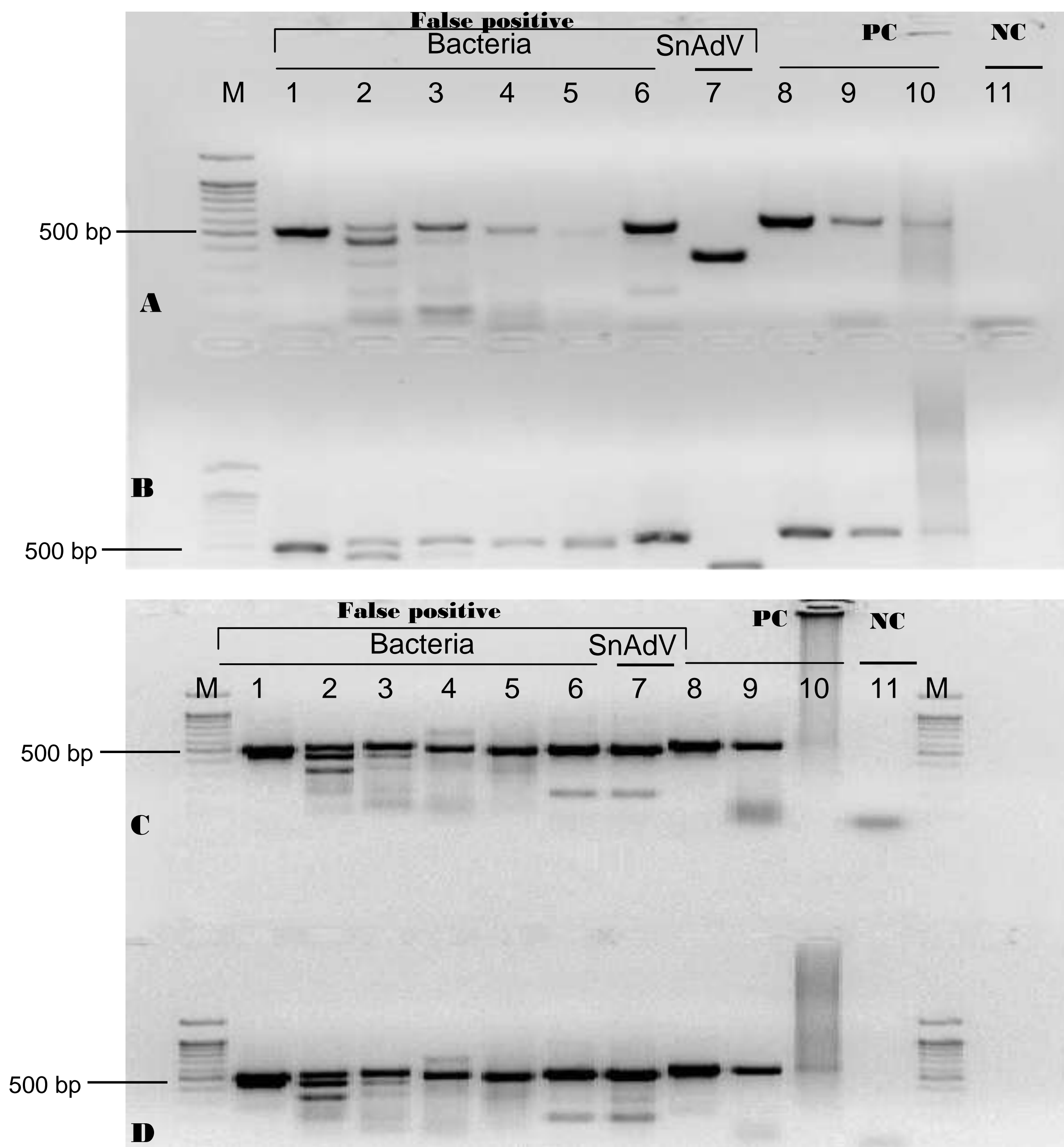
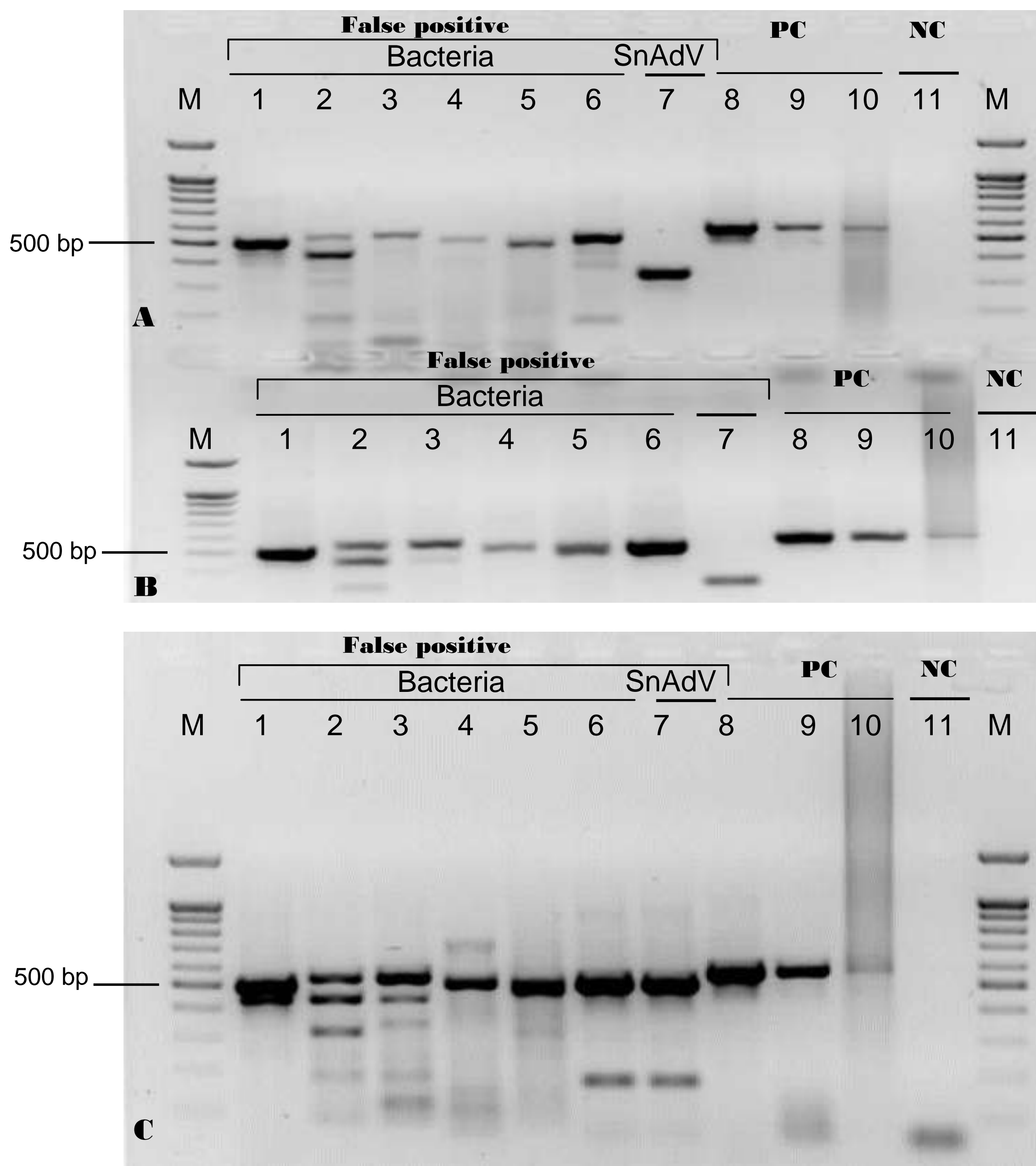
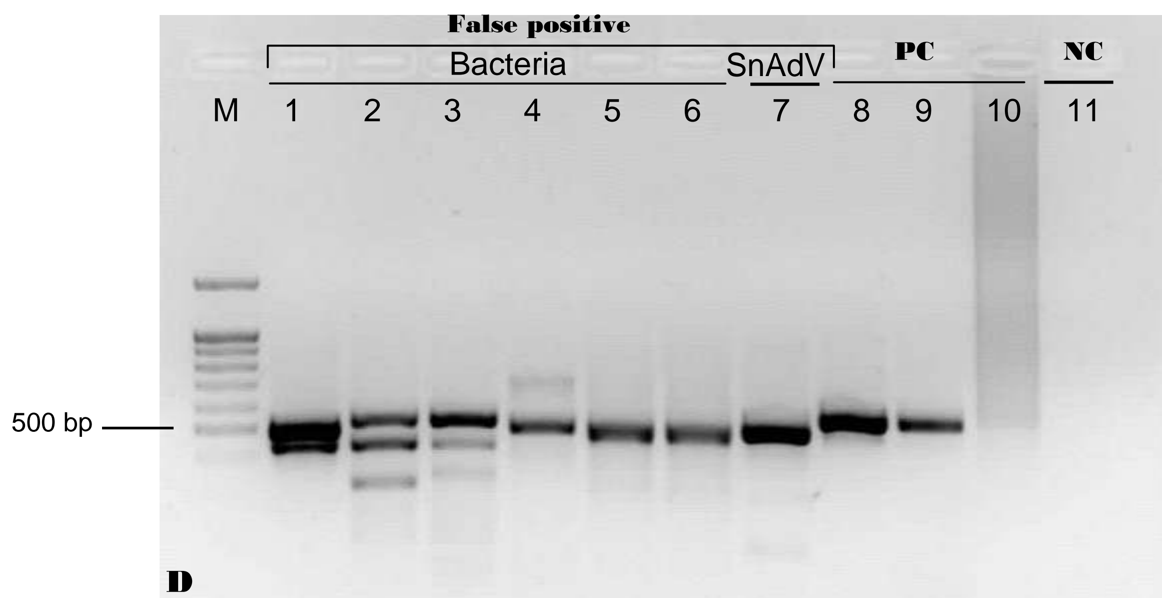


Figure 6.3: RT PCR targeting a portion of the L gene modified from Ahne et al. (1999) using an annealing temperature of 51 ºC with four different concentrations of MgCl2; (A) 1 mM, (B) 1.5 mM, (C) 2 mM, (D) 2.5 mM.





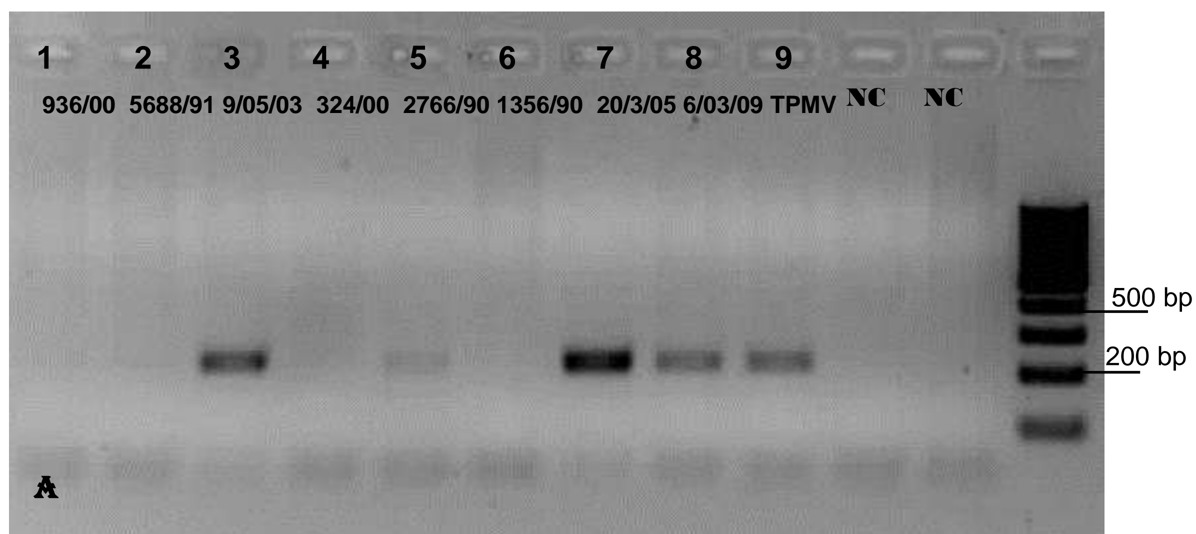
5.2.2: Designing new primers targeting partial L gene sequence

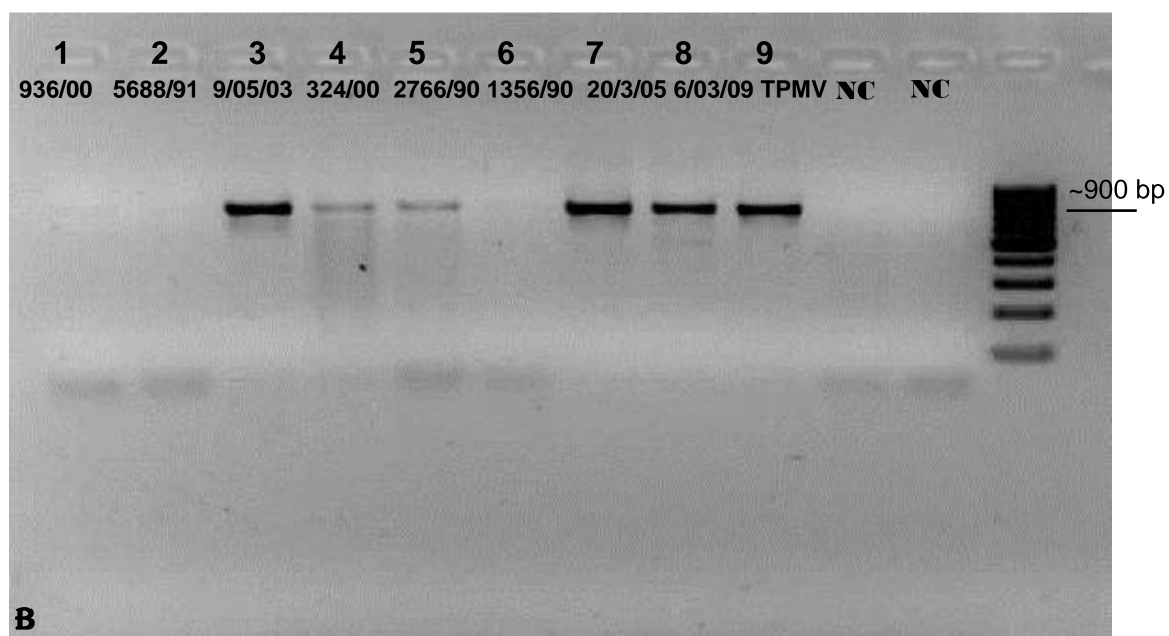
The following isolates: Dasy GER00, Pangut GER91, Crot GER03, Igu GER00, Vip GER90, Crot GER90, Orth GER05, Pangut GER09, Ther GER99 (Table 3.16) representing all genotypes of ferlaviruses known so far were used as PC for testing the new primers (Table 3.18). The most successful primer combinations of those recently designed (Table 4.3) which resulted in specific size products were; A- F2 in, R5 out in the 1st round and F3 in and R4 out in the 2nd round; B- F2 in, R6 out in the first round and F3 in and R5 out in the second round; and C F1, R6 in the 1st round and F3, R5 in the 2nd round.

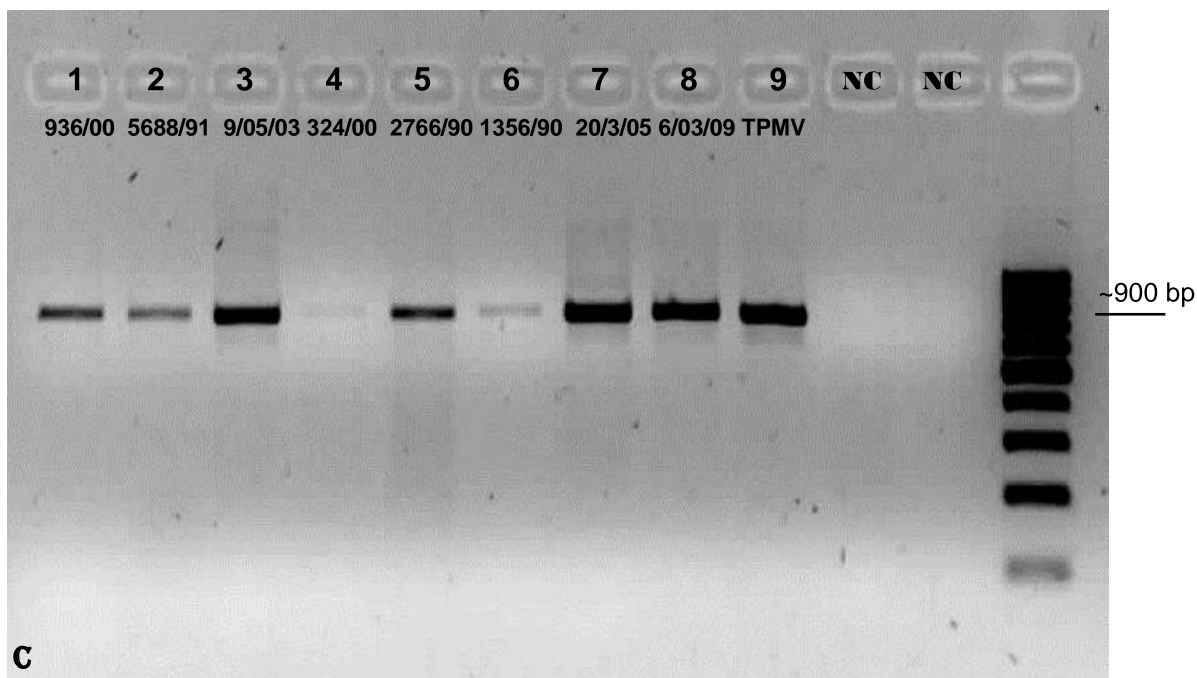
These nested combinations as shown in Figure 7.1 gave positive signals of the expected sizes (see in Table 4.3; ~200, ~800, ~900 bp products respectively). The rest of the primer combinations (D, E, F, G, H, I) either gave weak signals or failed to result in any amplicon. Among all primer combinations, C was the most sensitive as it was the only combination that resulted in positive amplicons from all ferlaviruses tested (100%) with the expected size product (929 bp). However, weak signals were detected for two isolates (324/00 and 1356/90). Primer combinations A and B only amplified RNA from some of the nine ferlaviruses tested: combination A amplified 44% and combination B amplified 66% of the nine isolates. No signals were detected for NC for all trials (Figure 7.1).

Figure 7.1: RT PCR targeting a portion of the L gene using primer combinations; A, B and C. using RNA from nine ferlaviruses isolates as templates.

(Primer combinations; see table 4.3)

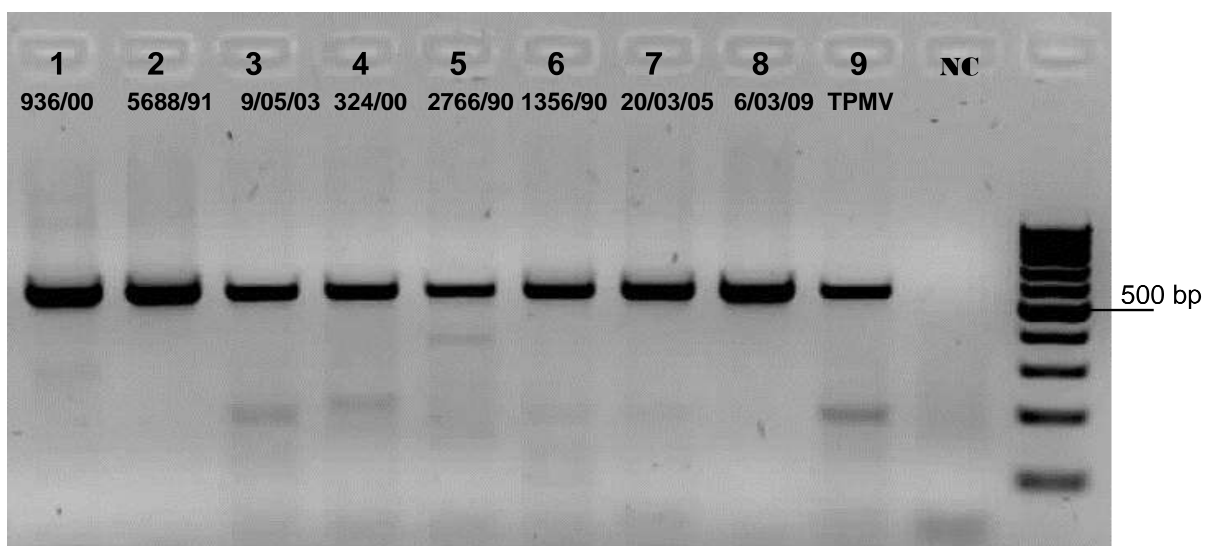






In order to compare the sensitivity of primer combination C with the already established RT-PCR described by Ahne et al. (1999) (illustrated in Table 4.2), the nested RT-PCR according to Ahne et al. (1999) was performed using the same RNA. Positive and strong signals (566 bp products) were obtained for all ferlaviruses as shown in Figure 7.2.

Figure 7.2: Nested RT PCR targeting a portion of L gene as described by Ahne et al. (1999) using RNA from a variety of ferlavirus isolates as templates.



5.3: Surface glycoprotein gene amplification

5.3.1: RT/PCR amplification and sequencing

Surface glycoprotein gene amplification was attempted for nine ferlavirus isolates (see Materials and Methods section 3.7.1 and Table 5.3.1). For the F gene 100% coverage was obtained for the following isolates: Orth GER05, Pangut GER09, Crot GER03, Igu GER00 and Crot GER90 and Vip GER90. For the rest of the isolates, the entire CDS region was obtained. However, the untranslated region located before the start codon is missing from these other isolates. Therefore, the coverage of the obtained F gene of these isolates are as follows: 98.83% Pyth GER01; 96.82% Var GER95; 97.66% Ther GER99 (supplementary data, Figure 2A in Appendix; deduced aa sequences of F gene). An overall coverage of 100% was obtained for the HN genes for all isolates (see supplementary data, Figure 2B) in Appendix; deduced aa sequences of HN genes).

The obtained sequences were concatenated based on overlapping sequences forming one continuous sequence including the F and HN as well as partial L gene sequences (supplementary data, Figure 1). The lengths of the concatenated sequences were as follows; 5334 bp for Var GER95, 5310 bp for Crot GER90, 5352 bp for Igu GER00, 5374 bp for Orth-GER05, 5366 bp for Vip GER 90, 5418 bp for Pyth GER01, 5430 bp for Crot GER03, 5370 bp for the Pangut GER09 and 5336 bp for the Ther GER99. The length of sequence that could be evaluated differed based on quality of sequence data of the L gene obtained. Sequence length of 5242 nt was used for phylogenetic analyses.

After importing the sequences into the BioEdit alignment program (Hall 1999), the beginning of the gene, start codon, CDS regions, end codon and intergenomic regions could be identified for each gene. The non-coding region of the F gene located subsequent to the stop codon was 95% conserved within the subgroup B isolates. However, this region showed only 67% similarity within subgroup A. For Pangut GER09, this region showed 57% similarity to squamate ferlaviruses (subgroups A and B) and 46% similarity to the corresponding region in Ther GER99.

The non coding region located before the start codon of the HN gene was 94% conserved within the subgroup B isolates, 70% within the subgroup A isolates.

Pangut GER09 (subgroup C) showed 83% similarity with isolates of subgroups A and B. However, the tortoise isolate showed only 30% similarity with the other isolates for this region. The non coding region located after the stop codon of the HN gene showed comparable results with that located after the stop codon of the F gene for all isolates. The non coding region located after the start codon of the L gene also showed comparable results to those of the HN gene. Gene starts and gene stops were 100% conserved among all ferlavirus isolates except for the gene start of the L gene in Pangut GER09 which was 88.89% conserved.

Three nucleotides were predicted to be intergenetic regions between the F, HN and L genes of the ferlavirus isolates. These regions usually consisted of CCT for all isolates except for the Pangut GER09 and the tortoise isolate which always had CTT as an alternative.

Any sequencing results showing any inconsistency were repeated at least once. All primer combinations resulted in the expected size products (see Materials and Methods section, Table 4.5). In the case of isolate Pangut GER09, new primers (F C) were designed and used for this isolate to amplify problematic regions. The tortoise isolate only gave positive results with F-HN primer combinations when Innu long taq polymerase (see Materials and Methods section Table 4.4) was used with the corresponding PCR conditions. New primer combinations (HN-L tortoise and HN tortoise) were designed and used as alternatives to the \`HN gene\` primers (Marschang et al., 2009) and \`HN-L\` primers which gave positive amplicons for all other ferlaviruses.

Table 5.3.1: Description of ferlavirus isolates analyzed in this study with the length of F, HN and L gene sequences obtained.

Isolate No/ year	**Isolate name**	**Sub group**	**Host**	**Species common name**	**Length of gene sequences obtained**		
					F	**HN**	**L**
3319/95	Var GER95	A	*Varanus prasinus*	Emerald tree monitor	1715 nt	1937 nt	1709 nt
368/01	Pyth GER01	B	*Python regius*	Ball python	1772 nt	1937 nt	1709 nt
9/5/03	Crot GER03	B	*Crotalus horridus*	Timber rattlesnake	1793 nt	1937 nt	1700 nt
324/00	Igu GER00	B	*Iguana iguana*	Green iguana	1793 nt	1937 nt	1622 nt
1356/90	Crot GER90	B	*Crotalus catalinensis*	Santa Catalina Island Rattlesnake	1793 nt	1937 nt	1580 nt
2766/90	Vip GER90	B	*Vipera palaestinae*	Palestine viper	1793 nt	1937 nt	1636 nt
20/3/05	Orth GER05	B	*Orthriophis taeniurus*	Beauty snake	1793 nt	1937 nt	1644 nt
tPMV/99	Ther GER99	-	*Testudo hermanni*	Hermann’s tortoise	1751 nt	1937 nt	1648 nt
6/3/09	Pangut GER09	C	*Pantherophis guttatus*	Corn snakes	1793 nt	1937 nt	1640 nt

5.3.2: Phylogenetic analyses

All ferlavirus isolates (Table 3.19) clustered together within the new genus *Ferlavirus* according to all phylogenetic analyses of both individual F (1728 bp) and HN (1937 bp) genes and the concatenated sequence of these genes with extended portions of the L gene (1544 bp) (supplementary data Figure 2 A&B, 1).

The obtained phylogenetic trees for both the F (Figure 8A) and HN (Figure 8B) genes demonstrate the same topology as in previous publications (Marschang et al., 2009; Abbas et al., 2011). Phylogenetic analyses using the complete sequence of the F gene showed the subgroup C isolate (Pangut GER09) clusters on a separate branch supported with high bootstrap values (74-100%) for all methods used. Using the UPGAMA method to analyze the HN gene led to the same results with a high bootstrap value of 96% for this branch, while the rest of the methods showed this isolate clustering with subgroup A isolates supported with lower bootstrap values (64-72%).

Phylogenetic analyses were conducted for the concatenated sequence of F, HN, and partial L genes. Depending on the methods applied, Pangut GER09 clustered either closer to subgroup B isolates (Bootstrap values 57-59%), or as its own subgroup, but supported with low bootstrap values (59%) (Figure 8C). Interestingly, when including members of other PMV genera in the analysis, Pangut GER09 clearly formed a separate subgroup supported with higher bootstrap values (98-100)% (Figure 8D). Regarding Ther GER99, all of the trees showed the same topology where this virus clustered within the genus as a sister group to the squamate ferlaviruses, supported with high bootstrap values (84-100%).

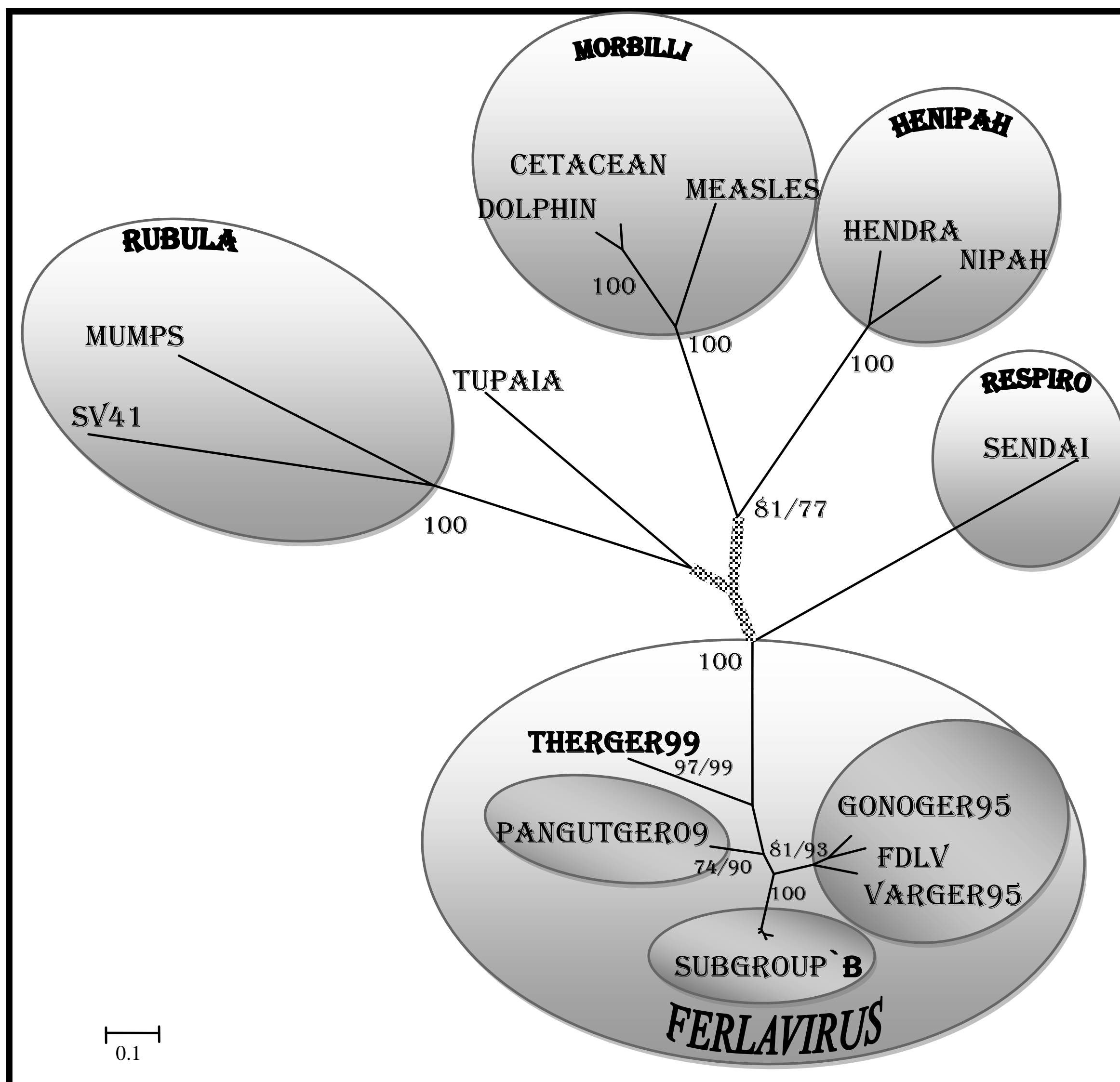


Figure 8A: Phylogenetic analysis of the full F gene CDS (1728 bp) using Maximum Likelihood analyses in Phylip program version 3.69.

Bootstrap values indicated on the branches are of both Maximum Likelihood and Neighbour Joining analyses which show the same topology and represented the percentage of 100, (500 for Neighbour Joining analysis) replicate trees respectively. Values higher than 60% are indicated at the branches, while branches supported with lower values are indicated with checkerboard lines. Subgroups A, B and C are framed with smaller circles. Ther GER99 is printed in bold. Full F gene CDS of FDLV AF251500 and Gono Ger 85 AY725422 were included to enhance the classification especially within subgroup A in reptilian PMV. Full F gene CDS from paramyxoviruses of different genera also used in the analysis: APMV-6/15072442, NDV |GI 259879797|gb|FJ772486.1|, Nipah |NV/MGI57282808, Hendra |GI29468603, Mumps SBL-1 gi|7672807|gb|AF143395.1|, (SV41) 55770820, Measles D-V/S gi| 162946413, Cetacean morbillivirus gi|520639 Dolphin morbillivirus gi|38490143, Tupaia gi|9634968, Sendai gi|11994992, HPIV-1 F gene,Oh-MVC11 gi|516851.

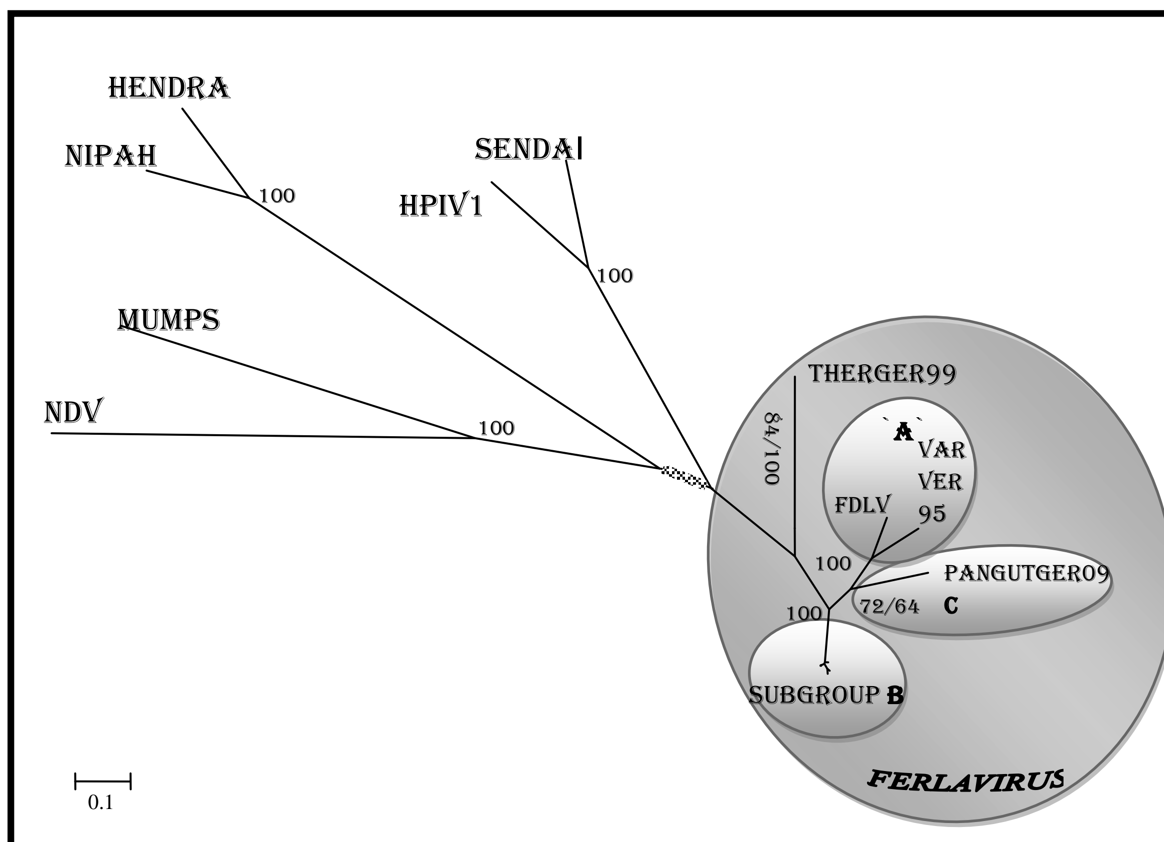


Figure 8B: Phylogenetic analysis of the full HN gene CDS (1937 bp) by Maximum Likelihood analysis in Phylip program version 3.69.

Bootstrap values indicated on the branches were calculated for 100 replicates and are for both ML and Fitch Margoliash analyses respectively. Values higher than 60% are indicated at the branches, while branches supported with lower values indicated with checkerboard lines. The *Ferlavirus* genus is framed with a large ovular circle with grey background. Subgroups A, B, and C are framed with smaller circles. Ther GER99 is printed in bold. Full CDS of FDLV NC_005084 is included. Full CDS of HN genes from paramyxoviruses of different genera were used as outgroups; Sendai NC_001552, HPIV1 NC_003461, Hendra NC_001906, Nipah NC_002728, Mumps NC_002200, NDV B1 NC_002617.1

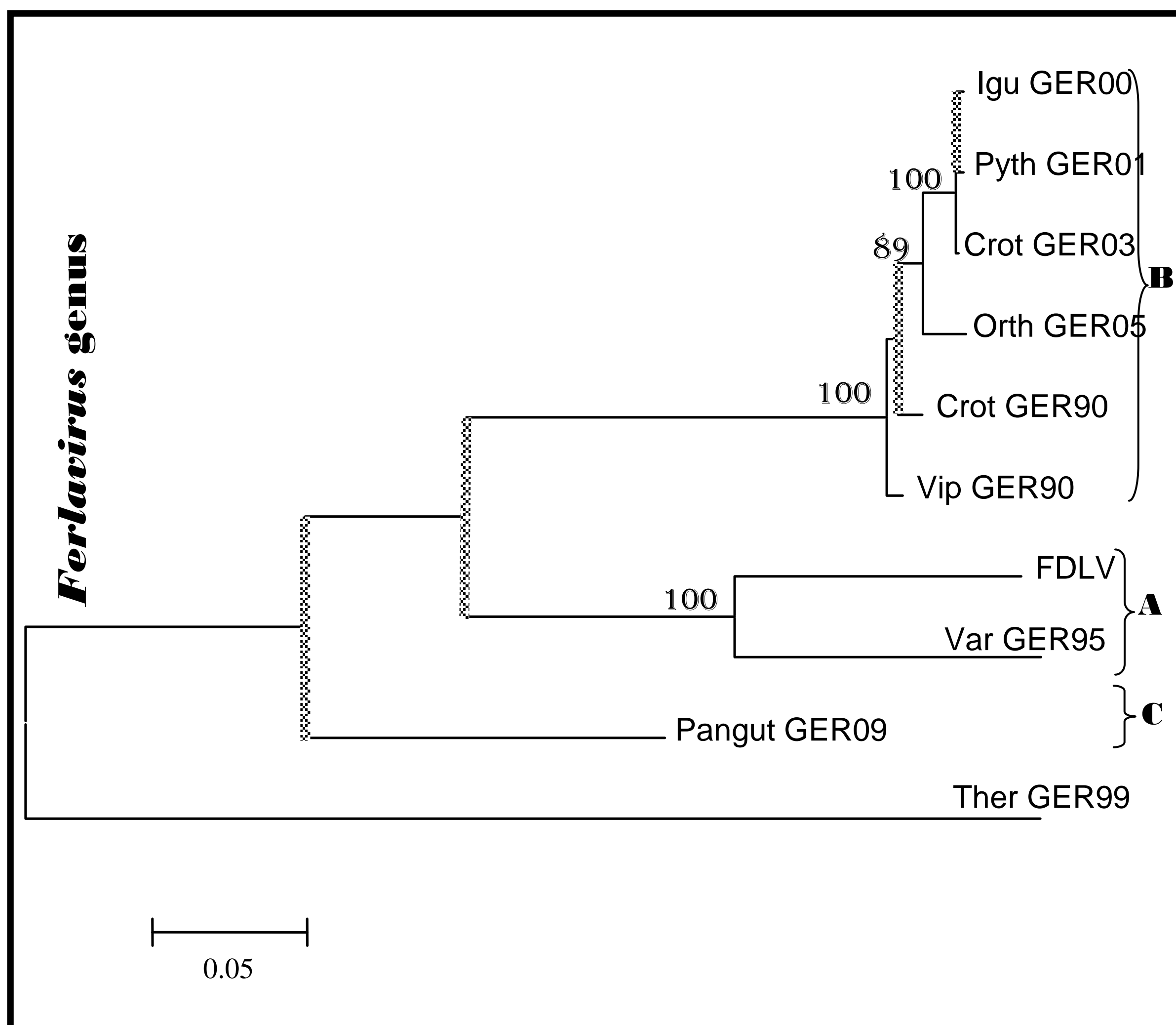


Figure 8C: Phylogenetic analysis of the concatenated sequence (5242 bp) of F, HN, and a large portion of L gene of 9 ferlaviruses.

The tree was created using the Maximum Likelihood analysis in the Mega 5.05 program. Bootstrap values indicated on the branches represent the percentage of 500 replicates with the same branches. Values higher than 60% are indicated at the branches, while branches supported with lower values are indicated with checkerboard lines

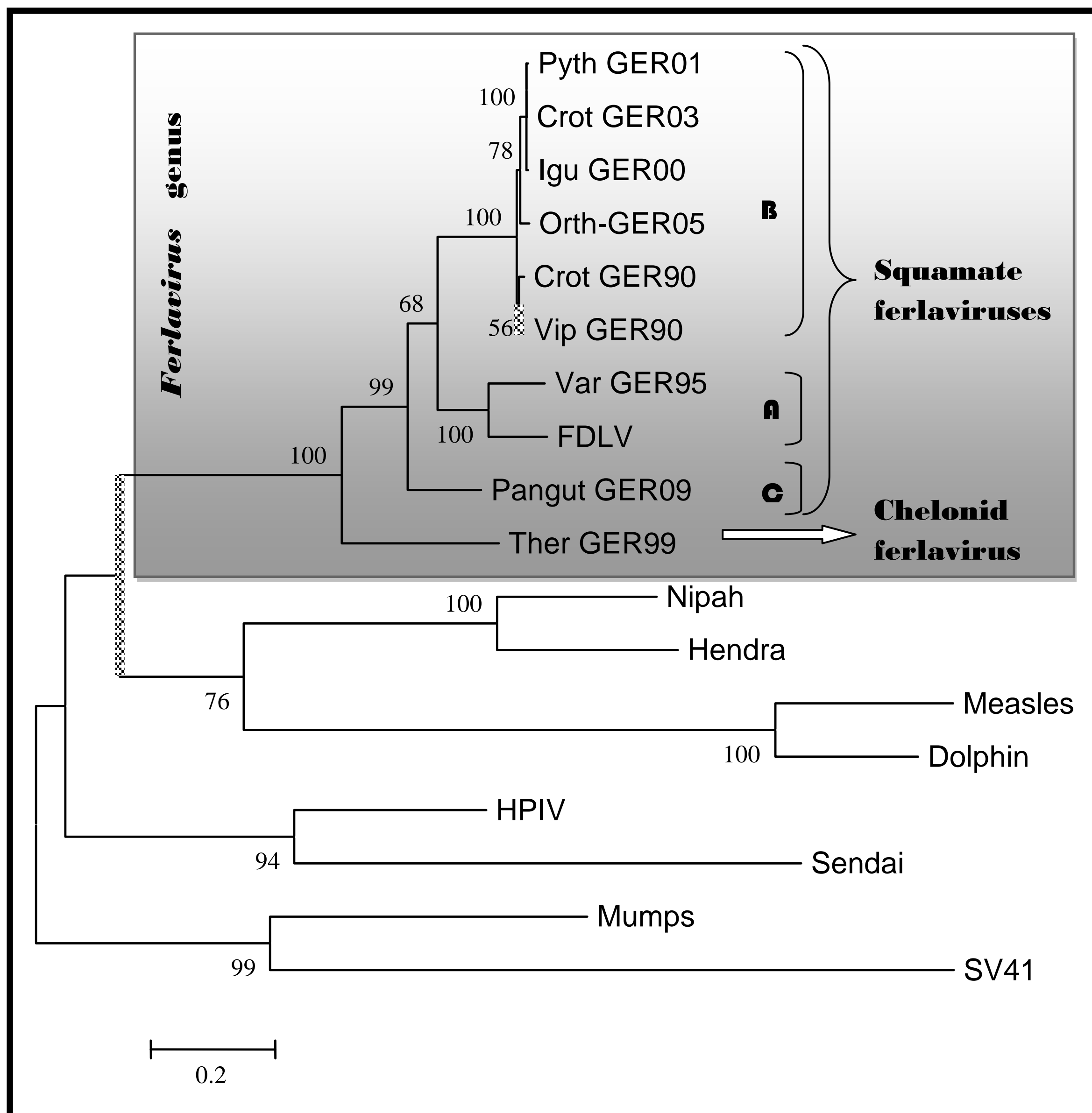


Figure 8D: Phylogenetic analysis of the concatenated F, HN, and L gene nt sequences (5242 bp).

Phylogenetic analysis of the concatenated F, HN, and L gene nt sequences (5242 bp) of nine ferlaviruses by ML method in Mega program version 5.05Bootstrap values above 60% are indicated on the branches and represented the percentage of 500 replicates with the same topology. Branches of lower values are drawn with checkerboard lines. GenBank accession numbers are as follows: Pangut GER09 (AY725422.1, HQ148085.1, HQ148084), Var GER91), FDLV (AY141760.2), Igu GER00(GQ277619- GQ277617), Crot-GER03 (GQ277611), Ther GER99 (GQ277615), Orth GER05 (GQ277620-GQ277616), Pyth GER01 (GQ277612), Crot GER90 and Vip GER90, Hendra virus (AF017149), HPIV-1 (AF457102), Dolphin Morbillivirus (AJ608288.1), Measles virus (AB016162.1), Mumps virus (AB040874.1), Nipah virus (AF212302.2), Sendai (AB039658.1), SV-41=Simian virus 41 (X64275.1).

5.3.3: Sequence identity matrixes

Sequence identity (ID) values were calculated for complete CDS and the deduced aa sequences of both F and HN genes for nine ferlavirus isolates: Var GER95, Pyth GER01, Crot GER03, Igu GER00, Crot GER90, Vip GER90, Orth GER05, THer GER99, Pangut GER09 (see Materials and Methods section Table 3.19) based on ClustalW alignment using the Phylip program package (version 3.69). The highest identity values for both genes were found between the squamate subgroup B isolates as follows: nt 96.6-99.8% and aa 98.8-100% for F gene (Table 5.3.2) and nt 97.1-99.8% and aa 99.1-99.8% for HN gene (Table 5.3.3). Identity values for F gene of subgroup A isolates showed moderate distances compared to subgroup B isolates (nt 78.3-79.1%; aa 91.9-92.1%) and subgroup C isolates (nt 77.1-78.1%; aa 90.8-91.1%). Comparable results were calculated for the HN genes of subgroup A isolates compared to subgroup B (nt 77.2-78.8; aa 90-91.3%) and C (nt 77.5-78.1%; aa 90-90.4%). For the Pangut GER09, identity values compared to subgroups A and B isolates were similar, supporting the classification of Pangut GER09 as the first representative of a third cluster within the *Ferlavirus* genus. ID values for the chelonid ferlavirus (Ther GER99) show equivalent distance to each of the squamate ferlavirus subgroups (A, B, and C) for both genes. However, these values are a bit higher for the F gene (nt 66.9-68.4%; aa 75.2-75.7%) than for the HN gene (nt 64.0- 65.6%; aa 70-72%).

ID values were also calculated for partial sequence of the L gene (1544 bp) for eleven ferlavirus isolates (Var GER95, Dasy GER00, Pangut GER91, Crot GER03, Orth GER05, Vip GER90, Pyth GER01, Crot GER90, Igu GER00, Pangut GER, Ther GER99) and found comparable to those found for F gene and higher than those for HN gene (Table 5.3.4). The squamate subgroup B had the highest identity values for L gene as follows: nt 97.3-99.8% and aa 98.8-100%. Identity values for partial L gene sequences among subgroup A isolates showed moderate distances compared to subgroup B isolates (nt 78.2-79.5%; aa 92.2-100%) and C isolates (nt 78.1-79%; aa 90.4-91.6%). ID values for the chelonid ferlavirus (Ther GER99) show equivalent distance to each of the squamate ferlavirus subgroups (A, B and C).

The U gene in squamate ferlaviruses encodes two ORFs (ORF-1 at position 68 aa and ORF-2 at position 87 aa) coding for 82 and 143/144 aa respectively (Papp et al., 2013 and Abbas et al., 2011). ID values were also calculated for the complete U gene CDS region of the nt sequence (246 and 429 bp) and the deduced aa sequence (82 and 143 aa) for ten ferlavirus isolates (Orth GER05, Igu GER00, Pyth GER01, Crot GER03, FDLV, Gono GER85, Dasy GER00, Xeno USA99, Biti CA98 and Pangut GER09). In general, nt and aa identity values between ferlaviruses are higher for the ORF-2 (77.4-99.5), (72.2-100)% than those for ORF-1 (76.7-100), (60.9-100)% respectively. For both, especially the aa ID values are lower than the corresponding values for the complete CDS regions of the F and HN genes and the partial sequences of the L gene (Table 5.3.5).

Table 5.3.2: Sequence identity matrix of ferlavirus F gene sequences.

Sequence identity values were calculated for the nucleotide (`Arial` letters) and the deduced amino acid sequences (`Times New Romans letters` and Italics) of the complete CDS of F gene (1728 bp) from eight squamate ferlaviruses representing subgroups A, B, and C and from the sole chelonid ferlavirus. All values are presented in percentage.

Subgroups	**A**	**B**	**C**
A	84.3	78.3-79.1	77.1-78.1
	94.8	*91.9-92.1*	*90.8-91.1*
B		96.6-99.8	
		98.8-100	
C		78.4-78.7	
		91.1-91.5	
Ther GER99	68.6-67.2	66.9-67.5	68.4
	75.2-76.1	*75.7-76.4*	*75.5*

Table 5.3.3: Sequence identity matrix HN gene

Sequence identity values were calculated for the nucleotide (`Arial ` letters) and deduced amino acid sequences (`Times New Romans letters `and Italics) of complete CDS of HN gene (1937 bp) from eight squamate ferlaviruses representing subgroups A, B and C and from the sole chelonid ferlavirus All values are presented in percentage.

Subgroups	**A**	**B**	**C**
A	84.4	77.2-78.8	77.5-78.1
	92.9	*90-91.3*	*90-90.4*
B		97.1-99.8	
		99.1-99.8	
C		77.5-77.7	
		90-90.6	
Ther GER99	64.4-65.6	64.3-65.0	64
	71.5	*71.8-72.0*	*70.6*

Table 5.3.4: Sequence identity matrix L gene

Sequence identity values were calculated for the nucleotide (`Arial ` letters) and deduced amino acid sequences (`Times New Romans letters `and Italics) of partial L gene (1544 bp) from ten squamatid ferlaviruses representing subgroups A, B and C and from the sole chelonid ferlavirus. All values are presented in percentage.

Subgroups	**A**	**B**	**C**
A	86.1-98.5	78.2-79.5	78.1-79
	96.3-99.8	*92.2-100*	*90.4-91.6*
B		97.3-99.8	
		98.8-100	
C		78.8-79.8	
		91.8-92.2	
Ther GER99	68.5-68.4	66.5-66.9	68.2
	72.7-73.3	*74.1-74.3*	*73.3*

Table 5.3.5: Sequence identity matrix U gene

Sequence identity values were calculated for both ORFs for the complete U gene CDS region of the nt sequence (ORF-1 246 and ORF-2 429 bp nt) (`Arial ` letters) and deduced amino acid sequences (ORF-1 82 and ORF-2 143 aa) (`Times New Romans letters `and Italics) from ten squamate ferlaviruses representing subgroups A, B and C. All values are presented in percentage. Normal print is for ORF-1, bold is for ORF-2.

Sub groups	A		B		C	
A	88.3-100	**87.5-99.5**	76.7-79.1	**77.4-78.6**	85.9-86.7	**81.6-83.8**
	71.9-100	***86.7-100***	*60.9-67*	***72.2-75***	*71.9-75.6*	***81.2-84***
B			95.9-100	**95.7-100**	77.1-77.9	**78.1-78.4**
			90.2-100	***97.2-100***	60.9-64.6	**75.6-76.3**

5.3.4 Fusion and attachment protein analyses

In order to assess the function of the predicted domains of the F and HN proteins, amino acid sequences of these proteins of 10 ferlaviruses including FDLV were aligned and compared (supplementary data, Figure 2 A&B).

5.3.4.1 Fusion protein analyses

Generally, subgroup A and B isolates each had a conserved sequence for each motif. However, the sequence motifs of each subgroup differed to some degree from those of the other subgroups. Pangut GER09 (subgroup C) revealed inconsistent results, occasionally showing the conserved sequence of subgroup A and/ or B. The chelonid ferlavirus revealed as well contradictory results for particular motifs, showing individual residues that are different from the sequences of the squamate ferlaviruses.

Signal peptide (SP) is the first motif predicted using the SignalP 2.0 software program (Table 3.21), at the N-terminus of the F protein at position 1-19 aa for FDLV

and Var GER95 (subgroup A). For all subgroup B and C viruses and the chelonid ferlavirus, this peptide was predicted at position 1-18 aa. In general, this motif was not conserved and had several hydrophilic residues for all ferlaviruses including those at the intermediate hydrophobic region at positions 7 and 8 for the Pangut GER09 and subgroup B isolates respectively where hydrophobic residues were substituted with hydrophilic ones.

The furin recognition site is the next predicted motif located at position 107-110 (labelled with a box) (supplementary data, Figure 2A) which consists of four amino acids upstream from the cleavage site (from -1 to -4) and one residue +1 downstream from that site. Fission of the C-terminus of this site would cleave the F0 into F1 and F2 subunits. This motif is conserved for all ferlavirus subgroups since all contain basic residues R, K and R at positions -1, -2, and -4 respectively. Contrary to all other ferlavirus isolates, the tortoise isolate has a strongly basic residue R at position-6, while members of subgroups A and B have slightly acidic amino acids (threonine and serine respectively) at the same position. At position +1 downstream from the cleavage site, all subgroups share the conserved hydrophobic and aromatic amino acid phenylalanine. In general, this motif is conserved and shows a high degree of similarity when compared with corresponding regions of members of the genera *Rubulavirus, Morbillivirus* and *Avulavirus.* However, henipah- and respiroviruses lack the paramyxovirus furin recognition site, instead they have a single basic residue at position -1 as illustrated in Figure 9.

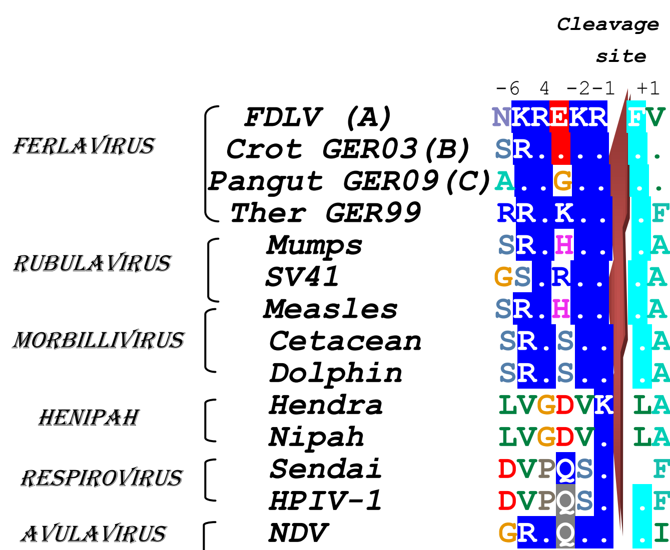


Figure 9: Furin recognition sites of F proteins of PMVs.

Deduced aa sequences of F gene of the squamate ferlaviruses (FDLV, Crot GER03, Pangut GER09) and the chelonid ferlavirus (Ther GER99) were aligned with corresponding regions of other PMVs. Members of the genera *Ferlavirus, Avulavirus, Rubulavirus* and *Morbillivirus* have identical furin recognition sites (RXKR), compared to members of the genera *Respirovirus* and *Henipahvirus* which lack this site.

The following motif is the highly conserved fusion peptide (FP) (Lamb & Kolakofsky, 2001) at position 111-135. As expected, this motif consists mainly of hydrophobic residues (V, A, L, I). At positions 112 and 118 (supplementary data, Figure 2A), Ther GER 99 has semi-conserved and conserved hydrophobic substitutions (F and I), while at position 114 this isolate has a non conserved substitution with an hydrophilic (T) residue compared to all other subgroups which have a hydrophobic residue there. In general, this motif is identical among all members of the squamate groups and shares a high degree of similarity with the corresponding sequences of other genera of the *Paramyxovirinae* (Figure 10).

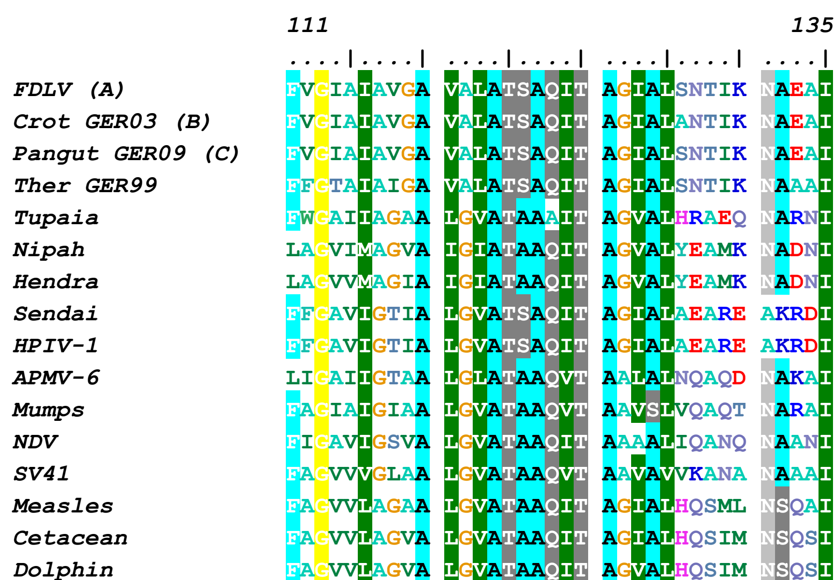


Figure 10: Fusion peptide regions of F proteins of several PMVs.

Deduced aa sequences of each subgroup of the squamate ferlaviruses (FDLV, Crot GER03, Pangut GER09) and the chelonid ferlavirus (Ther GER99) were aligned with corresponding regions of the FP of other PMV genera which consist mostly of hydrophobic amino acids.

HRA and HRB were predicted for all PMV isolates. These repeats were assumed to contain seven residues in the arrangement from (a) to (g) which was found to repeat six times at positions 145–186 and 461–502 respectively. A high level of similarity was noticed within HRA for all ferlavirus isolates, where it contains mainly hydrophobic and/or non-polar residues at positions a & d. At these regions HRB was also conserved and/or semi conserved for all ferlaviruses presenting non polar or hydrophobic residues with the exception of the tortoise isolate which had a hydrophilic serine residue instead of the hydrophobic alanin at position d of the fifth repeat. A leusine zipper (LZ) was detected within the HRB from 461~482 using the motif scan option and the leusine zipper prediction tools in the GENtle program. This motif was mapped to the hydrophobic residues (leucine and/or Isoleucine) at position (a) of this motif for all ferlaviruses (supplementary data, Figure 2A). As reported previously by Franke et al. (2006), all residue differences at positions other than a & d of both repeats were substituted with similar amino acids.

The last motif is the transmembrane domain TM which was predicted for all ferlavirus isolates at position 502-520 at the C- terminus end of the F protein using the

TMHMM Server 2.0 (2009). This motif had several changes for all squamate ferlaviruses, although most of them retain hydrophobic residues except for the chelonid ferlavirus isolate which has two hydrophilic residues (S and T) at positions 503 and 508.

Glycosylation site (N-G) detection was done using NetNGlyc 1.0 (2002) for the full length sequence of F protein for 10 ferlaviruses, including FDLV (as a reference). Three probable N-G positions were predicted for FDLV at locations 102, 436, and 443 aa. These results correspond with previous findings from Franke et al. (2006) when full length sequence of F gene from FDLV and Gono GER85 were analyzed and used to predict a model for the F protein of PMV of reptilian origin. The first N-G found at position 102 aa for FDLV was not detectable for any other ferlavirus isolates (Table 3.19). For Var GER95 (subgroup A), the second and the third N-G positions were detected at analogous sites compared to FDLV. For subgroups B, C, and the chelonid ferlavirus, these sites were also detected, though moved back to positions 435 and 442 respectively. The last N-G site predicted at position 442 aa for the chelonid ferlavirus did not pass the threshold. Nevertheless, these results are based on a computer modeling program and it is not defined whether these sites are truly glycosylated.

5.3.4.2 HN protein analysis

The first predicted motif in the HN protein was the cytoplasmic tail lying at the N-terminus which spans residues 1-25 aa. This domain was likely to be conserved except for the region 12-23 where many non-conserved substitutions were observed.

The next predicted domain using TMHMM Server 2.0 (2009) represents the transmembrane domain (TM) at positions 28 to 50 for all squamate ferlaviruses which is in agreement with previously published studies (Kurath et al., 2004). For the tortoise isolate, this motive was predicted at positions 31-49 using both TMHMM Server 2.0 (2009) and transmembrane prediction tools in the GENtle program. This motif was conserved containing hydrophobic residues for all ferlaviruses. For the chelonid ferlavirus, non conserved residues were observed at the N terminus of this domain, however, all remain hydrophobic.

According to the predicted model of NDV HN protein (Yuan et al., 2011), the stem can be mapped to the residues from 68 to 115. Sequence alignments of the HN proteins of several PMVs including nine ferlavirus isolates along with other PMV genera (respiro-, morbilli- and rubullaviruses) with the corresponding regions from NDV revealed that all substitutions in this region including those mapped to the outer surface and the hydrophobic core of the stalk were more conserved within PMV genera than between the genera. In general, all residues were conserved within the *Ferlavirus* genus compared to all other genera except for the tortoise isolate (Figure 11). Some non conserved residues were predicted for the chelonid ferlavirus especially at the fusion interaction sites (68, 80, 96) These sites correspond to aa 69, 81, and 97 of the NDV HN respectively.

Next to the stem region starting from 127 until the end of the HN protein is the head region which was highly conserved except in the chelonid ferlavirus at several positions (from 224 to 228, from 271 to 279, and from 345 to 360).

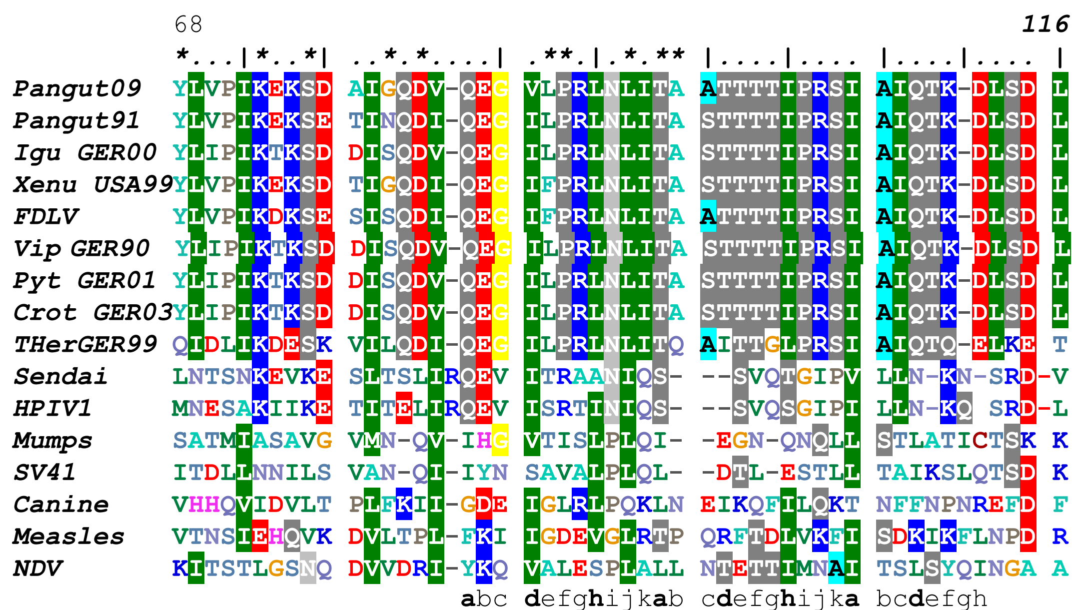


Figure 11: stalk regions of HN protein of several PMVs

Deduced aa sequences (HN gene) represent the predicted Stalk regions of squamate ferlavirus HN proteins (FDLV, Crot GER03, Pangut GER09, Pangut GER91, Igu GER00, Xeno USA99, Vip GER90, Pyt GER01) and the chelonid ferlavirus HN protein (Ther GER99). This region was aligned with the corresponding regions of other PMVs of other genera. Residues that mainly influence the fusion process by interfering with F-HN interaction are indicated by an asterisk. Residues a, d and j form the hydrophobic core of the 4HR of the NDV stalk region.

The subsequent position (246-251 aa) is the sialic acid binding site N-R-K-S-C-S. This site was found to be 100% conserved within HN proteins among several members of many PMV genera including: *Ferlavirus, Respirovirus, Rubulavirus* and *Avulavirus*, compared to henipaviruses (G protein) which revealed 40% similarity (Figure 12).

Genus	Virus	....|....| (5)
FERLAVIRUS	FDLV	RKSCSTVAQG
	pangut GER09	RKSCSTVAQG
	Var GER95	RKSCSTVAQG
	Pyt GER01	RKSCSTVAQG
	Igu GER00	RKSCSTVAQG
	Crot GER03	RKSCSTVAQG
	Orth GER05	RKSCSTVAQG
	Vip GER90	RKSCSTVAQG
	Crot GER90	RKSCSTVAQG
	Ther GER99	RKSCSTVALG
HENIPAH	Hendra	IHHCSSTYHE
	Nipah	VYHCSAVYNN
RESPIROVIRUS	HPIV-1	RKSCSVIAAG
	Sendai	RKSCSVVATG
RUBULAVIRUS	Mumps	RKSCSIATVP
AVULAVIRUS	NDV	RKSCSVSATP
	apmv 1	RKSCSVSATP
	apmv 3	RKSCSVTAGA
	apmv 2	RKSCSIVASK
	apmv 4	RKSCSVAAGS
	apmv 5	RKSCSIVATT
	apmv 6	RKSCSIIANP
	apmv 7	RKSCSIAATG
	apmv 8	RKSCSIVATQ
	apmv 9	RKSCSISATP

Figure 12: Putative sialic acid binding sites HN proteins of several PMVs.

Deduced aa sequences (HN gene) of squamate ferlavirus (FDLV, Crot GER03, Pangut GER09, Igu GER00, Vip GER90, Pyt GER01, Var GER95, Orth GER05, Crot GER90) and the chelonid ferlavirus (Ther GER99) were aligned to the corresponding regions of PMVs of other genera. The putative sialic acid binding sites R-KS-C-S found to be identical for all viruses except for the henipahviruses.

In the alignment of seven ferlavirus (FDLV, Pangut GER91, Xeno USA99, Igu GER00, Vip GER90, Pyt GER01 and Crot GER03) HN proteins with avian PMV (apmv) 1-9 HN proteins, a conserved region was noticed within the head structure as illustrated in Figure 13.

```
             247 .|....| ....|....| ....| 274
FDLV         RKSCSTVAQG RGAYLLCTNV VEDERTDY
Pangut GER91 RKSCSTVAQG RGAYLLCTNV IEDERTDY
Xeno USA99   RKSCSTVAQG RGAYLLCTNV VEDERTDY
Igu GER00    RKSCSTVAQG RGAYLLCTNV IEDERTDY
Vip GER90    RKSCSTVAQG RGAYLLCTNV IEDERTDY
Pyt GER01    RKSCSTVAQG RGAYLLCTNV IEDERTDY
Crot GER03   RKSCSTVAQG RGAYLLCTNV IEDERTDY
apmv 1       RKSCSVSATP LGCDMLCSKV TGTEEEDY
apmv 3       RKSCSVTAGA GVCWLLCSVV TESESADY
apmv 2       RKSCSIVASK YGCDILCSIV IETENEDY
apmv 4       RKSCSVAAGS GHCYLLCSLV SEPEPDDY
apmv 5       RKSCSIVATT EGCDVLCSIV TQTEDQDY
apmv 6       RKSCNIIANP NGCDVLCSLV KQTENEDY
apmv 7       RKSCSIAATG FGCDILCSVV TETENDDY
apmv 8       RKSCSIVATQ FGCDILCSIV IEKEGDDY
apmv 9       RKSCSISATP LGCDLLCAKV TETEEEDY
```

Figure 13: Deduced aa sequences represent the predicted head structure in the HN protein of several PMVs.

Deduced aa sequences (HN gene) of several ferlaviruses (FDLV, Crot GER03, Pangut GER91, Igu GER00, Vip GER90, Pyt GER01, Crot GER03) were aligned to the corresponding regions of nine aPMVs. The predicted head structure (HN protein) found to be conserved for all viruses.

In the aa alignment of the HN proteins of all ferlaviruses with members of the respiro, avula-, and rubula viruses, the sequence GAEGR, was conserved, showing 80-100% identity for all members except for the henipahviruses that showed only 20% similarity (Figure 14).

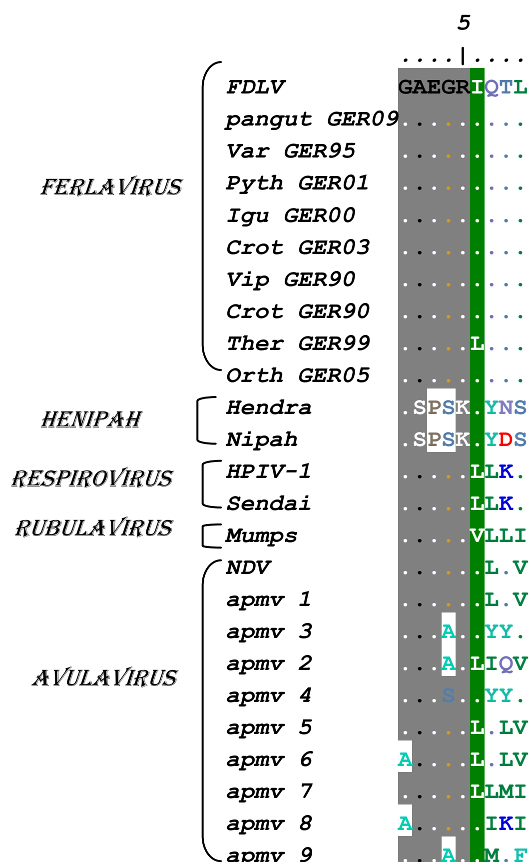


Figure 14: Deduced aa sequence GAEGR of HN gene of several PMVs

Deduced aa sequence of HN gene of several ferlaviruses (FDLV, Pangut GER09, Igu GER00, Vip GER90, Pyt GER01 and Crot GER03, Var GER95, Crot GER90, Ther GER99) were aligned with the corresponding regions of PMVs of other genera. The following sequence G-A-E-G-R showed a high degree of similarity.

For the prediction of potential N-glycosylation sites, the program NetNGlyc.1.0 was used. This led to the detection of two possible N-glycosylation sites for all ferlavirus isolates included in this study at positions 128 and 163 and two additional sites for the tortoise isolate, at positions 277 and 496.

6 Discussion

6.1 Prevalence of ferlaviruses in diagnostic samples

6.1.1 Ferlaviruses infecting snakes

The molecular diagnostic tool PCR/RT-PCR has enhanced our capability to identify and characterize pathogens. It has also enabled us to optimize diagnostic methods and understand specificity and sensitivity of these methods. Sequencing portions of viruses has contributed to taxonomic classification of new viruses and to widening our understanding of the possible factors involved in viral pathogenicity. It also provides the potential for understanding the genetic basis for presence or lack of species specificity.

There are actually a number of different factors that might lead to false negative results, including mutations in the primer binding sites and inhibitory substances in samples. Due to the possibility of RNA loss starting from the first step of the sampling procedure and then more specifically during RNA preparation, the present results might not reflect the real virus content. Therefore, the use of internal positive controls would be of great importance to examine the occurrence of inhibitory substances and to control RNA preparation as has been suggested by Vainionpa and Leinikki (2010)

In the current study, the infection rate (5.97 %) was low compared with other studies (Papp et al., 2010; Pees et al., 2010) which showed infection rates of 23% and 9% respectively. This might indicate that there are fluctuations in the infection rates in the snake population screened. However, the actual study populations of each study were not representative, as each used samples submitted to diagnostic laboratories. In the previously mentioned studies, the ultimate diagnosis was also based on RT-PCR results and not on sequencing results.

A study using virus isolation in cell culture for the diagnosis of ferlavirus infections in snakes showed highest recovery rate of ferlaviru*s* from the lungs of infected snakes (Blahak, 1995). Therefore, tracheal washes have been suggested for viral detection (Pees et al., 2010). In our study, the lung gave the most reliable positive results from the dead positive PMV snakes, followed by intestine which is consistent with the

previous results from Papp et al. (2010). The use of several different organs for the diagnosis of viral RNA in dead snakes, including lung and intestine, would possibly enhance the chances of virus detection. Interestingly, all ferlaviruses isolated in our study were from intestine and kidney, while no isolates were obtained from the lung compared to reoviruses, which were mainly isolated from lung, intestine and kidney of infected snakes.

Diseased snakes, showing mainly pneumonia and/or CNS signs were often documented without detectable ferlaviruses (supplementary data, Table 1) which corresponds to the previous findings from Papp et al. (2010). The majority of the snake samples tested in our laboratory were from clinically ill snakes that were collected by veterinarians and practitioners for analytic examination rather than random samples. Pees et al. (2010) has documented no significant correlation between the presence of PMV and clinical findings, when screening 100 viperid and boid snakes via RT-PCR. Asymptomatic seropositive and RT-PCR positive animals were documented in a recent study from Abbas et al. (2012) where healthy bearded dragons from a rescue centre in Munich were screened for the presence of ferlaviruses. The fact that not all ferlavirus infected squamates are sick could theoretically be dependent on several factors including; host species, viral pathogenecity factors, immune status, and time post infection.

The effects of sampling bias when screening only diseased snakes should therefore be considered when evaluating the prevalence of ferlaviruses in snake populations. Consequently, the infection rate obtained in these kinds of studies may not reflect the real occurrence of viruses.

Pees et al. (2010) underlined the significance of screening healthy collections of snakes for ferlaviruses and referred to the threat of unobserved contagious disease in these collections. A possible method to reduce the degree of sampling bias would be to screen both symptomatic and asymptomatic snakes for viruses, which can only be achieved by conducting entry examinations and screening for each newly established collection or when introducing new snakes into previously established groups.

In general, ferlavirus infections are clearly associated with respiratory disease in snakes, though CNS is also observed. Severe cases are most often doccumented in viperid snakes, though ferlaviruses have been detected in several snake families (Boidae, Pythonidae, and Colubridae) (Jacobson et al., 2007).

Koch's postulates, proving a relationship between the pathogen and the disease, have only been fulfilled once for ferlavirus infections in reptiles. A transmission study was carried out with a PMV in Aruba Island rattlesnakes (*Crotalus unicolor*) associated with pulmonary lesions. Snakes inoculated intratracheally with PMV isolated from Aruba Island rattlesnakes also developed disease and the virus could be reisolated from the infected animals. Two snakes of the test group in that study were found dead on day 19 and 22 post inoculation. Extensive hemorrhage was seen in the mouth and lung in one of these snakes (Jacobson et al., 1997).

In the case of multiple viral infections (PMV, reo and adeno viruses) in a group of captive corn snakes (Col-1, Col-1a~f), suspected transmission of ferlaviruses can be traced where six live snakes and one dead from a total of thirteen live corn snakes of one enclosure were found to be ferlavirus positive with the same sequence pattern (Pangut GER09) (Table 5.1.1). The source of the infection in this group might the addition of new untested animals to form new population. In the consecutive stock after dispersing all animals from the previous one and importing ten new snakes (54/09, supplementary data, Table 1), inappropriate disinfection of the terrarium could have played a role in transmitting the same types of non enveloped viruses (reo and adeno viruses) to the newly introduced animals whereas the environmentally less stable PMV was no longer present. In addition, concentrating on a particular pathogen (e.g. PMV in snakes) can lead to underestimation of the significance of other infectious agents. Multiple viral infections should therefore be considered when screening snakes for the presence of viruses (Abbas et al., 2011).

6.1.2 Sequencing and phylogenetic analyses

In the current study, all RT-PCR positive results were further verified by sequencing in order to discard all false positive results which indicate the presence of nonspecific products (other viruses, bacteria) as will be discussed in the next section (6.2) Identical sequences from different snakes from one enclosure (Col-1, 1a~f) & (Pyt-1a~c) and/or from several organs from one animal (Pyt-5) were obtained as previously documented by Ahne et al. (1999), Franke et al. (2001), and Papp et al. (2010). A ferlavirus 97% similar to FDLV Subgroup A (Acc. No: AY141760.2) was found in a single case (Pyt-2). This virus differed clearly from all other viruses detected in this study.

Several reports (Ahne et al., 1999; Marschang et al., 2009; Franke et al., 2001) have shown that ferlaviruses lack species specificity. In Marschang et al. (2009), ferlaviruses from lizards clustered within groups of snake isolates; Papp et al. (2010a), detected two ferlaviruses in a leopard tortoise clustered with those of squamate origin and did not form a separate cluster. In a recent study from Abbas et al. (2012), a ferlavirus was detetected in bearded dragons (*Pogona vitticeps*) (100% identical to Pyth GER01) that was originally isolated from a python. In another case from a python (Pyt-5), this snake was infected with a ferlavirus that was 100% identical to Igu GER00 which was originally isolated from an iguana indicating no species specificity. No signs of host species specificity were obtained when conducting phyogenetic analyses based on concatenated sequences of the full F, HN (CDC regions) and the extended portion of the L gene, where isolates from lizards could be found among both subgroup A and B members mixed with snake ones. In addition, the close genetic relation between ferlaviruses from such different hosts (snake, lizard, and tortoise) indicates that ferlaviruses may cross species barriers quite readily.

Of the PMV positive amplicons, except Pyt-2 (234 bp L gene), which was not included in the phylogenetic analyses, 60% (3 of the total 5 amplicons) clustered to subgroup B, while the remaining 40% clustered to the newly proposed subgroup C within the *Ferlavirus* genus (Figure 5A).

In one case (Pyt-2), the ferlavirus was 97% similar to FDLV (subgroup A). No other cases detected in this study belong to subgroup A despite the fact that viruses from both subgroups A and B have been detected in Germany. The only subgroup A viruses that have been described so far that orginated outside of Germany are Xeno USA99 and FDLV, which were originally found in the US and Switzerland respectively.

None of the detected ferlaviruses clustered in relation to geographical provenance or year of detection/isolation. There were also no epidemiological relations among the ferlaviruses detected in our study as they were all detected from diverse groups from different countries at different intervals.

In all methods used to analyse the L gene sequences obtained from the diagnostic samples, the first two representatives of the subgroup C isolates (Pangut GER09 and Hobuc HUN09) clustered together within the *Ferlavirus* genus supported with good bootstrap values (61-83%). These two isolates sometimes clustered close to subgroup B isolates or clustered as a seperate branch depending on the method applied. A similar inconsistency was noticed when assigning subgroup C isolates as in previous publications (Abbas et al., 2011; Papp et al., 2013) in which these viruses sometimes clustered with subgroup A or B viruses or in their own subgroup within the *Ferlavirus* genus depending on the gene targeted and the method applied. In general, this discrepancy was solved when conducting phylogenetic analyses based on the concatenated sequences of the F and HN genes and larger portion of L gene (as will be discussed in section 6.3).

Initially, the sole chelonid ferlavirus isolate was classified in the *Ferlavirus* genus as the first member of a chelonid group based on a portion of L gene sequence (Marschang et al., 2009). Unfortunately, in that study, no sequence data could be obtained from HN or from U genes of the Ther GER99 isolate which are of particular importance to assign the proper position of this isolate in relation with squamate PMVs. However, it was clear from the phylogenetic positioning of the partial L gene sequence that it clustered as an outgroup to the squamate PMVs within the new genus *Ferlavirus* and was never assigned to any of the existing subgroups. These phylogenetic results were further confirmed by later studies from Abbas et al. (2011) and Papp et al. (2013).

Several attempts have been carried out so far to amplify a portion of the U gene from the tortoise isolate, however, all trials gave no signal (Rösler, 2011). The lack of U gene amplification products might be attributed to either the lack of this gene in the tortoise isolate or to the high sequence differences at primer binding sites due to the higher variability in nucleotide sequences of this gene compared with HN and L genes as has been shown in our results and which corresponds to the previous findings from Marschang et al. (2009).

It is therefore still not clear whether the tortoise isolate does have the U gene to be further classified as the fourth cluster (subgroup D) within the *Ferlavirus* genus, or not and instead forms its own cluster (chelonid PMV) outside of the genus *Ferlavirus* within the subfamily *Paramyxovirinae*. Additional trials to discover the existence of this gene are of great value to reach the proper phylogenetic localization of the tortoise PMV in relation to the *Ferlavirus* genus and its groups.

6.2 Evaluation of the RT-PCR method and optimization trials

In 1999, Ahne et al. described a nested RT-PCR protocol targeting a portion of the conserved L gene of the ferlaviruses (FDLV). Since then, this protocol has been established as a standard molecular diagnostic technique for the detection of ferlaviruses in reptiles. Usually, this procedure results in amplicons of 566 bp that can then be used for subsequent sequencing, which opens possibilities for rapid categorization and provides a closer look into their taxonomic relationship and epidemiological investigations (Ariel, 2011). Previous studies (Papp et al., 2010; Pees et al., 2010) recommended using the previously established protocol for detecting ferlaviruses in snake populations due to its high sensitivity.

This RT-PCR protocol (L gene) was used for detecting PMV in several snake families in this study including Boidae, Pythonidae and Colubridae. Sequencing results indicate that this protocol is not very specific (29.23% of the apparently positive samples showed unspecific products of the expected size but not specific sequence). Including results of an RT-PCR targeting a different gene (e.g. the HN gene) as previously described (Marschang et al., 2009) as well as results of virus isolation in cell culture in addition to the results of this L gene RT-PCR protocol may support the

diagnosis. However, sequencing of each RT-PCR positive amplicon remains necessary for a final diagnosis.

Of the animals that tested negative for ferlaviruses by RT-PCR (L gene), unknown viruses were isolated on VH2 in two cases (Lab No; 3-2-09 and 106-09). In the first case, the snake suffered from pneumonia. Interestingly, of the animals that tested positive for ferlaviruses by RT-PCR and revealed no significant results after sequencing, unknown viruses were isolated from four of these samples (Lab No; 56-10, 60-1-10, 60-2-10 and 157-10) where the first animal suffered from CNS signs while the last suffered from CNS signs and pneumonia (supplementary data, Table 1). Of the samples that tested positive for feralvirus L gene by RT-PCR, 49.18% could not be sequenced while 19.66% could be sequenced. However, analysis of those sequences using BLAST, indicated non specific results (several kinds of bacteria/ unknown amplicons).

Changes in annealing temperature and $MgCl_2$ concentration did not lead to changes in the number of unspecific reactions detected. All sets of the degenerate primers used targeting partial L gene sequences showed lower sensitivity than the published protocol from Ahne et al. (1999). The L-gene RT-PCR described by Ahne et al. (1999) therefore remains the most senstivie method for the detection of ferlaviruses in reptiles. However, sequencing of PCR products is absolutely necessary to ensure the specificity of the reaction.

6.3 Surface glycoprotein amplification and characterization

6.3.1 Sequences and phylogenetic analyses

In this study, the full F and HN genes (CDS region) along with a longer portion of the L gene of 9 ferlavirus isolates have been successfully sequenced and analyzed to investigate their taxonomic relationships and to study sequence differences and their impact on viral properties.

It has been proposed in 2009 by Kurath to expand the subfamily *Paramyxovirinae* by including a new genus *‘Ferlavirus’* with the type species FDLV based on sequence analyses of the whole genome and the presence of the unknown U gene (Kurath et

al., 2004), which is missing in all other genera of the *Paramyxovirinae*. Earlier publications have characterized many PMV isolates based on sequence analyses of partial L and HN gene (Ahne et al., 1999) and L gene (Franke et al., 2001) sequences and revealed inconsistent clustering into two groups with intermediate isolates (Ahne et al., 1999) or into three groups (Franke et al., 2001). This contradiction in nomenclature was resolved in a later study by Marschang et al. (2009) by suggesting a revised grouping; a sensu lato A and sensu lato B. A recent publication from Abbas et al. (2011) has revealed the first representative of a new third PMV cluster C (Pangut GER09) which was equally distant from the other two squamate subgroups within the new genus *Ferlavirus*.

In the present study, the classification of isolate Pangut GER09 (proposed subgroup C) was finally determined only when using the continuous sequences obtained from the F, HN, and L genes of all PMV isolates combined with the corresponding sequences from members of other PMV genera within the subfamily *Paramyxovirinae*. Pangut GER09 clustered forming its own branch (subgroup C) within the monophyletic squamate ferlaviruses. Based on the concatenated sequences from all of these genes, subgroups A, B, and C always cluster together in all trials, forming single monophyletic groups in the novel genus *Ferlavirus* within the sub family *Paramyxovirinae* supported with high bootstrap values (98-100%).

The inconsistency in the clustering of subgroup C viruses within the *Ferlavirus* genus when using partial gene sequence was similar to the discrepant results obtained in previous studies (Rösler 2011; Papp et al., 2013; Abbas et al., 2011). In the latter study, the Pangut GER09 was compared with other PMVs based on partial L, HN, and U gene sequences revealing clustering of this isolate once with subgroup A using a portion of L gene sequence, second with subgroup B using a portion of HN gene sequence (bootstrap 72, 65% respectively) and third, alone forming its own subgroup using partial U gene sequences, although this was supported with lower bootstrap values.

Sequence identity values for this isolate have also revealed moderate distances to each subgroup A and B. Therefore, based on the phylogenetic analyses and Identity values obtained so far, we propose the taxonomy of the Pangut GER09 as the first agent of the third cluster subgroup C within the *Ferlavirus* genus.

By comparing the identity values from the full CDS regions of F, HN and U genes and the partial sequence of L (1544bp) of all ferlavirus isolates (Tables; 5.3.2, 5.3.3, 5.3.4 and 5.3.5), Identity values of F and L gene were comparable to each other and higher than those for the HN gene which indicate that the L and F genes are more conserved that the HN gene. Interestingly, the U gene was the least conserved gene. These findings are consistent with earlier findings from Marschang et al. (2009) in which partial U gene sequences were compared to partial sequences of L and HN genes of several ferlaviruses.

Interestingly, this is the first study to effectively augment and further characterize the whole F, HN and a large portion of the L genes of the tortoise PMV and several squamate PMV isolates (Table 3.19). In all of the trials, the tortoise isolate clustered as a sister group to the squamate PMVs, and was never allocated to any of the three subgroups predicted so far. In all phylogeny calculations, the tortoise isolate did form a larger monophyletic group within the new genus *Ferlavirus* supported with high bootstrap values, which ranged from 84 to 100% and never clustered to any other genus of the subfamily (Figures 7A, B, C and D). Furthermore, the identity values of the nt sequences of the F, HN and L genes of the tortoise isolate compared to corresponding genes of the squamate PMVs showed moderate distance to each subgroup. Therefore and despite the fact that no U gene could be detected in this isolate yet, all of the taxonomic analyses and identity values obtained so far confirm the tortoise PMV isolate to be a member of the *Ferlavirus* genus.

6.3.2 Fusion and attachment protein analyses

The envelope glycolproteins F and HN have an essential role in the viral immunogenicity and pathogenecity and they play multiple roles in virus entry and exit from the host. Klenk and Garten (1994) have demonstrated the important role of F protein in the virulence of mammalian and avian PMV. Lamb and Kolakovfsky (2001) have documented the vital role the attachment protein (HN) plays as the main antigenic determinant of PMV. Yuan et al. (2011), studied the functions of HN protein e.g attachment to sialic acid binding sites, activation of the fusion process and virus entry and then hydrolyzation of the sialic acid binding sites setting the progeny virions free. Yuan et al., 2011 has underlined the necessity of homotypic F and HN interactions in viral entry and the important role the HN protein plays for fusion

activation. Previous studies have highlighted the functional domains and important sites of F protein of several PMVs (Morrison, 2003; Franke et al., 2006; Lamb and Kolakofsky, 2001). Several studies (Lamb and Kolakofsky, 2001, Yuan et al., 2011) have revealed the structure of the HN protein which consists of the stalk and globular head. Sialic acid binding sites are mapped to the active sites of the neuraminidase domains in the head of the protein, while fusion activation residues are mapped to the stalk of the HN protein (Yuan et al., 2011).

As documented for respire- and morbilliviruses (Lamb and Kolakowisky, 2001) intergenic regions of three nucleotides long were detected in the current study for all ferlaviruses between the gene boundaries of F, HN and L.

Deduced amino acid sequences of F genes of all ferlavirus isolates contained all of the important domains detected by several software programs and predicted by aa sequence comparison with the homologous sequences of functional domains of the F proteins of reptilian PMV (FDLV and Gono GER85), avian PMV (NDV) and mammalian PMV (SV-5) as indicated by Franke et al. (2006); Morrison (2003); Lamb and Kolakofsky (2001) respectively.

The SP was the first domain detected for all ferlaviruses at the N- terminus of the F protein. Hunter (2001); Lamb and Kolakofsky (2001) described the important role the SP (especially its hydrophobic region) plays in the translocation machinery of the nascent synthesized protein to the membrane of the ER. All three regions of this motif for all ferlaviruses were not conserved and inconsistent in both length and aa composition which is similar to previous results from Von Heijne where a number of eukaryotic and prokaryotic signal sequence were studied and compared (1985). Von Heijne (1985) indicated that the intermediate region of the signal sequence extends from residues -7 to -14 in prokaryotes (*Escherichia coli*) and consists of highly hydrophobic residues. Substitutions of hydrophobic residues for one hydrophilic residue can be tolerated. This region remained mostly hydrophobic in all ferlaviruses and the single hydrophilic residue detected in this region for some ferlaviruses (Pangut GER09 and subgroup B isolates) (supplementary data, Figure 2A) should not affect the translocation process of the freshly translated polypeptides.

The next predicted region located at the C terminus of the SP is the furin recognition site. Klenk and Garten (1995) have documented that the number of basic residues at the furin recognition site and the sequence of this site play a role in the virulence of the viral strain. The furin cleavage site was conserved in all ferlavirus isolates having the basic residues (R-X-K-R) at positions -1, -2, and -4 correspondingly. All of these viruses should therefore induce infections that are not restricted to the respiratory tract, instead they can induce systemic infection which is in agreement with previous findings from Franke et al. (2006). In addition, this site was detected and found conserved for all rubula-, morbilli-, and avulaviruses compared to henipa (Hendra and Nipah) and respiroviruses (Sendai and HPIV1) which have a single basic residue at this site which corresponds to earlier results from Morrison (2003). Interestingly, henipah viruses induce infections in humans and horses mostly associated with the respiratory tract and the central nervous system (Chua et al., 2000).

At position -6 upstream from the cleavage site, subgroup A and B isolates shared slightly acidic amino acids (threonin and serine) which might decrease the viral virulence. Since at least three basic residues were predicted at positions -1,-2 and -4, the effect of these acidic residues might be reduced. Regarding the chelonid ferlavirus isolate, the basic residue (arginine) at this site might increase the virulence of this isolate due to the additive effect of this extra basic aa for cleavage efficiency (Watanabe et al., 1992). Interestingly, this site was detected and found conserved for all ferla-, rubula-, morbilli-, and avulaviruses compared to henipa and respiroviruses (Sendai and HPIV1) which lack this site which corresponds to earlier results (Rawling et al., 2011).

The next domain is the strongly hydrophobic FP which is the closest to the cleavage site (Lamb and Kolakofsky, 2001). By studying membrane fusion of influenza viruses mediated by HA protein, White (1992) has suggested that the FP inserts into the cellular membrane and starts membrane fusion which is important for CPE formation after infection (Bosch et al., 1989). For all ferlaviruses, this domain was conserved, except for the chelonid ferlavirus isolate where a sole non conserved hydrophilic aa (threonine) was detected at position 114. Sergel-Germano et al. (1994) have documented that the introduction of non conserved and charged aa in the FP of NDV may block the fusion process by interrupting the conformational modifications catalyzed by the HN protein.

Since all of the substitutions in this region were non charged and remained mostly hydrophobic, the fusion process of the chelonid ferlavirus should not be influenced. In the current study, this motif was 100% identical within the squamate ferlaviruses and shared a high degree of similarity with the chelonid ferlavirus. In general, this domain remained hydrophobic and shared a high degree of similarity when compared with the analogous sequences of henipa-, respiro-, morbilli-, avula- and rubulaviruses.

HR1 of NDV was found to be very important in fusion and for the initial folding of the protein (Morrison, 2003). Substitution of any leusine residues at position (a) of the leusine zipper motif of the F protein of NDV results in a minor decrease in fusion activity, while substitution of two or three leusine residues at this site abolishes the ability of the protein to induce fusion (Reitter et al., 1995). In that study Reitter et al. (1995) suggested that the leusine residues (as individual residues) do not perform a particular task, instead the repetitive leusine residues as a collection play a role in the fusiogenecity of the protein. In our study, HRA and HRB, especially the non polar side of the helix at positions a & d were conserved for all ferlaviruses. Furthermore, single residue substitutions on the nonpolar side of the helix should not interrupt the biological activities of the protein (Reitter et al., 1995). Therefore, the conserved polar residue (serine) at position d (492 aa) in HRB of the chelonid ferlavirus should not have a direct effect on membrane fusion.

Reitter et al. (1995) examined mutations at positions b, g and e of the LZ motif in the NDV fusion protein and their effect on fusion activity. All mutants showed fusion activity. However, a lower level of fusiogenecity was observed compared to the wild type NDV. For this reason the aa substitutions observed for the chelonid isolate on the polar side of the helix (positions; b,c,e,f and g) should not abolish the fusion activity, though it might decrease this function.

For all ferlaviruses, positions (a) of the HRB (461, 468, 475, 482, 489, 496 aa) constitute the LZ (supplementary data, Fig 1A) with the residues leucine or isoleucine which corresponds to earlier findings from Franke et al. (2006).

A transmembrane domain was detected for all ferlaviruses at the C- terminus of the F protein and showed a high degree of similarity within the squamate ferlaviruses. This domain remained mostly hydrophobic in the chelonid ferlavirus. The two hydrophilic

residues (serine and threonine) at positions 503 and 508 respectively should not have a huge effect on the hydrophobicity of this region.

Surface glycoproteins are post-translationally glycosylated in the rough ER. This process affects several characteristics of the glycoproteins including immunogenicity, antigenicity, protein folding, solubility and stability and intracellular transport (Olden et al., 1985; Panda et al., 2004). In the current study, the F proteins of subgroup A isolates displayed conserved N-G sites at positions 436 and 443, but not at position 102, where an N-G site was detectable only for FDLV. F proteins of subgroups B & C squamate ferlaviruses and the chelonid ferlavirus also displayed conserved N-G sites that were different in position (shifted back 1 aa) from subgroup A ferlaviruses. Moll et al. (2004) have studied the influence of losing N- glycan(s) in the F protein of nipah virus on proteolytic cleavage. They found that loss of the N- glycan located at position 99 aa of F2 and adjacent to the fusion peptide had little or no effect on cleavage efficiency. This site is analogous to the one detected for FDLV at position 102 aa. In contrast, Klenk and Garten (1994) have documented that N- glycan(s) located adjacent to the cleavage site might reduce cleavage efficiency by interrupting protease accessibility. Further studies are necessary to find out the real impact of N-G sites predicted for all ferlaviruses.

Deduced amino acid sequences of HN genes of all ferlavirus isolates contained all of the important domains detected by several software programs and predicted by aa sequence comparison to the corresponding sequences of the functional domains of HN protein of avian PMV (NDV) and mammalian PMV (SV-5) as indicated by Yuan et al. (2011) and Lamb and Kolakofsky (2001) respectively.

Overall, the HN proteins including their active sites were conserved for all ferlaviruses. However, different degrees of similarity were found, especially for the chelonid ferlavirus. The first domain detected was the cytoplasmic tail, this domain was mainly conserved especially within the squamate ferlaviruses.

The transmembrane domain was the next detected domain which remained hydrophobic for all isolates. A recent study by Yuan et al. (2011) predicted the HN structure of NDV and revealed that the hydrophobic core of the stalk region consists of four HBs formed by an 11- aa repeat. Residues at positions a, d, and h form the

hydrophobic core of the repeats. In the current study, this region was conserved among all ferlaviruses.

The predicted stem was conserved for all squamate ferlaviruses. For the chelonid ferlavirus, the non conserved residue predicted at the fusion interaction sites at position 80 aa was mapped to the interior core of the corresponding part of the NDV stalk. On the other hand, the nonconserved residue at position 96 aa in the same virus was mapped to the exterior surface of the of the corresponding part of NDV stalk. Yuan et al. (2011) indicated that non conserved residues at these sites should affect fusion activation. This may indicate a difference in pathogenicity or specific host cells between the chelonid ferlavirus and the squamate ferlaviruses.

The predicted sialic acid binding site NRKSCS as described by Lamb & Kolakofsky (2001) and the conserved region GAEGR (Morrison, 1988) were all conserved within ferla-, respiro-, avula-, and rubellaviruses, highlighting the relatedness of the ferlaviruses to other PMV genera and the possible value of these regions in the HN protein function and structure as suggested previously by Mirza et al. (1994) and Morrison (1988). Mirza et al. (1994) found that the motif NRKSCS is part of the neuraminidase active site in the HN protein of NDV. This may explain the lack of these motifs in the attachment proteins of morbilli- (G) and henipaviruses (H) together with the lack of neuraminidase activity in this protein in viruses from those genera.

McGinnes and Morrison (1994) have documented the role the cysteine residues of NDV HN play in protein folding and antigenic structure. In the alignment of ferlaviruses with several PMVs, all of these sites were totally conserved for respiro- and ferlaviruses (data not shown). Since all of these residues are conserved, this should not decreas the biological activities of the attachment protein as found by McGinnes and Morrison (1994).

Conserved N-G sites were predicted in HN proteins of all ferlaviruses which highlights the significant role they play in the protein functionality. Panda et al. (2004) predicted four N-G sites in the HN of NDV at residues 119, 341, 433, and 481. Loss of a carbohydrate side chain in these sites depending on their location might change virus virulence, fusion efficiency and intracellular protein transport. For instance, the loss of a glycan at residue 481 led to reduction in virus virulence and decreased

intracellular transport of the protein. Panda et al. (2004) indicated that the loss of N-G sites at aa positions 119 and 341 together increased virus fusogenicity, while the loss of the glycan at position 481 decreased fusogenicity. Panda et al. (2004) also showed that glycosylation probably does not have a significant effect on the attachment of NDV since mutant and parental viruses showed similar hemadsorption values. Despite the differences between the HN of ferlaviruses and NDV, it can be hypothesized that the two extra N-G residues found at positions 277 and 496 for the chelonid ferlavirus impair the second step of virus replication (fusion). This might in turn explain the slow growth of the chelonid ferlavirus in cell culture. Further studies are necessary to investigate the role of each N-G site.

Several studies have detected antigen binding sites on the F1 or F2 subunits of F proteins for several PMVs including HPIV 3, Sendai virus, human respiratory syncytial virus and NDV (Portner et al., 1987; Samson et al., 1986). These studies documented that the cysteine-rich region between the two HBR A and B of PMVs is rich with fusion-inhibiting epitopes. According to a previous study from Franke et al. (2006), one linear epitope has been detected for the F gene in ferlaviruses which was located within HRB. However, it was not clear if this epitope was associated with neutralization. In the current study this region was mostly conserved for all squamate felaviruses. For the chelonid ferlavirus, few non conserved residues were detected. Therefore antibodies binding to this region may differ among different ferlaviruses.

Although the attachment protein is the main antigenic determinant of ferlaviruses, no studies have examined antibody binding sites in these viruses so far. Thompson and Porter (1987) documented that in the HN protein of Sendai virus the region extending from 260-280 aa of the head may act as a potential antigenic binding site. The analogous region in Sendai virus to that in ferlaviruses is the region extending from 254-274 aa which was found to be 100% identical among all ferlaviruses and shows high similarity when compared with aPMV1-9 (Fig 13). Serological cross-reactivity of ferlaviruses with different aPMV serotypes has been frequently detected (Potgieter et al., 1987; Blahak, 1995). Therefore, this region might be an antigenic binding site for ferlaviruses. Loss of different N-G sites influences the configuration of antigenic epitopes (Panda et al., 2004). Therefore, the predicted N-G site at position 277 for the chelonid ferlavirus might enhance the functionality of this region as a potential antigenic binding site.

7 Conclusions

A low infection rate of ferlaviruses in snakes was documented for the last years (from the beginning of 2009 until the end of 2011). Based on previously documented prevalence rates for ferlaviruses within Germany, this may indicate a fluctuation in the infection rate. Diseased snakes with clinical signs considered typical for ferlavirus infection (pneumonia and/or CNS signs) were often found to be ferlavirus negative. The presence of PMV in snakes therefore did not correlate with the clinical investigations. Screening of both healthy and sick animals is important to understand the true prevalence of ferlavirus infection in a population and to avoid bias sampling.

The genetic diversity of ferlaviruses appears to be relatively high, with a total of 3 different genogroups documented among the squamate ferlaviruses. A single chelonid ferlavirus isolate is genetically distinct from these squamate subgroups and analysis of various viral genes and proteins support both phylogenetic differences and possible biological differences between these viruses.

Although the analysis of data on screening of diagnostic samples for the presence of ferlaviruses shows that the RT-PCR protocol published by Ahne et al. (1999) has the highest sensitivity of the methods tested, this protocol has been shown to be highly unspecific. Sequencing of PCR products is therefore necessary to ensure specific results. Extended studies are necessary to establish an alternative and more specific diagnostic assay.

Concentrating on a particular pathogen, for instance ferlaviruses in snakes may lead to underestimation of the significance of other infectious agents. Our findings therefore emphasize the importance of testing for several pathogens and different organs and tissues.

8 Summary

PMV are important pathogens for reptiles especially snakes and have been isolated from wild and private collections. During a period (2009-2011), a total of 495 clinical samples originating from 251 snakes of several families including Boidae, Pythonidae, and Colubridae were screened for the presence of PMV by RT-PCR described by Ahne et al. (1999) targeting a partial sequence of the L gene and virus isolation on the reptilian cell line viper heart cells (VH2).

Samples with positive amplicons (566 bp, L gene) were subjected to RT-PCR targeting partial sequence of HN as described by Ahne et al. (1999) and Marschang et al. (2009) and U gene as described by Marschang et al. (2009). All RT-PCR positive amplicons were subjected to sequencing in order to exclude false positive results. Phylogenetic analyses using several programs (Phylip 3.36 and Mega 5.05) were carried out to explore the associations between the viruses detected and to broaden our understanding of their taxonomic relationships.

Unspecific size products and specific size products with non specific amplicons were repeatedly obtained using the previously published protocol. Several trials were therefore carried out in an attempt to increase the specificity of the original RT-PCR protocol (Ahne et al., 1999) including optimization and sensitivity tests. Several concentrations of $MgCl_2$ (1, 1.5, 2, 2.5) mM and different annealing temperatures (45, 48 and 51) C^o were used in order to eliminate the unspecific size products. Sensitivity tests using several ferlavirus isolates were conducted using new degenerate primers targeting the conserved L gene. Changes in annealing temperature and MgCl2 concentration did not decrease the number of unspecific reactions detected. Sensitivity tests showed that the RT-PCR protocol described by Ahne et al. (1999) has the highest sensitivity. However, this protocol has been shown to be highly unspecific. Sequencing of RT-PCR products is therefore necessary to ensure specific results.

Ferlaviruses were detected in 5.97% of the snakes tested (15 of the 251 snakes screened). All ferlavirus positive snakes were from the families Colubridae and Pythonidae. The low infection rate might indicate a fluctuation in the infection rate.

A total of six different partial L gene sequences were obtained from 19 RT-PCR products using RT-PCR (L gene) and verified by sequencing. Three of these products clustered within subgroup B isolates. The one detected in an Indian python was 97% similar to FDLV (AY141760.2) (Subgroup A). Two (Pangut GER09 and Hobuc HUN09) were not assigned to subgroup A or B. However, they clustered together forming the first two representatives of the novel subgroup C within the Ferlavirus genus extending its classification into three squamate subgroups; A, B and C. Concurrent viral infections (PMV, reo and AdV) were detected in a group of corn snakes in Germany which highlight the significance for testing for different pathogens and different organs and tissues.

In order to assess the degree of genetic diversity within this group of viruses, complete CDS regions of the F and HN genes from nine ferlaviruses were sequenced and compared (on both nt and deduced aa sequence levels) with each other and with the corresponding sequences of other genera of the Paramyxovirinae. Phylogenetic analyses were conducted for each gene separately and for the concatenated sequences of F, HN and the extended portion (1544 nts) of L gene.

On a genomic level, squamate ferlaviruses are closely related; however they are distributed into three different genogroups (A, B and C). Deduced animo acid sequences of both F and HN genes of all ferlavirus isolates revealed conserved domains corresponding to those described for other members of the Paramyxovirinae. However, the chelonid ferlavirus showed for both genes and for some motifs some differences to the squamate (snake and lizard) group.

9 Zusammenfassung

PMV sind bedeutende Krankheitserreger bei Reptilien und konnten sowohl aus wildlebenden als auch in Gefangenschaft gehaltenen Populationen isoliert werden. Über einen Zeitraum von drei Jahren (2009-2011) wurden insgesamt 495 klinische Proben von 251 Schlangen verschiedener Familien wie Boidae, Phytonidae und Colubridae auf PMV untersucht, dabei wurde das Virus mittels RT-PCR nach Ahne et al. (1999), die auf das L-Gen des Virus abzielt nachgewiesen und Virusisolation durch Vermehrung in VH2 (Viper Herz Zellen) durchgeführt.

Proben mit positivem PCR-Ergebnis (566 bp, L-Gen) wurden weiterhin in RT-PCRs, welche auf die Gene HN und U abzielen, getestet. Alle positiven PCR-Ergebnisse wurden sequenziert um falsch-positive Resultate auszuschließen. Die erhaltenen Sequenzen wurden des Weiteren phylogenetische Analysen mit unterschiedlichen Programmen (Phylip 3.36 und Mega 5.05) unterzogen um die genetischen Relationen zwischen den Subtypen weiter zu erforschen und das Verständnis der taxonomischen Zusammenhänge zu erweitern.

Produkte mit unspezifischer Länge und Produkte mit spezifischer Länge aber unspezifischen Amplicons Sequencen wurden wiederholt getestet. Dafür wurden verschiedene Anpassungen des ursprünglichen Protokolls (Ahne et al, 1999) vorgenommen. Außerdem wurden Sensitivitätstests anhand neuer Primer, welche auf das konservierte L-Gen abzielten, durchgeführt. Veränderungen der Annealing Temperatur und der MgCl2 Konzentration hatten keinen Effekt auf die Anzahl unspezifischer Reaktionen. Sensitivitätstests zeigten, dass das RT-PCR Protokoll nach Ahne et al. (1999) die höchste Sensitivität aufweist. Da die PCR nach diesem Protokoll jedoch einen hohen Anteil falsch positiver Resultate aufweist, sollten, um spezifische Ergebnisse zu erhalten, die Produkte stets sequenziert werden.

Insgesamt wurden in 5.97% der getesteten Schlangen (15 von 251 Tieren) Ferlaviren nachgewiesen, dabei gehörten alle positiv getesteten Tiere den Familien Colubridae

und Phytonidae an. Die in der Studie gemessene geringe Infektionsrate könnte durch eine Schwankung in der Gesamtinfektionsrate zustande gekommen sein.

Aus den 19 RT-PCR Produkten wurden sechs unterschiedliche partielle L-Gen Sequenzen nachgewiesen. Drei dieser Produkte ließen sich dem Subtyp B zuordenen, eines aus einer indischen Phyton wies 97% Homologie zum FDLV (AY141760.2), (Subgruppe A), auf. Zwei Isolate (Pangut GER09 and Hobuc HUN09) konnten in keines der bestehenden Cluster der Subtypen A oder B eingeordnet werden. Sie bildeten zusammen eine eigene Gruppe und sind damit die ersten Mitglieder des neuen Subtyps C innerhalb des Genus Ferlavirus, welches nun erweitert und in die drei squamaten Subgrupen A, B und C unterteilt werden kann. Es wurden multiple Virusinfektionen (PMV, reo und AdV) in einer Gruppe von Kornnattern/Kornschlangen in Deutschland entdeckt, was die Bedeutung des Testens verschiedener Pathogene und unterschiedliche Organe und Geweben hervorhebt.

Um den Grad der genetischen Diversität innerhalb dieser neuen Virusgruppe einzuschätzen, wurden die kompletten CDS Regionen der Gene F und HN von neun Ferlaviren sequenziert und untereinander und mit entsprechenden Sequenzen anderer Mitglieder der Paramyxovirinae verglichen (sowohl auf der Ebene der Nukleotidsequenzen als auch der abgeleiteten Aminosäure-sequenz). Die phylogenetischen Analysen wurden dabei zum einen für jedes Gen separat und des weiteren anhand der aneinandergereiten Sequenzen der Gene F, HN und einem erweiterten Anteil des L-Gens (1544 Nuckleotide) durchgeführt. Dabei konnte gezeigt werden, dass die squamaten Ferlaviren auf genomischer Ebene eng miteinander verwandt sind, sich jedoch in drei verschiedene Genogruppen (A, B und C) aufspalten. Hergeleitete Aminosäuresequenzen der F und HN Gene aller Ferlavirusisolate in dieser Studie zeigten konservierten Domänen übereinstimmend mit denen anderer Mitglieder der Paramyxovirinae, allerdings konnten in beiden Genen und einigen Motiven Unterschiede zwischen dem cheloniden Ferlavirus und den squamaten Isolaten (Schlangen und Echsen) nachgewiesen warden.

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12 Appendix

Supplementary data, Table 1: Diagnostic samples tested negative for ferlaviruses by RT-PCR and sequencing.

Abbreviations: swab[§]= oral and cloacal swabs were collected together; [$]= adenovirus (AdV) PCR first described in Wellehan et al. (2004); PMV FP: False positive for PMV by RT-PCR; SeqR: Sequencing results; NS= not significant results.

Register No.	Species name	Tissue tested	Case history	Comments, Further findings (virus isolation, other PCRs)
1/09	common boa *(Boa constrictor)*	lung, kidney, Intestine		
3/4/09	common boa *(Boa constrictor)*	lungs, pancreas, kidney, intestine		
10//09	4 common boa *(Boa constrictor)*	four swabs[§]	respiratory problem	
11/2/09	common boa *(Boa constrictor)*	lung, pancreas, kidney, intestine		
12/09	common boa *(Boa constrictor)*	throat, tracheal, cloacal swab, lung, kidney, pancreas, intestine	respiratory problem	
22/09	common boa *(Boa constrictor)*	swab[§]	CNS signs	
45/5/09	Common boa *(Boa constrictor)*	throat, kloakal swab		
63/09	2 common boa *(Boa constrictor)*	lungs, kidney, intestine	stock problem animals died	
63/2/09	common boa *(Boa constrictor)*	lung, kidney, intestine	stock problem	
72/09	common boa *(Boa constrictor)*	swab[§]		
87/09	common boa *(Boa constrictor)*	throat, kloakal swab		

90/1/09	3 common boa *(Boa constrictor)*	three swabs§		
92-09	*(Boa constricor)*	lung, kidney, intestine, pancreas	snake died	
93-09	common boa *(Boa constrictor)*	lung, nieren, darm, pankreas	snake died	
94-09	(Common boa) *Boa constrictor*	lung, nieren, darm, pankreas	snake died	
96/09	common boa *(Boa constrictor)*	Paraffin cut ribbons	CNS and encephalopathy	
100/09	5 common boa *(Boa constrictor)*	Lung, kidney, intestine,pancreas		
102/09	common boa *(Boa constrictor)*	swab§		
106/09	common boa *(Boa constrictor)*	swab§		unknown virus isolated
112/3/09	common boa *(Boa constrictor)*	throat, cloacal swab		
113/ 09	common boa *(Boa constrictor)*	5 swabs§		
114/1/09	common boa *(Boa constrictor)*	throat, tracheal, cloacal swab		
4/10	common boa *(Boa constrictor)*	Pancreas, kidney, lungs, intestine	male	
17/1/10	common boa *(Boa constrictor)*	swab§		
28/10	madagascar ground boa *(Boa madagascariensis)*	lung		
45/10	common boa *(Boa constrictor)*	lung, kidney, intestine, pancreas		
57-10	common boa *(Boa constrictor)*		female, 8 years old	multiple small, dry papilloma lesions
73/10	common boa *(Boa constrictor)*	throat, kloakal swab	female, 13 years old	
82/10	madagascar ground boa (*Boa madagascariensis)*	throat, tracheal cloacal swab	respiratory problem	
88/10	common boa (*Boa constrictor)*	throat, cloacal swab	respiratory problem	
132/1/10	2 common boa *(Boa constrictor)*	two throat & cloacal swabs	male and fermale of one enclosure suffering from pneumonia	PMV FP: SeqR: *Acinetobacter baumanni*
147/10	common boa *(Boa constrictor)*	throat, tracheal cloacal swab	chronic anorexia, emaciation,	adenovirus PCR positive$

			mouth mucosa	
15/11	common boa *(Boa constrictor)*	oral swab	CNS signs, mortality observed in the same stock, skinning problem, anorexia	
35-11	common boa *Boa constrictor*	swab and trachea washing	dermatitis, snale mites were detected	adenovirus PCR positive$
74-11	common boa *Boa constrictor*	swab§		
76-11	common boa *Boa constrictor*	oral swab		
106-11	*5* common boa *Boa constrictor*	five swabs§	five female	
2/09	royal python *(Python regius)*	lung, kidney, intestine	animal died	
3/1/09	indian python *(Python molurus)*	throat, tracheal, kloakal, swab	respiratory problem	adenovirus PCR positive$
3/2/09	royal python *(Python regius)*	lung, pancreas, kidney, intestine	respiratory problem	unknown virus isolated. adenovirus PCR positive$ from intestine
3/3/09	green tree python (*Morelia viridis)*	lung, pancreas, kidney, intestine	respiratory problem	
03/5/09	carpet python (*Morelia spilota variegata)*	lung, pancreas, kidney, intestine	respiratory problem	
4/1/09	*6* royal python *(Python regius)*	lung, liver, intestine, kidney		adenovirus PCR positive$ from lung and intestine in four snakes
7/1/09	indian python *(Python molurus)*	tracheal washing& cloakal swab		PMV FP in tracheal washing. SeqR: NS
7/2/10	carpet python (*Morelia spilota variegata)*	brain		
8/1/09	indian python *(Python molurus)*	lungs, kidney, intestine	animal died	PMV FP in lung. SeqR: NS
8/2/09	royal python *(Python regius)*	lungs, kidney, intestine	animal died	
11/1/09	indian python *(Python molurus)*	throat, tracheal cloatal swab	respiratory problem	

18/09	carpet python *(Morelia spilota variegata)*	throat, cloacal swab		PMV FP. SeqR: NS
32-09	tieger python	brain, kidney, liver	stock PMV problem	PMV FP. SeqR: NS
44/09	carpet python (*Morelia spilota variegata)*	lung, intestine, kidney, pancreas		
45/09	4 indian pythons *(Python molurus)*	intestine, kidney, pancreas of four snakes		PMV FP in lung of one snake. SeqR: NS Same group as Pyt-1c
13/10	carpet python (*Morelia spilota variegata)*	Throat &cloacal swab		
47/09	green tree python *(Morelia viridis)*	lung, kidney, intestine, pancreas		adenovirus PCR positive[$] from lung, intestine and pancreas
64/09	angolan python *(Python anchietae)*	lung, liver, kidney	sub adult female with pneumonia, the animal is part of a group with chronic respiratory disease	Snake from international zoo veterinary UK
65/09	indian python *(Python molurus)*	throat, tracheal kloakal swab	CNS	
67/09	indian python *(Python molurus)*	intestine, kidney, pancreas, lung, swab[§]		
70/1/09	2 indian python *(Python molurus)*	two throat & kloakal swabs		PMV FP in one snake. SeqR: NS. Adenovirus PCR positive[$] in the same snake
109/09	children's Python (*Antaresia childreni*)	skin	skin problem, change the skin each 2 weeks	adenovirus PCR positive[$]
110/09	tiger python *(Python molurus)*	3 tracheal washing	respiratory problem	PMV FP. SeqR: NS
112/09	2 indian python *(Python molurus)*	two Throat & Kloakal swabs		
114/2/09	indian python *(Python molurus)*	lung, kidney, intestine,		PMV FP in lung, kidney

		pancreas		and intestine. SeqR: NS
114/3/09	indian python *(Python molurus)*	throat & cloacal swab		
5/10	green tree python (*Morelia viridis)*	lung, intestine, kidney		
6/10	carpet python (*Morelia spilota variegata)*	lung, kidney		
7/10	indian python *(Python molurus)*	intestine, lung, kidney		
8/1/10	8 carpet python (*Morelia spilota variegata)*	eight swabs		PMV FP in two snakes. SeqR: NS
9/1/10	indian python *(Python molurus)*	throat, tracheal cloacal swab		
17/2/10	monty python	swab§		
18/10	royal python (*Python regius)*	throat & cloacal swab	female noticed with respiratory problems and CNS	microbiological investigations from tracheal washing revealed the prevelence of *klebsiella pneumonia* and *psudomonas aeroginosa*
23/1/10	5 indian pythons *(Python molurus)*	lung, kidney, pancreas, intestine		PMV FP in three snakes from lung, kidney and intestine. SeqR: NS
25/10	indian python *(Python molurus)*	throat, tracheal cloacal swab		
27/10	green tree python (*Morelia viridis)*	throat, cloacal swab		
34/10	indian python *(Python molurus)*	throat, cloacal swab		
39/10	bismark ringed python (*Bothrochilus boa)*	lung, kidney, intestine		
56/10	royal python (*Python regius)*	throat, kloakal swab	CNS signs	PMV FP. SeqR: NS. Unspecified virus isolated
60/10	2 indian python *(Python molurus)*	lung, kidney, pancreas, intestine		PMV FP in both snakes. SeqR: NS.

				Unknown virus isolated from from both snakes
86/1/10	8 royal pythons (*Python regius)*	lung, kidney, intestine of 1 snake and 7 swabs[§]		
103/10	tiger python *(Python molurus)*	swab[§]		
123/10	Royal python (*Python regius)*	swab[§]	stock respiratory problem,	adenovirus PCR positive[$]
132/3/10	royal python (*Python regius)*	swab[§]	662g, male with respiratory problem	
138/10	royal python Python regius	lung, Intestine, kidney, pankreas		PMV FP. SeqR: NS
139/10	8 royal python Python regius	8 swabs[§]		
150/10	indian python *(Python molurus)*	lungs, kidney, pancreas, intestine		adenovirus PCR positive[$] from intestine
151/10	royal python (*Python regius)*	throat, Cloakal swab		adenovirus PCR positive[$]
157/10	royal python (*Python regius)*	throat, tracheal cloacal swab	CNS, Respiratory problem	PMV FP. SeqR: NS. Unknown virus isolated
160/10	royal python (*Python regius)*	trachea washing		PMV FP. SeqR: NS
1/1/11	*Python brongersmai*	lung, trachea, kidney, liver, swab		adenovirus PCR positive[$] in swab
1/2/11	*Python brongersmai*	lung,trachea, kidney, liver, swab		adenovirus PCR positive[$] in kidney
1/3/11	royal python *(Python regius)*	lung, trachea, kidney, liver, kidney, swab		adenovirus PCR positive[$] in lung
6/4/11	royal python *(Python regius)*	heart, head	group of python of about 40 snake died of fatal pneumonia. the group was treated with Gentamycin that had no effect. Septicaemia was found after	adenovirus PCR positive[$] in head

			dissection	
10/11	royal python *(Python regius)*	pancreas, intestine, kidney		
11/11	royal python *(Python regius)*	oral, cloacal swab		
12/11	royal python *(Python regius)*	oral, cloacal swab		
13/11	royal python *(Python regius)*	oral, cloacal swab		PMV FP. SeqR: NS
14/11	7 royal python *(Python regius)*	7 head,		adenovirus PCR positive[$] in one snake. Same group as 6/11
17 & 18/11	2 royal python *(Python regius)*	two swabs[§]		PMV FP in both snakes SeqR: NS
19 & 20/11	2 red albino blood python *(Brongersmai albino)*	two swabs[§]		
21/111/11	indian python *(Python molurus)*	lung, Kidney, Intestine, Pancreas Brain		adenovirus PCR positive[$] in intestine in one snake
23/11	royal python *(Python regius)*	lung, kidney, intestine		adeno virus isolated. adenovirus PCR positive[$]
25/11	royal python *(Python regius)*	Intestine, kidney	animal died, Enteritis noticed	
26/11	reticulated python *(Python reticulatus)*	brain, lung, kidney, intestine	animal died enteritis	
31/11	5 royal python *(Python regius)*	five swabs		adenovirus PCR positive[$] in one snake
55/11	blutpython *(Python brongersmai)*	swab[§]		
60/11	python, unknown species	swab[§]		adenovirus PCR positive[$]
41/11	*royal python (Python regius)*	swab[§]		
101/11	python burmese *(Python bivittatus)*	lung	Male with CNS,	PMV FP in brain, kidney and lung. SeqR: NS

				(*Acinetobacter aceti*) in all tested organs
107/11	2 python Burmese *(Python bivittatus)*	two swabs[§]		same animals as 101-11
112/11	royal python *(Python regius)*	swab[§]	anorexia, parasitosis, emaciation	
113/11	3 carpet python *(Morelia spilota variegata)*	three swabs[§]		
115/11	royal python (*Python regius)*	20 swabs[§]		
6/1/09	corn snake (*Elaphe guttata)*	lung, kidney, intestine		
6/2/09	corn snake (*Elaphe guttata*)	lung, kidney, intestine		
14/09	6 corn snake (*Elaphe guttata*	six swabs[§]		reovirus isolated from three snakes
17/1/09	2 garter snake (*Thamnophis sirtalis)*	2 swabs[§]		PMV FP. SeqR: NS
33/09	garter snake (*Thamnophis sirtalis*		CNS signs, Animal died problem in single animal	
42/09	corn snake (*Elaphe guttata)*	Tracheal swab	pneumonia	
54/09	10 corn snake (*Elaphe guttata)*	Ten Throat, Kloakal swab		adenovirus positive[$] PCR in three snakes. Reovirus isolated in two snakes. This group is the consequtive group to the captive corn snakes (Col-1, Col-1a~f) which was established after dispersing the first group of snakes. Both

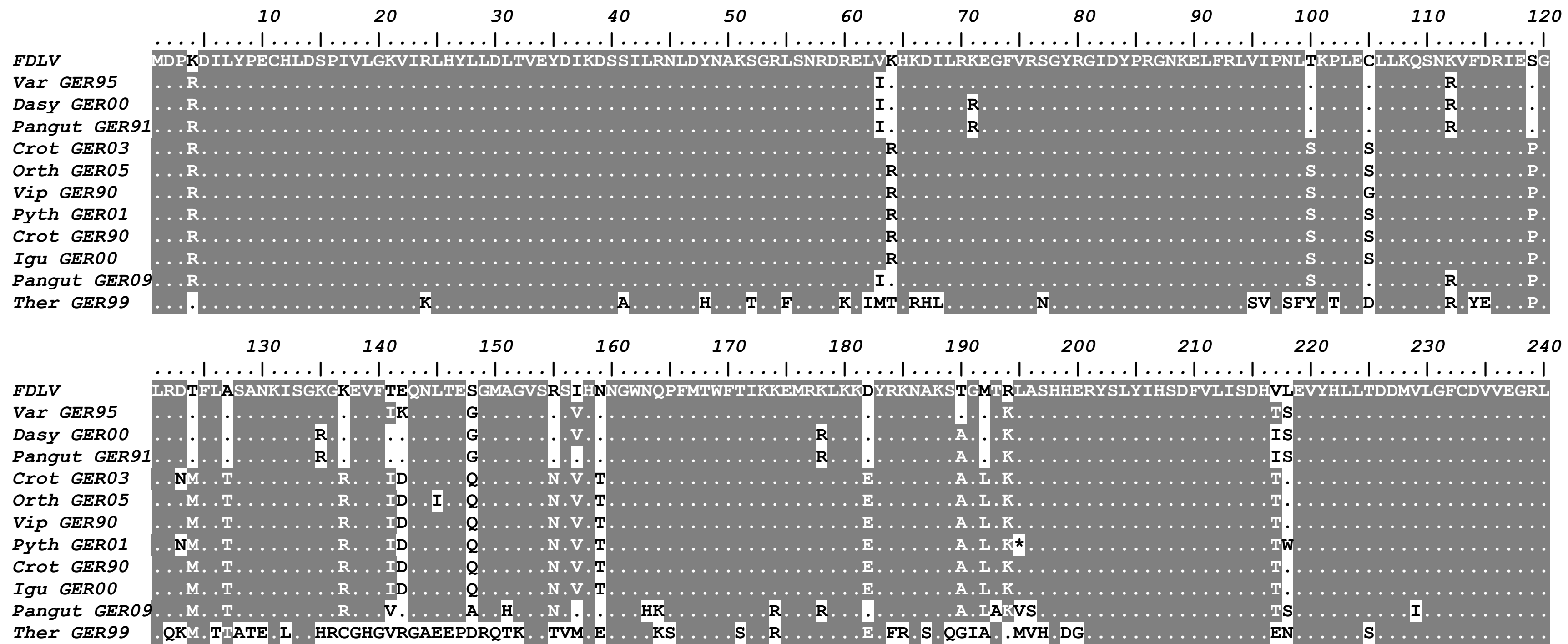
				groups belong to the same owner.
55/09	aesculapian Snake (*Elaphe longissima)*	lung, kidney intestine pancreas		
59/09	corn snake (*Elaphe guttata)*	lung, kidney, intestine		adenovirus PCR positive$
60/09	corn snake (*Elaphe guttata)*	lung, kidney, intestine	animal died suddenly	PMV FP in lung and kidney. SeqR: NS. adenovirus PCR positive$ in lung and kidney
66/09	2 corn snake (*Elaphe guttata)*	two Throat, cloacal swabs		adenovirus PCR positive$ in 66-1-09
86/09	unknown	lung		
95/09	garter snake *(Thamnophis sirtalis)*	lung, kidney, intestine, pancreas	animal died suddenly	
142/10	dice snake *(Natrix tessellata)*	lung and liver		
95/09	garter snake *(Thamnophis sirtalis)*	lung, kidney, intestine, pancreas	animal died suddenly	
101/09	2 corn snake (*Elaphe guttata)*	two swabs§		PMV FP in one snake. SeqR: NS, *Salmonella spp*
29/10	corn snake (*Elaphe guttata)*	lung, kidney, intestine	stock problem, snake died	
95/09	garter snake *(Thamnophis sirtalis)*	lung, kidney, intestine, pancreas	animal died suddenly	
101/1/09	2 corn snake (*Elaphe guttata)*	two swabs§		PMV FP in one snake. SeqR: NS
97/2/09		swab§		adenovirus PCR positive$
42/10	corn snake (*Elaphe guttata)*	small intestine	stock of animals died,Trichomonas and enteritis observed	
70/10	corn snake (*Elaphe guttata)*	throat swab and cloacal swab	male	

84/10	corn snake (*Elaphe guttata)*	throat, tracheal cloacal swab	CNS signs	
152/10	corn snake (*Elaphe guttata)*	throat, tracheal cloakal swab	respiratory problem	adenovirus PCR positive$
154/10	corn snake	throat and cloacal swab	emaciation, parasitosis, vomitus, and bacterial infection noticed. This snake represents a single case within established stock	
155/10	corn snake *(Pantherophis guttatus)*	throat and cloacal swab	CNS, vomits, high amount of Flagella in cloaca flashing. This snake represents a single case within established stock	
54/11	corn snake *(Pantherophis guttatus)*	swab§	emaciation, enteritis were observed	
61/11	corn snake *(Pantherophis guttatus)*		male, CNS signs	PMV FP. SeqR: NS, *Pseudomonas putida*
67/11	corn snake *(Pantherophis guttatus)*	swab§	8 years old, CNS	
24/11	corn snake *(Pantherophis guttatus)*	lung, intestine, kidney	enteritis	
30/11	corn snake *(Pantherophis guttatus)*	lung, kidney, intestine	4 years old male, stock problem	adenovirus PCR positive$ in lung
104/11	5 corn snake *(Pantherophis guttatus)*	five swabs§		PMV FP in four snakes (104-1,2,4,5-11). SeqR: NS, *Enterobacter cloaca*. adenovirus PCR positive$ in three snakes (104-1,4,5-11)
136/1/10	4 eyelash Viper	four throat,		adenovirus

	(Bothriechis schlegelii)	kloakal swab		PCR positive[$] In two snakes
116/11	horned viper *(Vipera ammodytes)*	kidney, lung, intestine	stock problem	
121/11	*Dinnik's viper (Vipera dinniki)*	cloacal swab	respiratory problem	
52/09	ground rattlesnake (*Sistrurus miliarius)*	intestine, kidney, lungs, pancreas, brain		
16/1/11	unspecified species	Brain Trachea Lung Heart Liver Kidney Pancreas Intestine Colon		PMV FP in brain, lung and heart. SeqR: NS adenovirus PCR positive[$] in liver
80/11	unspecified species	swab[§]		
122/11		swab[§], Brain, Lung, Pancreas, kidney, intestine		
97/1/09	unspecified species	lung		PMV FP in lung. SeqR: NS. adenovirus positive[$] PCR in kidney, intestine and pancreas

Supplementary data, Figure 1: Deduced amino acid sequences of L gene of twelve ferlaviruses.

Deduced amino acid sequences of L gene of twelve ferlaviruses (FDLV, Var GER95, Crot GER03, Igu GER00, Orth GER05, Pyth GER01, Pangut GER09, Ther GER99, Vip GER90, Crot GER90, Dasy GER00, Pangut GER91). Identity shading refers to 80% among ferlaviruses. Dots indicate identical letters to that of the reference sequence (FDLV)



```
                       250       260       270       280       290       300       310       320       330       340       350       360
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FDLV         MVSAVASMDPNYVNIGIRGEQLWSLIDSLFILLGNKVYDVVGMLEPLALSYIQLRDPIKEIRGAFLYYNLSELDQLLKEDTILSTGEVKDFIELISEIINMGSVSEISEIFSFFRSFGHP
Var GER95    .I...............K...................................................................................D..................
Dasy GER00   .................K...................................................................S..................................
Pangut GER91 .................K...................................................................S..................................
Crot GER03   .I...S......A..SVK............V......................................M..............VD..N.............N.................
Orth GER05   .I...S......A...VK............V......................................M..............VD..N.............N.................
Vip GER90    .I...S......A...VK............V......................................M..............ID..N.............N.................
Pyth GER01   .I...S......A...VK............V......................................M..............VD..N.............N.................
Crot GER90   .I...S......A...VK............V......................................M..............VD..N.............N.................
Igu GER00    .I...S......A...VK............V......................................M..............VD..N.............N.................
Pangut GER09 .I...............K.......................................L...........M....H......L..AE..H........L...DN.................
Ther GER99   .I..TSEL....TQ..L......G......TIM..Q.....................VA.V.........N...V..R.N.M.TNE..S...QAL.N.V..DK.................

                       370       380       390       400       410       420       430       440       450       460       470       480
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FDLV         VLEAVTAADKVRDHMCKPKVLDYETLQKGHAVFCSMIINGFRERHGGSWPPCFLPDYASSELKAVMANHAAIPYELSVNNWESFIGFKFDKFEEVNLDEDLTIFMKDKALSPIRAEWDTV
Var GER95    ...................I....I....................................I..................................................V.......
Dasy GER00   ...................I....I...........................S........I..........................................................
Pangut GER91 ...................I....I...........................S........I..........................................................
Crot GER03   I..................I..........................................................S........R..........................S.....
Orth GER05   I..................I..........................................................SS.......R..........................S.....
Vip GER90    I..................I..........................................................S........R..........................S.....
Pyth GER01   I..................I..........................................................S........R.................R........S.....
Crot GER90   I..................I..........................................................S........R..........................S.....
Igu GER00    I..................I..........................................................S........R..........................S.....
Pangut GER09 I..................I....V...........................S......P.....A.............H....V..R.........................KS.....
Ther GER99   I..................I...K.I.Q........................I..EH..ADI..I.V..S....DIC.RD.........E.......................KS.....

                       490       500       510
             ....|....|....|....|....|....|....
FDLV         YPQENMQYSPGRSSTSRRLVDVFLEDMEFNPQDI
Var GER95    ...........K.......I..............
Dasy GER00   ..................................
Pangut GER91 ..................................
Crot GER03   ...........K......................
Orth GER05   ...........K......................
Vip GER90    ...........K......................
Pyth GER01   ...........K......................
Crot GER90   ...........K......................
Igu GER00    ...........K......................
Pangut GER09 ...........K......................
Ther GER99   ........PT.KG..............D.D.NN.
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Supplementary data, Figure 2: Deduced amino acid sequence of F and HN genes (CDS region) of ten ferlaviruses.

Deduced amino acid sequence of F and HN genes (CDS region) of ten ferlaviruses (FDLV, Var GER95, Crot GER03, Igu GER00, Orth GER05, Pyth GER01, Pangut GER09, Ther GER99, Vip GER90, Crot GER90) and the important motifs. Threshold for shading with dark and light grey is 80%. Dark grey refers to identical residues among all ferlaviruses. Light grey refers to similar substitutions. Dots indicate identical letters to that of the reference sequence (FDLV). Abreviations for the consensus sequence: * = identical or conserved residues among all ferlaviruses, : = indicates conserved substitutions, . = indicates semi-conserved substitution.

Supplementary data, Figure 2A: Deduced amino acid sequence of F gene (CDS region)

```
                                                                                                            F2 <--  --> F1
                 [Signal peptide]                                                           [Furin recognition site]  [Fusion peptide]
                          10        20        30        40        50        60        70        80        90       100       110       120
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....RXKR.x...|
FDLV (A)         MTRITILQIILTLTLPVMCQVSFDNLEQVGVMFDKPKFLKITGPASTATMIIKLIPTLGTMESCGTSAVNEYKKTLDTILVPLRDTINKLSTDITVVEGTSNISNKREKRFVGIAIAVGA
Var GER95 (A)    ..KTI.F..V...AA.A................................I..............................I...................KV.T...............
Crot GER03(B)    .-KV.T.L...I.ITSIL..I...........................................................I...............L...PKTLSR..............
Igu GER00 (B)    .-KV.T.L...I.ITSIL..I...........................................................I...............L...PKTLSR..............
Crot GER90(B)    .-KV.T.S...I.IT.TL..I...........................................................I...............L...PKTLS...............
Vip GER90 (B)    .-KV.T.S...I.IT.IL..I...........................................................I...............L...PKTLSR..............
Orth-GER05(B)    .-KV.T.P...I.ITSIL..I...........................................................I...............L...PKTLSR..............
Pyth-GER01(B)    .-KV.T.L...I.ITSIL..I...........................................................I...............L...PKTLSR..............
pangut GER09(C)  .-SGAMPY.VVV.AV.T...I...S..........................................................K...D........L...LKTPA..G............
Ther GER99       .-EK...LFLWGFIIS.SG.I...Q...I..VY.R...........K............A.A...N...Q...S..E...G......D.IK...S.I...RLSNRR.K...F.T...I..
Consensus        *       ::  :   .   *:***.***:**::*:***********.**:*********:* ***.***:***.**:*** **:***:*:.***:*:***      :* ***.* ***:**

                 [Fusion peptide]               [Heptad Repeat A]
                          130       140       150       160       170       180       190       200       210       220       230       240
                 ....|....|....|....|....abcdefg abcdefg abcdefg abcdefg abcdefg abcdefg...|....|....|....|....|....|....|....|....|....|
FDLV(A)          VALATSAQITAGIALSNTIKNAEAIESIKSS IQASNQA IQKVIDA QGRTVTV INGIQDH INSVINPALNQLGCDVAKNTLAISLTQYFSKLSLLFGPNLRNPVEQPLSVQAIAGLMDGDI
Var GER95(A)     ............................... ....... ....... ....... ....... .............................................................
Crot GER03(B)    ...............A...........L... .....K. ....... ....... ....... .............................................................
Igu GER00(B)     ...............A...........L... .....K. ....... ....... ....... .............................................................
Crot GER90(B)    ...............A...........L... .....K. ....... ....... ....... .............................................................
Vip GER90(B)     ...............A...........L... .....K. ....... ....... ....... .............................................................
Orth-GER05(B)    ...............A...........L... .....K. ....... ....... ....... .............................................................
Pyth-GER01(B)    ...............A...........L... .....K. ....... ....... ....... .............................................................
pangut GER09(C)  ...........................L... .....K. ....... ....... ....... .............................................................
Ther GER99       ......................A....L.A. ....... ....... .....I. ....... ...................................A.I.......................
Consensus        ***************:****** ****:*:* *****:* ******* *****:* ******* ***********************************:*:***********************
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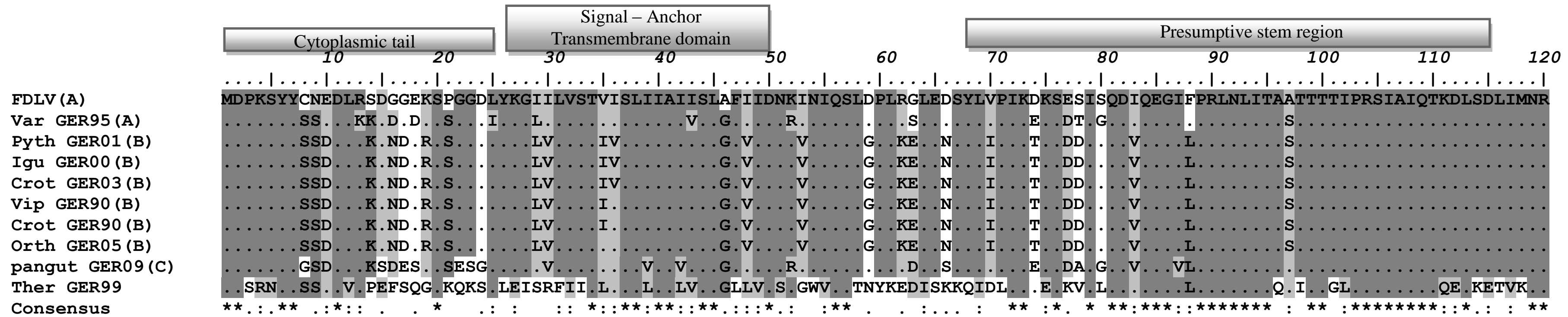
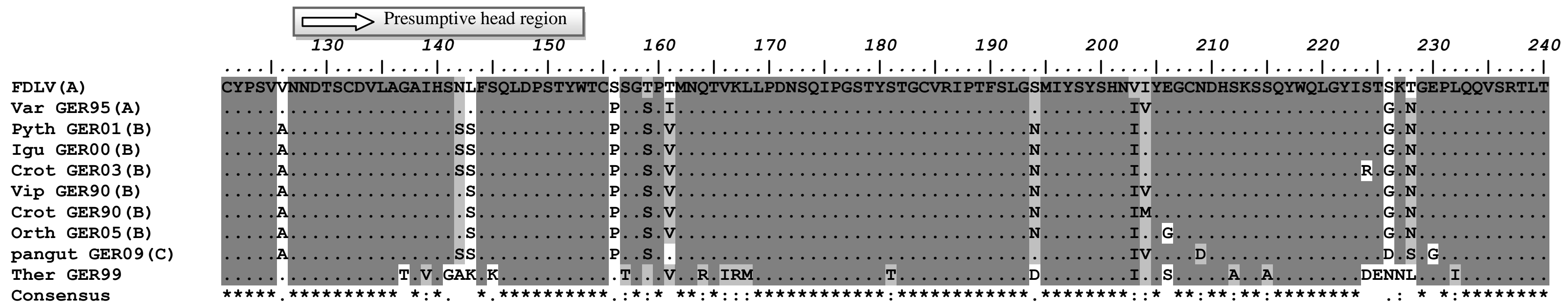
                 250       260       270       280       290       300       310       320       330       340       350       360
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FDLV(A)         NAVVSQLGYTQSDLLDLLSTESIVGTVTAIDMVNYMIQIEMSFPQYITIPDTKVLEGHKITFNDKGSEWQTQVPSTIAVRDILIAGVDPDGCSITSTSYICKNDPTYAMSEVLTNCFRGN
Var GER95(A)     .................................................................................................E.....................K
Crot GER03(B)    .........P...............V.......................R.....R.........................V............................
Igu GER00(B)     .........P...............V.......................R.....R.........................V............................
Crot GER90(B)    .........P...............V.......................R.....R........G................V............................
Vip GER90(B)     .........P...............V.......................R.....R.........................V............................
Orth-GER05(B)    .........P...............V.......................R.....R.........................V............................
Pyth-GER01(B)    .........P...............V.......................R.....R.........................V............................
pangut GER09(C)  .........P...........................I...........................N...............V............................
Ther GER99       .S.......SS..............GV..R.......................I.......NA....A.............NSE........P...........L...M.E..K.R
Consensus        *:*******: ***************** .:** *******************:****:****: *****:***.***********:.:***:*.************:***:*:**:*.

                                                                                                                     Heptad Repeat B
                 370       380       390       400       410       420       430       440       450       460       470       480
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|abcdefg abcdefg abcdef
FDLV(A)         TQECPRARITSTFATRFAIARSTVIANCVAAVCLCGDPGIPVVQKAEVTLTAMTLDQCSLITVDGLQIKPSKSIANVTANFGNITLGPVVSVGDLDLSAELTKVQSD LKEAQDK LDESNA
Var GER95(A)     .......................................T.......I.........N.....................................................
Crot GER03(B)    .......................................T.........................L..............................  .....R .....S
Igu GER00(B)     .......................................T.........................L..............................  .....R .....S
Crot GER90(B)    .......................................T.........................L..............................  ....GR .....S
Vip GER90(B)     .......................................T.........................L..............................  ....GR .....S
Orth-GER05(B)    .......................................T.........................L..............................  .....R .....S
Pyth-GER01(B)    .......................................T.........................L..............................  .....R .....S
pangut GER09(C)  .......................................T...............V.I.......N.......D......................  ....E. ......
Ther GER99       .EN.........N...VV..I.......S.........I.....K..S..VTND.G.........G.FLG.I.S....VS...II..DS.........QE ..T..SY ..K..G
Consensus        *::***********.***:.** ******** ******* *:***** **:**. ::*.*:*:*******.* :.*:*::***::***::**..***********.: ** **  **:**.

                                                                  Tansmembrane anchor
                       490       500       510       520       530       540
        G abcdefg abcdefg abcdefg..|....|....|....|....|....|....|....|
FDLV(A)         I LQGINNK ILTAPTS IALIVVSVVVILLIIGMISWLVWLTKAVRRSNTRSERVTPSAYNNLGFIK
Var GER95(A)     . ....... ....... .......IV.....I...IF..R..K..SI..............T.......
Crot GER03(B)    . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
Igu GER00 (B)    . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
Crot GER90(B)    . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
Vip GER90(B)     . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
Orth-GER05 (B)   . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
Pyth-GER01 (B)   . ....... ....... ......IIV.I...I...A.......K..SI....I..NT.......
pangut GER09(C)  . ....... ..S.... .....II.V.F...VV.......R..K..S.....I...T.......
Ther GER99       . NNL..Q. ..KSSV. ....IFLSIA..T..CL.L.....R.IK.I.QKP..I...T......L.
 Consensus       * *:.:**: **.:..* ****:.  : :* ** :: ** :**:*::* . :.**:**.:******:*
```

Supplementary data, Figure 2B: Deduced amino acid sequence of HN gene (CDS region)





```
                    Sialic acid binding site
                     NRKSCS
                        250       260       270       280       290       300       310       320       330       340       350       360
FDLV(A)            LNNGLNRKSCSTVAQGRGAYLLCTNVVEDERTDYSTEGIQDLTLDYIDIFGAERSYRYTNNEVDLDRPYAALYPSVGSGTVYNDRILFLGYGGLMTPYGDQAMCQAPECTSATQEGCNSN
Var GER95(A)       ..........................................V.................S.................................V............R......DD....
Pyth GER01(B)      ..D.......................I.................................S...................I..............K..............T.........
Igu GER00 (B)      ..........................I.................................S...................I..............K..............T.........
Crot GER03 (B)     ..........................I.................................S...................I..............K..............T.........
Vip GER90(B)       ..........................I.................................S...................I..............K..............T.........
Crot GER90(B)      ..........................I.....................V...........S...................I..............K..............T.........
Orth GER05(B)      ..........................I.................................S...................I..............K..............T.........
pangut GER09(C)    ..........................................I.....V.....N.........................I....V........V............G.P........N.
Ther GER99         ..D.I.........L.........H.I...V...RSN.T.........L..THK....SDR.INF...............I.............IS........N..L.PTVN.DD..R.
Consensus          **:*:********* *********:*:*** ***  ::* *** *****:**:.:.***:: *::***************:****:********:.********:** *.:..*:.** *

                        370       380       390       400       410       420       430       440       450       460       470       480
FDLV(A)            QLIGYFSGRQIVNCIIEIITVGTEKPIIRVRTIPNSQVWLGAEGRIQTLGGVLYLYIRSSGWHALAQTGIILTLDPIRISWIVNTGYSRPGNGPCSASSRCPAQCITGVYTDIFPLSQNY
Var GER95(A)       .................V................................................................K.........R..P......V.................
Pyth GER01(B)      ....W.........................K...................................................E.........N...........................
Igu GER00 (B)      ....W.........................K...................................................E.........N...........................
Crot GER03(B)       ....W.........................K...................................................E.........N...........................
Vip GER90(B)       ....W.........................K...................................................E.........N..P........................
Crot GER90(B)      ....W.........................K...................................................E.........N...........................
Orth GER05(B)      ....W.........................K...................................................E.........N...........................
pangut GER09(C)    ....W..............K........K.....................................................E.........S..P......V.................
Ther GER99         .F........V...L..V.II.....L.K.T..............L........I..............M............E.........E..P......S.......N....I....
Consensus          *:**:*****:***:**:*  :*****:*:* *************:********:*************:*********** ********* **.****** *******.****:****

                                                                                 Presumptive head region  <==
                        490       500       510       520       530       540       550       560
FDLV (A)           GYLATVTLLSGVDRVNPVISYGTSTGRVADSQLTSSSQVAAYTTTTCFTFNQKGYCYHIIELSPATLGIFQPVLVVTEIPKICS-
Var GER95 (A)      ...........I..I........P...........P...............................................-
Pyth GER01(B)      ............N......................A............I...................V......A.....V..-
Igu GER00 (B)      ............N......................A............I...................V......A.....V..-
Crot GER03(B)       ............N......................A............I...................V......A.....V..-
Vip GER90(B)       ............N......................A............I...................V......A.....V..-
Crot GER90(B)      ............N......................A............I...................V......A.....V..-
Orth GER05(B)      ............N......................A............I...................V......A.....V..-
pangut GER09(C)    ................................................I..........................A.....V..-
Ther GER99         .Q.........NT....T.....P......K.I.TAE......M....VLAER.F.............L......A.......QP
Consensus          * *********  *:**.*****.******.*:*:..****** ****  :  ::*:*************:******.*****:*.
```

Affirmation

I hereby declare that I have completed the dissertation independently, and this research is original. I have not been supported by a commercial agent in writing this dissertation. Additionally, no aids other than the indicated sources and resources have been used. This work has not been previously used neither completely nor in parts to achieve any other academic degree.

Maha Diekan Abbas

Stuttgart-Hohenheim, December 2013

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