Szent István University
Postgraduate School of Veterinary Science

Detection and characterisation of adeno-, irido- and paramyxoviruses in reptiles

Ph.D. dissertation

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Table of contents

Abbreviations

aa	amino acid(s)
AB	antibiotics
AdV	adenovirus
Aps.	adenovirus positive sample
AtAdV	atadenovirus
bp	basepair(s)
CIV	Chilo iridescent virus (syn. IIV-6), IIV from a rice stem borer moth
CDS	coding sequnce
CNS	central nervous system
CPE	cytopathic effect
C_t	threshold cycle (in real-time PCR)
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate mix
DMEM	Dulbecco's modified Eagle's medium
dpi	days post infection
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
EM	electron microscopy(ic)
FAdV	fowl adenovirus
FAM	6-carboxy fluorescein
FDLV	Fer de Lance virus
FV3	frog virus 3
GbIV	Gryllus bimaculatus iridovirus (syn. cricket iridovirus)
HA	haemagglutination
HAdV	human adenovirus
HI	haemagglutination inhibition
HRSV	human respiratory syncitial virus
IBD	inclusion body disease
ICTV	International Committee on Taxonomy of Viruses
IGR	intergenic region
IHC	immunohistochemistry
Ips.	iridovirus positive sample
IPTG	isopropyl β-D-1-thiogalactopyranoside
Ir.iso.	iridovirus isolate
ISH	*in situ* hybridisation
IIV	invertebrate iridovirus
IV	iridovirid – member of the family *Iridoviridae*
kb	kilobasepair(s)
kDa	kilodalton
nt	nucleotide(s)
LB	Lysogeny broth
LCDV	lymphocystis disease virus

MCP	major capsid protein
MGBNFQ	minor groove binder non-flurescent quencher
MST	mean survival time
NCLDV	nucleo-cytoplasmic large DNA viruses
NDV	Newcastle disease virus
NGS	new generation sequencing
nPCR	conventional PCR (normal PCR)
oPMV	ophidian (snake) paramyxovirus(es)
ORF	open reading frame(s)
PCR	polymerase chain reaction
PjIV	Popillia japonica iridovirus (from Japanese beetle)
PMV	paramyxovirus
qPCR	real-time PCR (quantitative PCR)
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	revolutions per minute
rPMV	reptile paramyxovirus
RT-PCR	reverse-transcription followed by PCR
ss	single-stranded
TAE	tris-acetate-EDTA
$TCID_{50}$	median tissue culture infective dose
TP	terminal protein
UTR	untranslated region(s)
VAB	viral antibiotic peptide
var	variant
VN	virus neutralisation
WIV	Wiseana iridescent virus (syn. IIV-9) IIV from a grass moth
X-gal	5-bromo-4-kloro-3-indolil-β-D-galactopyranoside

1. Summary

Among reptiles, adenoviruses (AdVs) have most often been identified in squamates (lizards and snakes) associated with gastroenteritis and hepatitis or central nervous signs. These viruses have been isolated and/or genetically characterised only in a very few cases. We detected and characterised 5 types (9 sequence variants) of squamatid atadenoviruses (AtAdVs). From bearded dragons (*Pogona vitticeps*), an emerald monitor (*Varanus prasinus*) and an asp viper (*Vipera aspis*) only PCR detection was successful. However, from samples of two Gila monsters (*Heloderma suspectum*) and a Mexican beaded lizard *(H. horridum*), AdVs could be propagated on cell culture, yielding the first report of lizard AdV isolates (type 1 and 2; LAdV-1 & -2). Partial genome analysis of LAdV-1 & -2, from these two closely related hosts, along with the phylogenetic analysis of the other detected types, contributed to the hypothesis of coevolution and reptilian origin of genus *Atadenovirus* members. The partial genomes (17 kb, 13 kb) of the two LAdVs were most alike each other, and revealed highest similarity to the snake AdV-1 sequence from GenBank. Some genes found at the right end of these genomes, however, differed significantly from those in SnAdV-1. Most interesting of these differences was the presence of a second fiber gene in both LAdV types. Apparently both fibers are functional, and thus LAdVs are the first AtAdVs reported with more than one fiber.

Iridoviruses (IVs) are important pathogens of poikilotherm vertebrates and various invertebrates. In our studies we tested the hypothesis on the potential of invertebrate iridoviruses (IIVs) infecting reptiles. We established a sensitive and specific qPCR test and an *in situ* hybridization (ISH) probe as new methods for the detection of these viruses. Cricket iridovirus-like (GbIV) variants were detected and isolated from several lizards, as well as a scorpion and crickets. In *per os* and *per coelom* infection trials with bearded dragons (*Pogona vitticeps*), we could not fulfill the Koch's postulates on the pathogenicity of an IIV isolate in lizards. Although, the most sensitive detection methods (qPCR, nPCR, isolation) detected IIVs occasionally in non-digestive organs of *per os* infected lizards, no symptomatic, pathological or histopathological (including ISH, EM) finding supported the propagation of the IIVs in the lizards. In cricket bioassays, the Koch's postulates were fulfilled with 3 different isolates. Although the bioassays showed some difference between the isolates, the partial genome sequence analysis (15 genes, 14 kb) revealed very limited (up to 0.4%) variance between the different isolates. However, the sequence comparison with the

homologous parts of Chilo iridescent virus (CIV), to which cricket IIV is considered to belong as a variant, showed longer insertions/deletions and two spots of recombination, not reported before. Based on these results, the taxonomical reclassification of GbIV, to a separate type, should be considered.

The first reptilian paramyxovirus (rPMV) was described in 1972 with respiratory or CNS signs. The isolated virus (Fer de Lance Virus - FDLV) is considered the type species of all rPMVs. Phylogenetic analysis of this and further squamatid PMVs justified the establishment of a new genus *Ferlavirus*. But subgrouping within this genus remained controversial, and prevalence of the PMVs in captive populations was not surveyed and no genetic information was available from non-squamate ferlaviruses. In our study, previously uncharacterised ferlavirus isolates from six captive snakes, three lizards and a tortoise were compared based on sequences of three genes (L, HN, U). The tortoise ferlavirus clustered as the most ancient branch in the new genus, while the other squamatid isolates separated in three groups. The established new groups “A” and “B” were in *sensu lato* extensions of groups from earlier reports, however, the new group “C” members (from a corn snake [*Pantherophis guttatus*] from Germany and a masked water snake [*Pantheropsis buccata*] from Hungary) were very distinctly related to any other previously described squamatid PMV. The genus characteristic U gene was identified in all squamatid ferlaviruses but could not be detected in the tortoise isolate. In a PCR survey of PMV infection of snakes, fifteen different sequence variants were identified either belonging to “group A” or “group B” squamatid ferlaviruses. The observed prevalence (27.5%) was surprisingly high, and concurrent infection with more than one PMV type was recorded in different organs of the same snake and/or in different snakes originating from the same populations. Similarly, in a leopard tortoise (*Geochelone pardalis*) with severe respiratory distress, 3 different squamatid ferlavirus types were identified in four different organs. These findings along with a recent report on a non-ferlavirus snake PMV from Australia underline the importance of further PCR and serological PMV surveys in both captive and wild reptile populations.

2. Introduction

Reptile virology dates back approx. 50 years, and began by surveying zoonotic arboviruses (flaviviruses, togaviruses) in potential reptile reservoir hosts (e.g. Thomas & Eklund, 1960; Whitney et al., 1968). These investigations discovered numerous positive examples, ascertained temperature dependence of viremia in reptiles and have continued up until today with intermittent intensity.

Discoveries of reptile specific viruses started about a decade later. The most important pathogens among the reptile viruses turned out to be diverse for the different reptile taxa. In chelonians (turtles and tortoises) rana- (*Iridoviridae*) and herpesviruses, in crocodilians poxviruses, in ophidians (snakes) paramyxo- and reoviruses, and in lacertilians (lizards) reo- and adenoviruses were the predominantly diagnosed illness-related agents (Essbauer & Ahne, 2001; Marschang, 2011; Ariel et al., 2011).

Among reptiles, adenoviruses (AdVs) have most often been identified in lizards, but have also been detected in various species of snakes, chelonians, and a few crocodiles. AdV infection appears to have a world-wide distribution in captive populations and wild collected snakes have also been tested positive for antibodies against an AdV (Marschang et al., 2003). In squamates, gastroenteritis and hepatitis are most commonly associated with AdV infection (Jacobson, 2007), and central nervous signs also occur occasionally (Raymond et al., 2003). The associated virus has been isolated and/or genetically characterised in a very few cases only.

Iridoviruses (family *Iridoviridae*) are important pathogens of lower vertebrates (poikilotherm vertebrates) and various invertebrates (Jancovich et al., 2011). Of the five accepted genera, members of two genera (*Iridovirus* and *Chloriridovirus*) had earlier been described in invertebrates only, and are shortly named as invertebrate iridoviruses (IIV). At the end of the 1990's, two research groups in Germany isolated and characterised a new IIV from crickets (Kleespies et al., 1999; Just & Essbauer, 2001). In both cases, the animals derived from commercial breeders that produced crickets as food for reptiles. Later, these IIVs were described in lizards by two German research groups (Just et al., 2001; Marschang et al., 2002a). A host-switch of this virus from prey insects to the predator lizards was hypothesized. The virus was characterised phenotypically and genetically, and considered a variant of Chilo iridescent virus (CIV or IIV-6), type species of the *Iridovirus* genus (Jakob et al., 2002b).

The first reptilian paramyxovirus (rPMV) was described in 1972 from a viper collection, and was named after its host: Fer de Lance Virus (FDLV). It has been associated with respiratory- and CNS signs, and mortality. Further squamatid (snake and lizard) ferlaviruses have been detected and characterised since, a number of them by partial gene sequences. Phylogenetic analysis and the unique genome-organisation (Kurath et al., 2004) both justified the establishment of a new genus *Ferlavirus* for these rPMV within the *Paramyxovirinae* subfamily (Kurath, 2009). But subgrouping within this genus remained controversial (Ahne et al., 1999; Franke et al., 2001), prevalence of the PMVs in captive populations was not surveyed properly and very little information with no genetic sequences was available from non-squamate ferlaviruses.

The objectives in our studies were to further characterise these important and common reptile viruses, to describe new types and to extend our understanding of their prevalence, host specificity and phylogenetic relationships.

3. Review of literature

3.1. Reptile virology

3.1.1. Viruses occurring in reptiles

Reptile virology is a relatively young field that has undergone rapid development over the past few decades. Early studies beginning in the 1960's dealt mainly with the role of reptiles as intermediate hosts for economically important arboviruses and their potential as reservoirs for zoonotic agents (e.g. flaviviruses and togaviruses). These studies have shown that various reptile species are susceptible to different arboviruses, that persistent infection and overwintering can occur and that temperature affects the development of viremia in these animals (Hayes et al., 1964; Whitney et al., 1968; Shortridge et al., 1974; Oya et al., 1983). Later, at the end of the 20th century as West Nile virus emerged in the Americas, the interest in these infections revivified (Kuno 2001; Steinman et al., 2003; Klenk et al., 2004; Nevarez et al., 2008).

Other studies focused on the role of viruses as pathogens in reptiles. In many cases, however, Koch's postulates have not been fulfilled, so the connection between virus and disease were often postulated based on clinical, pathological and histological observations (Marschang, 2011). Transmission studies establishing pathogenicity and cofactors were also scarce, possibly due to the relatively low commercial importance of reptiles, difficulties with the availability of animals and permits for statistically sound experiments, difficulties with housing of reptiles in an experimental setting or the inability to propagate some viruses in cell culture to sufficient titres for transmission studies (Ariel, 2011). The viruses that have been most commonly detected in reptiles are various for the different reptile taxa. In chelonians (turtles and tortoises) herpes-, rana- and picornaviruses, in lizards and snakes adeno- and reoviruses have clinical relevance, whereas in crocodiles poxvirus infection can cause severe damage (Table 1). Viruses as threatening agents for wild and endangered species, such as herpesviruses in chelonians, have refocused attention on the primary characterisation of their pathogenesis (e.g. Jacobson et al., 1991; Lackovich et al., 1999; Greenblatt et al., 2004) as well as on developing efficient diagnostic methods for their detection (Quackenbush et al., 2001).

Table 1. Viruses detected in reptiles

		Lacertilians (lizards)	Ophidians (snakes)	Chelonids (turtles & tortoises)	Crocodilians
dsDNA	***Adenoviridae***	+	+	+	+
	Iridoviridae				
	Ranavirus genus	+	+	+	
	Iridovirus genus	+			
	Erythrocytic virus	+	+		
	Poxviridae	+		+	+
	Herpesviridae	+	+	+	+
	Papillomaviridae	+		+	
ssDNA	***Circoviridae***			+[t]	
	Parvoviridae	+	+		
ds RNA	***Reoviridae***	+	+	+	
ssRNA(-)	***Paramyxoviridae***	+	+	+	
	Rhabdoviridae	+[a]			
	Bunyaviridae	+[a]		+[a]	
	Arenaviridae		+[c]		
ssRNA(+)	***Caliciviridae***		+		
	Flaviviridae	+[a]	+[a]	+[a]	+[a]
	Picornaviridae		+	+	
	Togaviridae	+[a]	+[a]	+[a]	
ssRNA RT	***Retroviridae***	+[e]	+	+[e]	+[e]

Major pathogens are featured with bold crosses, groups further discussed in this dissertation are highlighted in red. (Modified and updated from the original by Essbauer & Ahne, 2001.)

Abbreviations: “a” – arbovirus, “c” – candidate ethiological agent for IBD, “e” – endogenous retrovirus, “t” – including “Tornovirus”, a novel circular ssDNA virus, which was found in turtle fibropapillomas, and which might represent a novel virus family

3.1.2. Detection of viruses in reptiles

Generally, diagnosis of reptilian viruses can be similar to that of all other viruses. A wide range of tools is available including classical virological methods, as well as molecular tools. Histopathology can give the initial indication of a viral infection and most infections are described alongside the pathological changes they induce. Several diseases have been described and named after the observed histological changes only, long before their initial viral background was clarified, e.g. inclusion body disease (IBD) in snakes (*Retroviridae* or *Arenaviridae*) (Schumacher et al., 1994b; Jacobson et al., 2001b; Stenglein et al., 2012) or erythrocytic necrosis viruses in lizards (*Iridoviridae*) (Stehbens & Johnston, 1966). Herpes- and adenoviruses have been detected for a long time based on the inclusion bodies they cause in tissues of infected reptiles (Frye et al., 1977; Jacobson et al., 1985). Specific detection of viral nucleic acid by *in situ* hybridisation (ISH) or viral proteins by immunohistochemistry (IHC) on the histology slides is also an applicable diagnostic tool to detect some of these viruses (e.g. Perkins et al., 2001; Teifke et al., 2000).

Virus isolation in cell culture is another classical method for virus detection but with decreasing popularity due to its time and labour costs, although it has numerous advantages that should not be neglected. With this method the viral agent(s) can be unselectively amplified for easier identification and characterisation, and propagated for use in transmission studies or for production of specific antisera (Jacobson & Origgi, 2002; Johnson et al., 2010). Some viruses induce cytopathic effects (CPE), whereas others do not cause CPE or do not grow at all in cell culture. For those cell lines that support propagation of a particular virus, the temperature regime for both cell and viral growth will usually be lower as for mammalian systems due to the poikilothermic nature of reptiles. Isolation of zoonotic viruses from reptiles dates back as far as 1939, when Western equine encephalitis virus was isolated from South American pit vipers (*Bothrops alternatus*) (Rosenbusch, 1939) and it was followed by several other cases of flavi- and calicivirus detection from snakes, tortoises and alligators using either mammalian or mosquito cell lines (Lee et al., 1972; Smith et al., 1986; Drury et al., 2001). Specific reptilian viruses can also be isolated on heterologous cell lines e.g. Vero cells or in cell lines derived from fish (Vieler et al., 1994; Ariel et al., 2009), but homologous reptile derived cell lines are usually more susceptible. The first tissue explants were made from an apparently healthy iguana, from which a herpesvirus could be isolated (Clark & Karzon, 1972). Since then several permanent reptilian cell culture systems have been established and are now available from the American Type Culture Collection (ATCC) or other cell culture banks. Of the adherent epithelial cell

types available from the ATCC, box turtle (*Terrapene carolina*) heart cells (TH-1; CCL-50), iguana (*Iguana iguana*) heart cells (IgH-2; CCL-108), tokay gecko (*Gekko gecko*) lung cells (Gekko lung-1; CCL-111) and viper (*Vipera russelli*) heart cells (VH-2; CCL-140) originate from healthy organs, whereas viper (*Vipera russelli*) spleen cells (VZW; CL-129) originate from a metastatic tumour and produce a gammaretrovirus.

Electron microscopy (EM) is a time, cost, and labour consuming method, but it has been an ultimate diagnostic tool for reptile viruses, from the early stages of reptile virology (e.g. Ippen et al., 1978) up until recently (e.g. Hughes-Hanks et al., 2010). The obvious advantage of this unselective method is the morphologic description of new agents, and as a primary tool it also may direct the diagnostician toward selecting the best molecular diagnostic tools (primers and/or probes) to be used in the following tests.

Polymerase chain reaction (PCR) has become the most preferred method for diagnostics in virology. Numerous conventional (nPCR) and a few real-time PCRs (qPCR) have been described for various virus genera/families occurring in reptiles (e.g. Ahne et al., 1999; Marschang et al., 2005; Wellehan et al., 2004, 2009). Subsequent sequencing of parts of the viral genome opens possibilities for fast characterisation, insight into phylogenetic relationships and epidemiological investigations. The drastic decrease in the cost of sequencing has further turned diagnostic attention to molecular methods and enabled metagenomic studies with discovery of new agents in reptile diseases with complex viral background (Ng et al., 2009). High-throughput new generation sequencing (NGS) methods are also becoming more and more popular and they are already in use to characterise new viruses found in reptiles (Hyndman et al., 2012a; Stenglein et al., 2012).

Indirect detection of viral exposure of individuals and/or screening collections with the help of serological methods has also been repeatedly used in reptiles. These tests were mostly developed in academic research laboratories and few are commercially available. Such tests have been developed for exposure of marine turtles (Coberley et al., 2001) and terrestrial tortoises (Origgi & Jacobson, 1999; Origgi et al., 2001) to herpesviruses. These enzyme-linked immunosorbent assays (ELISA) have high sensitivities and specificities, however, in the indirect format they require specific anti-reptile immunoglobulins which limits their applicability to one or a few related species. Simpler and more widely applicable methods are the virus neutralisation test (VN) that is also frequently used in herpesvirus serodiagnostics (Marschang et al., 2001) as well as in AdV surveys (Marschang et al., 2003), and the haemagglutination-inhibition (HI) test which is applicable particularly with PMVs (Jacobson et al., 1992; Gravendyck et al., 1998).

3.2. Adenoviruses

3.2.1. General introduction to the family *Adenoviridae*

Adenoviruses are medium-sized (70–90 nm), non-enveloped, double-stranded DNA viruses with an icosahedral capsid symmetry. AdVs were first described in 1953 by Rowe et al., from spontaneously degenerating human pharyngeal tonsillectomy cultures and at the same time from tracheal phlegm of people suffering from acute respiratory illness (Hilleman & Werner, 1954). Later the kinship of these two isolates was recognised, giving the denomination adenovirus to the agents for their adenoid origin (Enders et al., 1956). The first animal AdV isolate was found in cattle in 1959 (Klein et al., 1959), and numerous further isolations followed during the next four decades from various bony vertebrates, representing all major classes from fish to mammals (Russell & Benkő, 1999). Today the family *Adenoviridae* has five accepted genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus* (Harrach et al., 2011), with altogether approx. 45 recognised virus species comprising over 250 different serotypes of which 67 were described from humans (Harrach et al., 2012).

In the following structural descriptions (Fig. 1), the most studied human AdV serotype HAdV-2 acts as the model (Russell, 2000; 2009) and the roman numbers indicate major polypeptides in order of their decreasing molecular weight (Maizel et al., 1968). The main capsid proteins, the hexon (II), the penton base (III) and the fiber (IV) form the icosahedrons of an AdV. Additional minor structural components contribute to the stability of the capsid (IIIa, VI, VIII, IX) (Fig. 1/B). From the penton base a fiber protrudes to form the complex of a penton capsomer at the vertices. A single fiber is located at each vertex of most AdVs except for members of the *Aviadenovirus* genus, in which two protrude from each penton base (Gelderblom & Maichle-Lauppe, 1982). The number of the protruding fibers is not always in direct connection with the coding genes present in the viral genome. Extra copies (paralogs) of the fiber gene can be found in several, but not all aviadenoviruses (Chiocca et al., 1996; Kaján et al., 2010, 2012) (Fig. 2), in a few mastadenoviruses (HAdV-40 and 41, certain simian AdVs) (Kidd & Erasmus, 1989; Kidd et al., 1990; Davison et al., 1993; Kovács et al., 2005) and in the sole examined ichtadenovirus genome (Doszpoly, 2011).

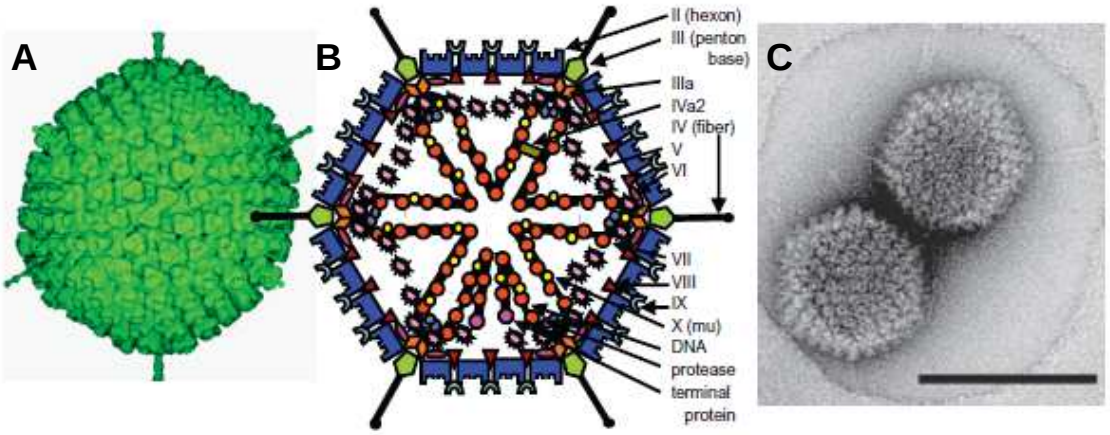

Figure 1: (A) Cryo-electron reconstruction of a particle of an HAdV-2. **(B)** Section of a mastadenovirus particle. For the description of the nucleocore components see above text. **(C)** Negative contrast electron micrograph of a FAdV-9 particles.
The bar represents 100 nm (Harrach et al., 2011).

In the core of an AdV, the linear genome is in complex with polypeptide VII, a histone-like protein and mediator of virus DNA import into the nucleus and with proteins X and V (the later one only in mastadenoviruses). The terminal protein is covalently linked to the 5' end of each strand of the viral genome (Fig. 1).

Adenoviruses have a genomic arrangement of a conserved middle part and variable ends (Davison et al., 2003). A central block of conserved genes (L region), whose transcription is driven by the major late promoter, as well as the early regions (E2A & E2B) downstream on the complementary strand, contain 18 genes that are present in all known AdVs and code either for structural proteins or for enzymes essential for the viral replication. The flanking regions are genus-specific, and contain the ORFs that are conserved within a certain genus, but have no homologues in other genera (Fig. 2).

AdVs can be assigned to genera based on their genome organisation and phylogenetic relatedness. Three of the five genera have a defined host species spectrum as well: *Mastadenovirus* members have all been isolated from mammals, *Aviadenovirus* members all from birds, and the genus *Ichtadenovirus* contains the only AdV found in fish. However, the other two genera have a mixed host origin. Atadenoviruses were found in birds, squamates, ruminants and even a marsupial (Thomson et al., 2002). Siadenoviruses have been detected in several bird species, in a leopard frog and in Sulawesi tortoises (Rivera et al., 2009).

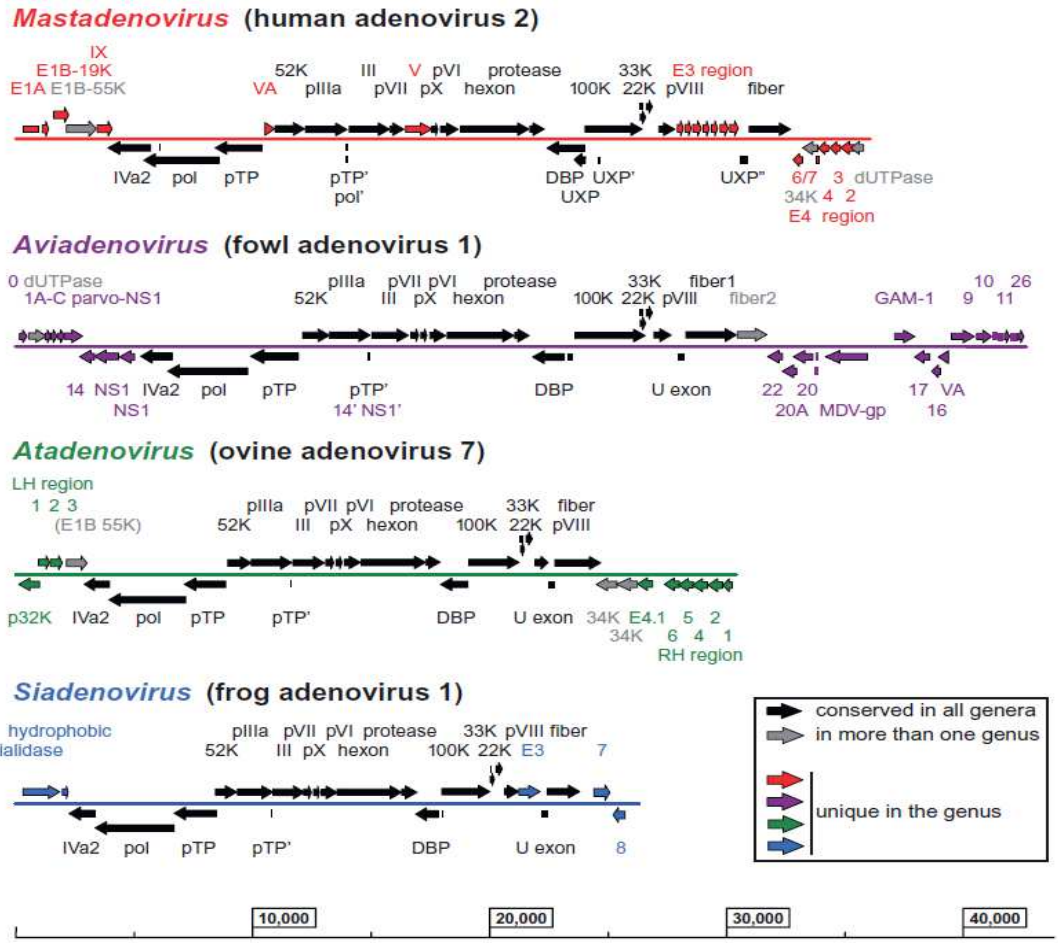

Figure 2. Schematic illustration of the different genome organizations found in members of the four established genera for AdVs from tetrapod hosts. Black arrows depict genes conserved in every genus, grey arrows show genes present in more than one genus, coloured arrows show genus-specific genes (Harrach et al., 2011).

The specific characteristics of certain bovine AdV types was first recognised in Hungary by Bartha (1969), before the era of molecular genetics. The new methods later confirmed his early findings (Benkő et al., 1988, 1990; Harrach et al., 1997) and subsequently led to the revisal of the taxonomy of the family *Adenoviridae*, with the establishment of a third genus *Atadenovirus* (AtAdV) (Benkő & Harrach, 1998; Dán et al., 1998; Benkő et al., 2000). New findings on AtAdVs from various other hosts led to the development of a coevultion hypothesis with occasional host switches (Harrach, 2000; Benkő & Harrach, 2003).

In the last decade, the Hungarian veterinary virologists have also pioneered in the detection and characterisation of AdVs from non-domestic animals (Sainsbury et al., 2001; Farkas et al., 2002, 2008; Kovács et al., 2003, 2004, 2010; Doszpoly, 2011; Jánoska et al., 2011; Kovács, 2011; Kovács & Benkő, 2011) among them a snake AdV (for further details see the following sections). Many of these studies substantiated the establishment of the fourth and fifth genera: *Siadenovirus* and *Ichtadenovirus*.

3.2.2. Characteristics of *Atadenovirus* genus members

Atadenoviruses are serologically and phylogenetically distinct from AdVs of other genera and their genomic organization is also different. AtAdVs have been detected in a broad range of hosts, including scaled reptiles (order Squamata) several bird and ruminant species and also a marsupial (Benkő et al., 2002; Harrach et al., 1997, 2011). Virions are relatively heat stable compared to mastadenovirions. The genome size of sequenced isolates ranges from 29.5 to 33.2 kb. For the earlier characterised ruminant, marsupial and avian atadenoviruses, the G+C content of the genome was found to be low (33.6 to 43.0%). The corresponding high AT content gave the name for the genus. However AtAdVs originating from reptiles seem to have a balanced G+C content (Farkas et al., 2002, 2008; Wellehan et al., 2004). AtAdVs have several unique proteins, and some that show very little similarity to their suspected counterparts in other AdV genera. The central part of the genome of AtAdVs is similar to that of mastadenoviruses (except that there are no protein V and IX genes), while the extremities of the genomes are different. The right-hand end of the AtAdV genome for example contains several shorter genes similar to each other. There are two E4 34K homologue genes, and 2 to 4 RH homologues. Genes LH3 and E4.3 (and its homologue the E4.2) show slight similarity with mastadenovirus proteins E1B 55K and E4 34K, respectively. No immunomodulatory genes such as those found in the mastadenovirus E3 region have been found in AtAdV. Some members of the genus have extra transcriptional units in their genome, e.g. duck AdV-1 (DAdV-1; previously egg drop syndrome virus: EDS) has a unique region at the far right-hand end with seven uncharacterised ORFs with supposed host-specific in functions.

Applying the general virus species concept (Regenmortel, 1992) to AdV, serologically distinguishable AtAdV serotypes have been grouped into species by the ICTV study-group under Hungarian leadership. The data available at the beginning of our studies suggested the separation of several species within the genus *Atadenovirus*, but 4 of them had been approved at that time only (Benkő et al., 2005).

3.2.3. Adenovirus infections in reptiles

In reptiles, AdV infections have been detected by light and electron microscopy (EM) examination or by *in situ* hybridization (ISH) (Ramis et al., 2000; Perkins et al., 2001) of histopathological sections in a number of different species of the Diapsida class, including one crocodile species from the Archosauria subclass (Jacobson et al., 1984) and also in chelonians (Wilkinson, 2004). AdVs are the viruses most commonly

identified in lizards, especially bearded dragons (*Pogona vitticeps*). AdV infection has also been reported in several other agamid, varanid and chameleonid species as well as 10 snake species from the Squamata order of the Lepidosauria subclass (Essbauer & Ahne, 2001). Associated pathological lesions varied from enterohepatic inflammation (hepatitis, oesophagitis, enteritis) to splenitis, nephritis, pneumonia or encephalopathy. The primary pathogenic role of these viruses was questioned in many cases in which they were detected without signs of concurrent disease (Jacobson & Kollias, 1986; Jacobson & Gardiner, 1990; Ogawa et al., 1992; Schumacher et al., 1994a). However, the pathogenicity of an AdV for reptiles was demonstrated in one case by an experimental transmission study (Jacobson et al., 1985).

In spite of the numerous detections of reptilian AdV infection by EM, ISH or, more recently, by PCR, there were very few reported cases in which the virus was successfully isolated. Jacobson et al. (1985) obtained AdV from a boa constrictor (*Boa constrictor*) while Ahne and his co-workers isolated an AdV strain from a royal python (*Python regius*) (Ogawa et al., 1992) and from a moribund corn snake (*Pantherophis* [*Elaphe*] *guttatus*) showing clinical signs of pneumonia (Juhasz & Ahne, 1992). This corn snake isolate was later randomly cloned (Benkő et al., 2002) and completely sequenced (Farkas et al., 2002, 2008) and thus serves as a prototype for reptilian AdVs. A sequence comparison of partial IVa2 and DNA polymerase gene sequences of this prototype virus with those of three AdV isolates from other German snakes proposed that they were identical (or very similar, as the examined genes were conserved) (Marschang et al., 2003). Although adenovirus infections are frequently described in lizards, no virus was isolated from a lizard in cell culture until the beginning of our studies.

Wellehan et al. (2004) designed two degenerate primer pairs based on the consensus sequence of the DNA polymerase genes of different adenovirus types from three genera. This nested PCR system has been shown to be an efficient tool for surveying for adenovirus infections of all genera in a wide range of animals, among them reptiles (Benkő et al., 2006). In spite of the degenerate primers, direct sequencing of the products is possible, and phylogenetic analysis of the sequences can help to determine the virus type. Wellehan and co-workers have used this system to describe 6 novel lizard adenoviruses from seven host species (eublepharid geckos, tokay gecko, Gila monster, blue-tongued skink, bearded dragon and mountain chameleon). In both of the above mentioned studies, the phylogenetic analysis of the short (ca. 300 bp) DNA polymerase segments clearly clustered all squamatid (lizard and snake) AdVs within the genus *Atadenovirus*, giving further support for the coevolution theory of AtAdVs, that this AdV lineage coevolved with the (squamatid) reptiles (Harrach, 2000).

AdVs of chelonians, however, which so far have been analysed based on short DNA polymerase gene sequences, have all clustered either into the genus *Siadenovirus* (Rivera et al., 2009) or outside of the existing genera (Farkas & Gál, 2009).

3.3. Iridoviruses

3.3.1. Iridovirids, general introduction to the family *Iridoviridae*

Iridovirids belong to the nucleo-cytoplasmic large DNA viruses (NCLDV), a group of DNA viruses that also include mimiviruses, phycodnaviruses, African swine fever virus, and poxviruses. Although the genome size of NCLDVs varies greatly (between 100 kb and 1200 kb), they appear to form a monophyletic group based on a subset of about 30 conserved genes (Filée et al., 2008). Following the suggestion of Vetten and Haenni (2006), members of the family *Iridoviridae* will be referred to as iridovirids (or in short: IV) in the dissertation to distinguish them from members of the genus *Iridovirus* (*sensu stricto* invertebrate iridoviruses, IIV).

The word “irido” is derived from Iris who was the Greek goddess of the rainbow. This is due to the "rainbow like" iridescence observed in heavily infected insects as mature virions accumulate within the cytoplasm of their infected cells in large paracrystalline arrays. Pelleted samples of invertebrate iridoviruses can also show this characteristics (Fig. 3).

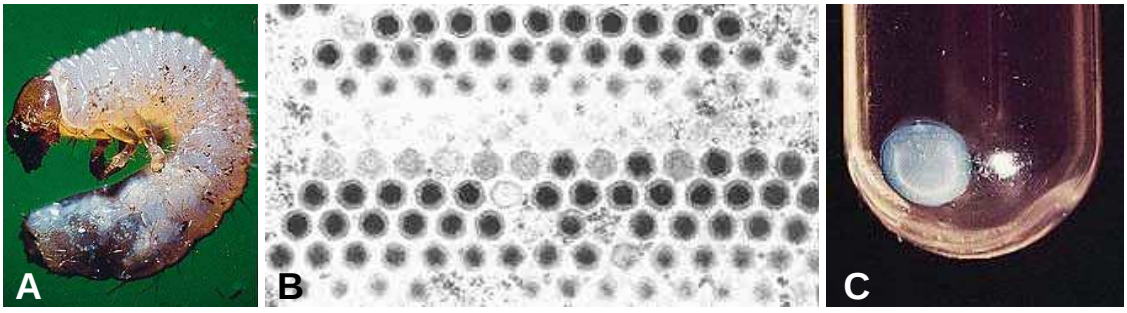

Figure 3. Bluish iridescence often caused by the members of the genus *Iridovirus*. **(A)** Larvae of the grass grub *Costelytra zealandica* displaying blue colouration of the hindgut due to iridovirus infection. **(B)** Paracrystalline array of virus particles within infected cell. This array gives rise to the iridescent phenomenon. **(C)** Pellet of purified Tipula iridescent virus. (Webby at al., 1998, 2012)

The family *Iridoviridae* is currently divided into five genera (*Irido*-, *Chloriridо*-, *Rana*-, *Megalocyti*- and *Lymphocystivirus*) of which the first two occur in invertebrates only (*sensu lato* IIV), whereas members of the latter three infect ectothermic

vertebrates (fish, amphibians, reptiles) (Jancovich et al., 2011). Morphologically, iridovirids are large, icosahedral viruses (120–200 nm in diameter) that possess an internal lipid membrane located between the viral core and outer capsid (Fig. 4).

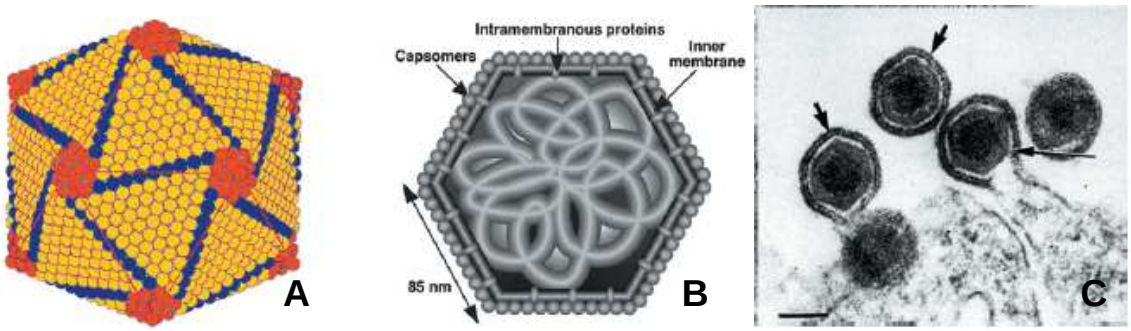

Figure 4. (A) Outer shell of invertebrate iridescent virus 2 (IIV-2). **(B)** Schematic diagram of a cross-section of an iridovirus particle, showing capsomers, transmembrane proteins within the lipid bilayer, and an internal filamentous nucleoprotein core. **(C)** Transmission electron micrograph of particles of frog virus 3 (FV-3), budding from the plasma membrane. Arrows and arrowheads identify the viral envelope; the bar represents 200 nm. (Jancovich et al., 2011)

Particles can be released by budding from the cell membrane and acquire an envelope, but in contrast to other virus families, this envelope is not required for infectivity, and many virions remain cell-associated and are released as naked particles following cell lysis. Members of the family possess linear, double-stranded DNA genomes, which vary in size from approximately 140 kb (genus *Ranavirus*) to over 200 kb (genus *Iridovirus*). Iridovirid genomes are unique among animal viruses in that they are circularly permuted and terminally redundant (Goorha & Murti, 1982; Delius et al., 1984). Because the terminal repeat region accounts for 5%–50% of the genome length, the total size of each genome can be much longer than the length of the unique region (e.g. ~105 kb for a typical ranavirus).

Since their isolation nearly 50 years ago, iridovirids have been overshadowed by other DNA viruses of medical or veterinary importance, specifically adeno-, herpes- and poxviruses (Chinchar et al., 2009). However one family member, lymphocystis disease virus (LCDV), has been known for over a century by the wart-like disease it causes in several species of salt- and fresh-water fishes (Weissenberg, 1965). A second family member, frog virus 3 (FV3), was also extensively studied (Chinchar, 2002) for its ecological impact. The relative absence of commercial, agricultural or medical damage caused by iridovirid infections limited the study of this diverse virus family (Williams et al., 2005). However, within the last 20 years the increased recognition of vertebrate IVs as important pathogens infecting commercially and ecologically important fish and amphibian species has attracted renewed interest in the impact of iridovirids in ectothermic vertebrates (Hyatt et al., 2000; Chinchar, 2002;

Williams et al., 2005; Mendelson et al., 2006). For example, members of the genus *Ranavirus* were identified as the causative agent in approximately half of the documented cases of amphibian mortality reported in the USA between 1996 and 2001 (Green et al., 2002). In addition, viruses in the genus *Megalocytivirus* have been responsible for numerous outbreaks of severe disease in fish farming facilities throughout Asia (Nakajima et al., 1998). Despite the growing impact of iridovirid diseases worldwide and the increased use of molecular approaches to elucidate their phylogeny and life cycle (Williams, 1996, 1998; Chinchar, 2002; Williams et al., 2005), it appears that this virus family is not yet receiving considerable scientific recognition everywhere.

3.3.2. Invertebrate iridescent viruses, characteristics of the genus *Iridovirus*

The first invertebrate iridescent virus (IIV) was reported in the mid 1950's from insects (Smith & Xeros, 1954; Williams & Smith, 1957). Further similar viruses were found later and assigned to the genera *Iridovirus* and *Chloriridovirus*. This latter genus has one accepted member only (mosquito iridescent virus, IIV-3), which was considered outlying of the genus *Iridovirus* based on differences in phenotypic traits (particle size, iridescence colour) and on its narrower host range (Jancovich et al., 2011). However recent phylogenetic analysis supports that IIV-3 is close relative of other IIVs of the genus *Iridovirus* (IIV-9) thus the taxonomic separation of this genus should be revised (Wong et al., 2011). Members of the genus *Iridovirus* (*in sensu stricto* IIV) infect a wide range of invertebrates, mainly arthropods, but there have also been a few reports from other taxa (molluscs, an annelid and a nematode) (Williams, 2008a, 2008b). The name of the hosts from which the IIVs had been first isolated was later used in the nomenclature of these viruses. To accommodate the growing number of hosts reported with IIV infections these viruses were assigned type numbers based on the chronological order in which they were reported (Tinsley & Kelly, 1970). As such, IIV-6 (synonym: Chilo iridescent virus, CIV) is the type species of the genus *Iridovirus*. This genus currently comprises two accepted species (IIV-1 and IIV-6), and eleven candidate (earlier: tentative) species of interrelated viruses with a dehydrated particle diameter in the range of 110-160 nm (Jancovich et al., 2011). In comparison, IIV-3 member of genus *Chloriridovirus,* has a particle size of 180 nm. There are many records of iridoviruses from invertebrate hosts which have not been or have been poorly characterised.

IIVs have been isolated from a wide range of arthropods and have a global distribution. In nature, the host range appears to vary but there is evidence, for some viruses, of natural transmission across insect orders and even phyla. Therefore several IIVs have been suggested to use as pest control agents (e.g. Kleespies et al., 1999; Hernandez et al., 2000; Henderson et al., 2001; Jakob et al., 2001). Patent infections are often displayed in iridescence in the animals (Fig. 3) and are mostly lethal. The non-lethal covert infections can also be common in certain hosts. No evidence exists for transovarial transmission. Where horizontal transmission has been demonstrated, it is usually by cannibalism or predation of infected invertebrate hosts (Williams, 2008b). In many early reports, IIV infection was suspected based on either the apparent iridescence or on EM observation only.

The majority of the IIV sequences currently available in GenBank are from the major capsid protein (MCP) gene. Approximately 80% of these data date back more than 10 years and were published in a single study (Webby & Kalmakoff, 1998). In that study, a molecular comparison of fragments of the MCP gene of eighteen diverse isolates revealed that IIVs of the Iridovirus genus cluster into three groups/clades. Group I contained two isolates (IIV-31 and PjIV, *Popillia japonica* IV), which were closely related phylogenetically, but had been isolated at distant locations (USA and the Azores) from evolutionarily distant host species (an isopod and a beetle). Group II contained a single member, the CIV (Chilo iridescent virus), the type species of the genus isolated from a stem borer lepidopteran in Japan. All other isolates, which were obtained from different dipteran, lepidopteran, coleopteran, hymenopteran, and isopod hosts collected on five continents, clustered into Group III. Later studies added a few further sequences to this tree. An isolate from a New Zealand isopod was a close relative of the other two in Group I (Sadler et al., unpublished; GenBank accession AF297060). A new crustacean isolate from Madagascar formed a new group by itself (Tang et al., 2007). In addition, a new isolate has been added to Group II as well. This latter IIV was originally detected in insects bred for the pet trade in Europe and named cricket iridovirus (CrIV) or *Gryllus bimaculatus* iridovirus (GbIV) after its first known host (Kleespies et al., 1999; Just & Essbauer, 2001), and its wide host range has been demonstrated amongst different insect orders (Kleespies et al., 1999). In addition to the primary findings in crickets, closely related viruses have been repeatedly detected in lizards (Just et al., 2001; Marschang et al., 2002a). It has been hypothesized that lizards become infected with the virus when fed IIV infected prey insects.

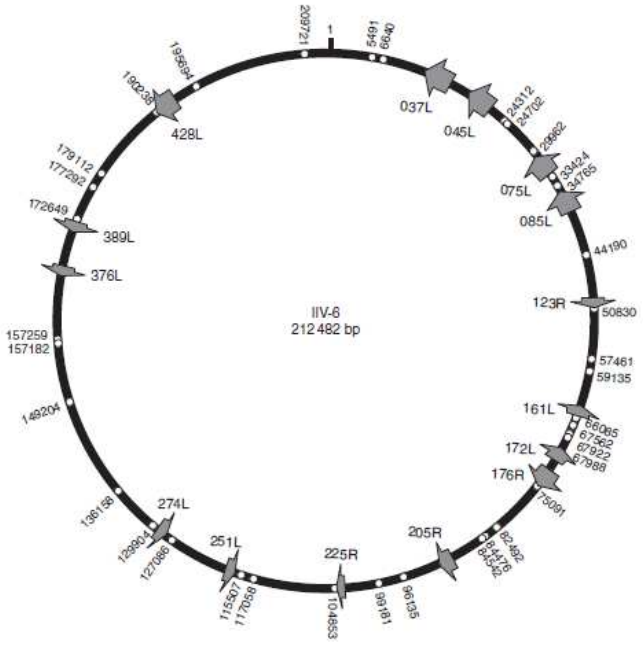

Figure 5. Schematic circularised illustration of the linear genome of IIV-6 (CIV). Position of selected ORFs (some listed in the text below) are indicated with arrows with their actual orientation (L or R). White dots indicate EcoRI cleavage sites. (Jakob et al., 2002a.)

The IIV genome is not methylated in contrast to virtually all vertebrate iridoviruses (Williams, 2008a). At the beginning of our studies, two IIV genomes have been completely sequenced only: IIV-3 and IIV-6. The genome of IIV-6 is 212 kb (unique portion) with 28.6% G+C content and comprises 468 ORFs, of which 234 are nonoverlapping. No collinearity was observed between the genomes of IIV-3 and IIV-6. Core IIV genes include those involved in 1.) replication, including DNA polymerase (037L), RNA polymerase II (176R, 428L), RNAse III (142R), a helicase (161L), and a DNA topoisomerase II (045L); 2.) nucleotide metabolism, such as ribonucleotide reductase (085L, 376L), dUTPase (438L), thymidylate synthase (225R), thymidylate kinase (251L), and thymidine kinase (143R); and 3.) other proteins of known function including inhibitor of apoptosis (157L, 193R), and the major capsid protein (MCP or 274L). Other notable putative genes identified in IIV-6 include an NAD-dependent DNA ligase (205R) and a putative homologue of sillucin (160L), a cysteine-rich antibiotic peptide, the first described viral antibiotic (VAB) (Fig. 5).

3.3.3. Iridovirid infections in reptiles

Iridovirids have been described as possible pathogens of reptiles since the 1960's. The IVs that have been described and partially characterised in reptiles include

ranaviruses in chelonians, lizards and snakes, erythrocytic necrosis viruses in lizards and snakes, and invertebrate iridoviruses (IIV) in lizards (Marschang, 2011).

Ranaviruses as important pathogens of ectothermic vertebrates have been regularly isolated from reptiles since the late 1990's. They have been mostly described in chelonian species world-wide, including terrestrial tortoises (Mao et al., 1997; Marschang et al., 1999; Blahak & Uhlenbrok, 2010), and fresh-water turtles (*Terrapene spp., Pelodiscus [Trionyx] sinensis, Trachemys scripta elegans*) (Chen et al., 1999; De Voe et al., 2004; Johnson et al., 2007; Huang et al., 2009). In these species, viral infection has been associated with lethargy, anorexia, nasal discharge, conjunctivitis, severe subcutaneous cervical oedema, ulcerative stomatitis, and "red-neck disease". Histologically, infected animals have been found to have hepatitis, enteritis, and pneumonia. In a transmission study Koch's postulates have also been fulfilled (Johnson et al., 2007). The complete sequence of the soft-shelled turtle virus has been determined (Huang et al., 2009), showing a high degree of sequence conservation and a collinear arrangement of genes with frog virus 3 (FV3), and suggesting transmission of an amphibian virus to reptiles.

In lizards, ranaviruses have been described in a gecko (*Uroplatus fimbriatus*) in Germany (Marschang et al., 2005) and a mountain lizard (*Lacerta monticola*) in Portugal (Alves de Matos et al., 2011). In the gecko, infection was associated with granulomatous lesions in the tail and liver. In the mountain lizard, no overt disease was documented. The origin of infection in reptiles has not been documented, and characterisation of the detected viruses generally relied on partial sequences of the major capsid protein (MCP) gene. The detected viruses are closely related to FV3, the type species of the genus *Ranavirus*. Ranavirus infection has also been described in green pythons (*Chondropython viridis*) in Australia (Hyatt et al., 2002). The snakes showed ulceration of the nasal mucosa, hepatic necrosis and severe necrotizing inflammation of the pharyngeal submucosa.

A study comparing genome sequences from a range of ranaviruses from different hosts suggested that the ancestral ranavirus was a fish virus and that several recent host shifts have taken place, leading, among others, to infection of reptiles (Jancovich et al., 2010).

Viral erythrocytic infections associated with irido-like viruses have been described in lizards, snakes, and turtles (Wolf, 1988). Pathology associated with erythrocytic necrosis virus infections in reptiles is unclear, but morphological changes in infected erythrocytes have been documented. A transmission study conducted with lizards showed that infection with these agents can, in some cases, become systemic and may lead to death (Alves de Matos et al., 2002). Recently, a PCR was successfully

used to detect an iridovirid in a ribbon snake (*Thamnophis sauritus*) with erythrocytic inclusions in Florida, USA (Wellehan et al., 2008). A lizard (*Lacerta monticola*) erythrocytic virus from Portugal was also PCR-sequence characterised and found 65.2/69.4% (nt/aa%) similar to the ribbon snake virus (Alves de Matos et al., 2011). Ultrastructural differences between the viruses were also detected by EM. These studies supported the classification of the erythrocytic necrosis viruses of reptiles in a new genus in the family *Iridoviridae*.

In 2001, a German group reported the isolation of IIV-like viruses from the lung, liver, kidney, and intestine of two bearded dragons (*Pogona vitticeps*) and a chameleon (*Chamaeleo quadricornis*) and from the skin of a frilled lizard (*Chlamydosaurus kingii*) on viper heart cells (VH-2) at 28°C (Just et al., 2001). The frilled lizard showed pox-like skin lesions and one of the bearded dragons had pneumonia. The other lizards had died with non-specific signs. Part of the MCP gene of the isolates was sequenced and had 97% identity to the nucleotide sequence of Chilo iridescent virus (CIV or IIV-6), the type species of the genus *Iridovirus*, and 100% identity to the nucleotide sequence of the cricket iridovirus (GbIV). A host-switch of this virus from prey insects to the predator lizards was postulated (Just et al., 2001).

3.4. Paramyxoviruses

3.4.1. General introduction to the family *Paramyxoviridae*

Paramyxoviruses (PMV) are negative sense single stranded RNA (ssRNA(-)) viruses with a helical nucleocapsid (13-18 nm diameter) packaged in an envelope (≥150 nm) (Fig. 6). Family *Paramyxoviridae* includes some of the important and ubiquitous disease-causing viruses of humans, including the leading cause of vaccine-preventable children deaths, that has been targeted by the World Health Organization for eradication (measles), and some of the most prevalent viruses known (respiratory syncytial virus, parainfluenza virus and mumps virus). Important animal viruses that have a major economic impact on poultry and animal rearing also belong to this family (Newcastle disease virus and rinderpest virus) (Lamb & Parks, 2007).

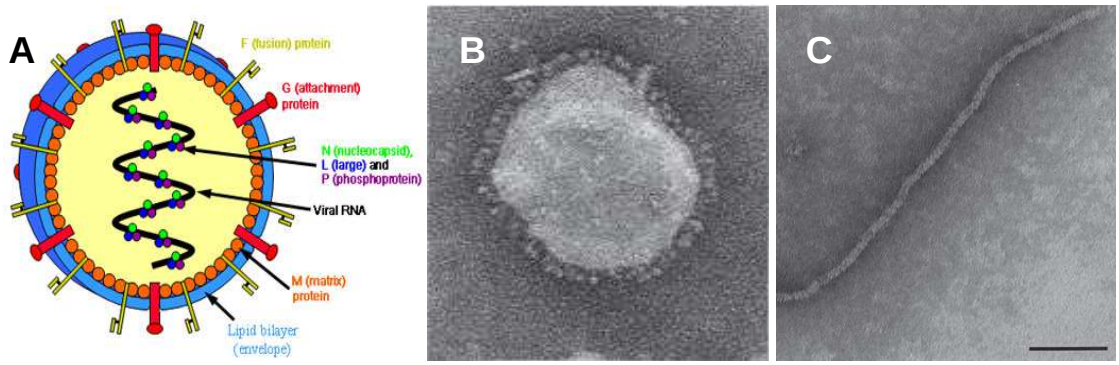

Figure 6. (A) Schematic diagram of a henipavirus particle in cross-section. (www. drugdiscoveryopinion.com) **(B)** Negative contrast electron micrographs of intact parainfluenza virus 5 (PIV5, previously simian virus 5 [SV5]) particles and **(C)** the PIV5 nucleocapsid after detergent lysis of virions. The bar represents 100 nm. (Wang et al., 2011)

PMVs are members of a separate family along with three others (*Borna*-, *Filo*-, and *Rhabdoviridae*) in the order *Mononegavirales*. Members of the family *Paramyxoviridae* have genomic organisation and strategy of gene expression and replication similar to those of other families in the order, especially the families *Rhabdo*- and *Filoviridae*. PMVs are defined by having a protein (F) that causes viral-cell membrane fusion at neutral pH. The family was divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae,* with five and two accepted genera, respectively, at the beginning of our studies (Lamb et al., 2005).

The linear non-segmented PMV genome contains 6 to 8 transcriptional elements each of them encoding a single mRNA (with a consensus order of: 3' N-P-M-F-G/H/HN-L 5' in members of the anterior subfamily) with the exception of the P element (Fig. 7). The P element is transcribed into an exact-copy and an edited mRNA encoding the P and V proteins alternatively. Three of the proteins translated from these mRNAs are nucleocapsid associated: RNA-binding protein (N), phosphoprotein (P) and large RNA polymerase protein (L) whereas the other three proteins are membrane associated: matrix protein (M), fusion protein (F) and attachment protein (G, H or HN). These latter two membrane protruding proteins are considered major epitopes of these viruses and are responsible for the fusogenic, haemagglutinating and neuraminidase activities found in many (but not all) PMVs. Two of the genes coding for nucleocapsid-associated proteins (N and L) are more conserved and used for resolving phylogenetic relations between genera, while genes coding for the type specific surface epitops are better for resolving relationship within the different genera (Wang et al., 2011).

Beyond basic similarity, however, there is divergence between different genera in genome length, existence of overlapping ORFs within the invariant genes, length and sequence of untranslated regions (UTRs) and intergenic regions (IGRs), etc. In all members of the subfamily *Paramyxovirinae* the genome length must be a multiple of 6

nt for efficient genome replication (the “rule of six”), perhaps reflecting the precise packaging of the genome by a nucleocapsid protein subunit (Kolakofsky et al., 2005).

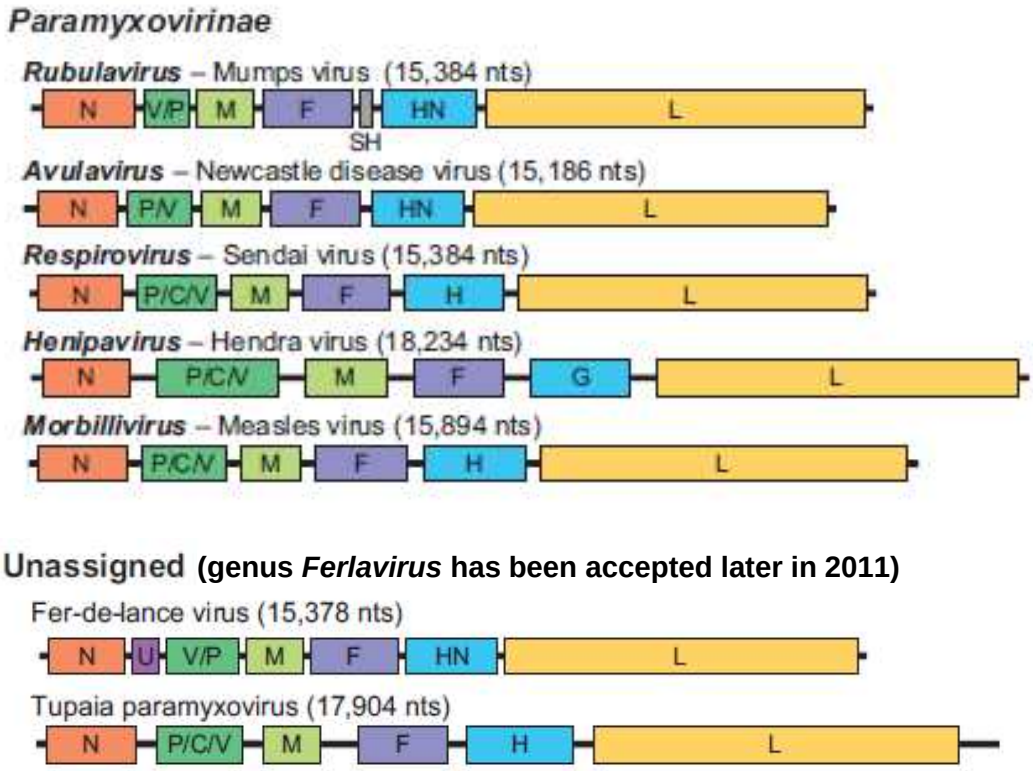

Figure 7. Maps of genomic RNAs (3’-to-5’) of viruses in the subfamily *Paramyxovirinae* and in two “unassigned” species. Each box represents a separately encoded mRNA, approximately to scale; multiple distinct ORFs within a single mRNA are indicated by slashes. For rubula- and avulaviruses, the intergenic sequences are variable (1–190 nt long). In the group of unassigned new viruses, there is a 3-nt intergenic region similar to those observed in the genera *Morbillivirus*, *Respirovirus* and *Henipavirus*. There are also novel genes present in these viruses (such as the U gene in Fer de Lance virus) (Wang et al., 2011).

3.4.2. Paramyxovirus infections in reptiles

The first reptilian paramyxovirus (rPMV) was described in a serpentarium in Switzerland in 1972 (Fölsch & Leloup, 1976) from common lancehead vipers (*Bothrops atrox*) with severe respiratory distress, lethargy, CNS signs and 30% mortality. The virus isolated (named after its host: Fer de Lance Virus – FDLV; Clark et al., 1979) is considered the type species for this group of reptilian PMVs and is the only one which has been fully sequenced to date (Kurath et al., 2004). Its 15,378 nt long genome has an extra transcriptional unit between the N and P genes with 2 predicted overlapping ORFs coding for short proteins of unknown function (U gene; Fig. 7). This feature along with unique gene start patterns and the calculated phylogenetic relatedness of FDLV to other known PMVs were the major justifications for the establishment of a new genus

"*Ferlavirus*" for this unassigned virus within the *Paramyxovirinae* subfamily (Kurath, 2009).

PMV are an important cause of disease in snakes in both private and zoologic collections and have been described in many different parts of the world (Jacobson et al., 1992; Essbauer & Ahne, 2001; Ariel et al., 2011; Marschang, 2011). Ferlavirus infections have also been detected in a number of snake species by different methods (virus isolation, histology, EM, IHC, ISH, serology - Ahne et al., 1987; Potgieter et al., 1987; Jacobson et al., 1992; Blahak, 1995; Homer et al., 1995; Richter et al., 1996; Manvell et al., 2000; Orós et al., 2001; West et al., 2001; Kolesnikovas et al., 2006). Clinical signs in infected snakes are similar to those described in the first case, mostly the respiratory tracts were involved, but CNS signs have also been observed regularly (Jacobson, 2007).

Isolation of PMVs from lizards is relatively rare (Ahne & Neubert, 1991; Gravendyck et al., 1998; Marschang et al., 2002b) and clinical signs of pneumonia have been described once only (in PMV infected caiman lizards; *Draecena guianensis*) (Jacobson et al., 2001a). However, antibodies against PMVs have been detected in both wild-caught and captive lizards (Gravendyck et al., 1998; Marschang et al., 2002b; Lloyd et al., 2005). In turtles (chelonians), descriptions of PMV infections are even rarer (Oettle et al., 1990; Zangger et al., 1991) and have been associated with dermatitis (Zangger et al., 1991). There were no published accounts of the isolation of a PMV from a chelonian at the beginning of our studies.

A number of RT-PCRs have been described for the detection and genetic characterisation of ferlaviruses based either on the RNA dependent RNA polymerase (L) or haemagglutinin-neuraminidase (HN) or fusion protein (F) genes (Kindermann et al., 2001; Sand et al., 2004). Phylogenetic analyses of partial gene sequences have shown that all of these snake PMVs are related to each other and to FDLV, but distinct from other PMVs, giving further support for the establishment of a new "*Ferlavirus*" genus. Subgrouping within the proposed genus was not congruent between the different analyses as some (Franke et al., 2001) did not include the results of others (Ahne et al., 1999).

3.4.3. Characteristics of the members of genus *Ferlavirus*

The characterisation of the new genus *Ferlavirus* is based on the complete genome data acquired from the FDLV (Kurath et al., 2004). Its genome contains the six invariant paramyxovirus genes in the standard order with a length following the "rule of six". In addition, the 55 nt 3' leader sequence length, complementarity between the 3'

and 5’ genomic termini, transcription start and stop sequences, and pattern of conserved and divergent domains within the L protein are features FDLV shares with all other paramyxoviruses. The lack of an M2 protein, evidence for expression of multiple proteins from the P gene locus, conserved V protein carboxyl terminal domain, and uniformly low amino acid identities with human respiratory syncitial virus (HRSV) proteins indicate conclusively that within the *Paramyxoviridae* family FDLV is not in the subfamily *Pneumovirinae* but is a member of the subfamily *Paramyxovirinae* (Lamb & Parks, 2007; Wang et al., 2011).

Within the subfamily, various features of the FDLV genome comprise a mosaic with regard to similarity to known genera. The 3’ terminal genomic sequence and transcription start and stop sequences are most similar to henipaviruses, the trinucleotide IGR regions are similar to henipaviruses, morbilliviruses, and respiroviruses, the ATP-binding motif in the L protein is identical to that of rubulaviruses and avulaviruses. Phylogenetic analyses of the FDLV proteins also indicate that FDLV is not consistently more closely related to any known paramyxovirus genus or species than to others, as an indication for classification into a separate genus.

The most notable unique feature is the presence of the novel U gene between the N and P genes. The U gene was shown to be present, with 84 to 89% nucleotide identity, in two further snake PMVs (Gono-Ger85, Biti-CA98; Ahne et al., 1999) that appeared to represent different viral species, suggesting that the U gene is most likely a feature common to all ferlaviruses.

4. Aims of the studies

Aims of the studies were:

1. to survey captive squamatid reptiles for the presence of adenoviruses in order to isolate or detect new reptilian AdV types and preliminary characterise these viruses by partial sequencing.

2. to determine the pathogenic potential of newly isolated invertebrate iridoviruses for lizards by transmission studies and surveys.

3. to compare IIVs isolated from crickets, lizards and other terrarium pets on the basis of partial genome sequences and pathogenic characteristics using a cricket bio-assay at different temperatures.

4. to develop sensitive and specific diagnostic tests (particularly a quantitative real-time PCR) for the detection of IIVs in diagnostic samples from lizards.

5. to survey captive squamatid reptiles for the presence of paramyxoviruses in order to isolate or detect new reptilian PMV types, and to estimate distribution of ferlavirus types known from earlier reports.

6. to molecularly characterise ferlavirus isolates and compare these with those from earlier reports in order to resolve taxonomical grouping incongruence.

7. to gain sequence and deduced phylogenetic information about ferlaviruses occurring in chelonians.

5. Materials and methods

5.1. Samples

5.1.1. Samples for adenovirus screening

Table 2. Adenovirus **p**ositive **s**amples (Aps) with short case histories

	Species	Id. Nr.	Origin	Age	Case history	Sample type
Squamata, **Lacertilia**	**bearded dragon** *Pogona vitticeps*	**Aps.1** 04/06	A	?	Imported from the USA, CNS signs, opisthotonus, spasms	o./cl. swabs
		Aps.2 07/06	A	?	No report	o./cl.swabs
		Aps.3 19/06	B	?	No report	organs, feaces
		Aps.4 35/06	C	ad.	Abnormal blinking; inactivity, apathy, hiding, anorexia, dark colouration, rounded back with fallen tail, tachycardia, dyspnoea, exitus	liver, lung, kidney, intestine
		Aps.5 64/06	A	ad.	Chronic gastrointestinal problems	blood, cl. swab
		Aps.6 65/06	D	ad.	Paralysis of the hind limbs and tail, trembling, hyperesthesia of the tail and of the back	blood, o./cl. swabs
	Mexican beaded lizard *Heloderma horridum*	**Aps.7** 23/06	E*	juv.	Kept together with Gila monsters and leopard tortoises. Outbreak with several deaths among the *Heloderma spp*.	intestine, liver, heart
		Aps.8a, b 29/1-2/06	E*	juv.	Swab taken 2 weeks later from the two survivors of the above group (both pos.)	2 o./cl. swabs
	Gila monster *Heloderma suspectum*	**Aps.9** 29/3/06	E*	juv.	Swab taken 2 weeks later from a survivor Gila in the same enclosure	oral swab
		Aps.10a, b 73/1-2/06	E*	juv.	Swabs taken half a year p. ob., from 2 new animals which had no contact with the others (both positive)	2 oral swabs
		Aps.11a to -11v 08/07	E*	juv.	Swab taken a year p. ob., from 21 Gila and 7 beaded lizard, all new animals, no contact with the others (12 Gila positive)	21 oral swabs
	emerald monitor *Varanus prasinus*	**Aps.12** 5/3/07	F	?	Died, hepatitis	liver, spleen, kidney
Ophidia	**asp viper** *Vipera aspis*	**Aps.13** 61/06-A1	F	?	Group of nine snakes, this one died	pooled organs, (liver)
	common boa *Boa constrictor*	**Aps.14** 968/00	NA	juv.	Isolate from an earlier study, served as a positive control	isolate on VH-2 cells

Laboratory register numbers are indicated in grey. Abbreviations: ad.= adult, cl.= cloacal, CNS= central nervous system, Id. Nr.= identification number, juv.= juvenile, o.= oral, NA= not applicable, p.ob.= post outbreak, squa.= squamata, A= Mainz, B= Fellbach, C= Kiel, D= Hannover, E= Zoo Kgs Lyngby, Denmark, F= Leipzig, *= from the same population.

Routine diagnostic samples from a total of 72 lizards, snakes and tortoises with case histories considered suspicious for AdV infection were sent to our laboratory during a course of two years. Twelve samples sent for other virus tests were surveyed “blind” for the presence of AdVs as well. DNA extracts of different organs or swabs

were pooled for the same animal or same batch and treated as one. Positive animals are listed in Table 2, negatives are listed in the Appendix Suppl. Table 5.

In the case of the Gila monsters (*Heloderma suspectum*) and Mexican beaded lizards (*Heloderma horidum*) (Aps.7-11 in Table 2), follow-up tests were performed at the Danish zoo in which these animals were kept after the detection of AdV. Five consecutive tests were performed in the group consisting of Gila monsters, Mexican beaded lizards and leopard tortoises (*Geochelone pardalis*). First intestine, heart, liver and liver-swab samples from a dead Mexican beaded lizard (Aps.7) were tested when an outbreak started among a group of juveniles. Two weeks later oral and cloacal swabs were taken from some survivors. Another two months later a Gila monster and two leopard tortoises from the same animal group were swabbed. Six months after the outbreak, two asymptomatic young Gila monsters from a separate enclosure were swabed for a follow up study (Aps.10). Finally, another half year later 21 newly introduced Gila monsters and 7 fresh Mexican beaded lizards were orally swabbed, before introducing them to the facility (Aps.11).

5.1.2. Samples for iridovirus studies

Eight different IIV isolates, originating from four different lizard species, a scorpion and the prey cricket species fed to these pets were used in this study (Table 3).

Table 3. Origin of IIV isolates.

	Host of virus isolation	Id. Nr.	Owner	IIV positive organs*	Case history
Squamata, **Lacertilia**	**high-casqued chameleon** (*Chamaeleo hoehnelii*)	**Ir.iso.1** 100/2001	A	kidney, liver, spleen, *lung,* intestine	emaciation, kerato-conjunctivitis
	bearded dragon (*Pogona vitticeps*)	**Ir.iso.2** 66/2003	B	*lung,* heart, tongue	unknown
	bearded dragon (*Pogona vitticeps*)	**Ir.iso.3** 64/2003	B	*lung,* brain, tongue, stomach, intestine	unknown
	spiny tailed lizard (*Uromastyx sp.*)	**Ir.iso.4** 08/2004	B	*skin*	hyperkeratosis
	four-horned chameleon (*Ch. quadricornis*)	**Ir.iso.5** 626/2000	C	*liver*	emaciation, several animals died suddenly
	green iguana (*Iguana iguana*)	**Ir.iso.6** 1125/2000	D	*skin*	hyperkeratosis
Arthropoda	**house cricket** (*Acheta domesticus*)	**Ir.iso.7** 52/2003	E	*whole body*	prey insects, died suddenly
	emperor scorpion (*Pandinus imperator*)	**Ir.iso.8** 25/2006	F	*abdominal organs*	loss of UV colouration

*From all positive organs listed, the origin of the isolates used in the sequence comparison is printed in italics. Isolates compared in a cricket bioassay as well, are printed in red.

All these viruses were isolated at the Institute of Environmental and Animal Hygiene at the University of Hohenheim in Stuttgart, Germany, partly by me or by colleagues as described elsewhere (Marschang & Becher, 2002; Weinmann, 2007). These isolates were used for molecular biological comparison and for bioassays.

Further diagnostic samples (45 lizards, 27 crickets) arriving at the laboratory were also tested for the presence of IIVs by virus isolation as well as by conventional PCR (nPCR) and real-time PCR (qPCR) methods. Those tested positive are listed in Table 4.

Table 4. Irido **p**ositive **s**amples

	Host of virus isolation	Id. Nr.	Owner	IIV positive organs*	Case history
Squamata, **Lacertilia**	**common agama** (*Agama agama*)	**Ips.1** 74/2008	A	fibrome biopsy	Small white proliferations disseminated in the skeletal musculature.
	bearded dragon (*Pogona vitticeps*)	**Irps.2** 91/2008	B	tissue proliferation on right back leg, right shoulder, & carpus	Myxoid spindle cell sarcoma
	bearded dragon *(Pogona vitticeps)*	**Ips.3** 86/2008	B	cloacal swab	Liver lesions, apathy, CNS signs
	bearded dragon (*Pogona vitticeps*)	**Ips.4** 85/2008	C	oral swab, cloacal swab	CNS-signs
	bearded dragon (*Pogona vitticeps*)	**Ips.5** 57/2009	B	oral swab, cloacal swab	CNS signs, opisthotonus
	bearded dragon (*Pogona vitticeps*)	**Ips.6** 71/2009	B	oral swab, cloacal swab	Acute bloody stool, hypoglycemia, sclerotic liver
	collared lizard (*Crotaphytus* sp.)	**Ips.7** 9/2009	B	femoral pore biopsy	Enlarged femoral pores, stomatitis
	spiny-tailed lizard (*Uromastyx acanthinura*)	**Ips.8** 88/2008	C	skin-crust	Cheilitis
	spiny-tailed lizard (*Uromastyx sp.*)	**Ips.9** 24/2009	B	skin	Cheilitis
	ocillated lizard (*Timon lepidus*)	**Ips.10** 5/2009	B	femoral pore biopsy	Hyperkeratosis of the femoral pores
	green iguana (Iguana iguana)	**Ips.11** 94/2008	C	skin, oral swab, cloacal swab	Fungal infection of the skin in several animals in this group.
	three horned chameleon (Chamaeleo jacksoni)	**Ips.12** 10/2010	D	swab	Survivor from a population of three. History of IIV infection in the dead animals.
Arthropoda	**5 crickets** (*Gryllus spp.*)	**Ips.13a to 13e** 87/2008	B	whole body	Purchased at a pet shop. Several crickets died within a few days.
	6 crickets (*Gryllus spp.*)	**Ips.14a to 14f** 11/2010	D	whole body	Samples from two pet shops, where several crickets died. The prey for lizard Ir.12 was purchased in these shops.

Diagnostic samples from pet lizard and prey insect tested positive for IIV by nPCR & qPCR methods. Original source of the samples: A= Münster, Germany; B= Hannover, Germany; C= Mainz, Germany; D= Amberg, Germany.

5.1.3. Samples for paramyxovirus studies

PMV from six snakes, three lizards and one tortoise were included in a genetic comparison study based on 3 genes. Background information on the animals from which the viruses were obtained is listed in Table 5. Except for the two most recent field samples from a German corn snake and a Hungarian masked water snake case (PanGut-GER09 & HoBuc-HUN09), the viruses were isolated in pure culture. Isolates were grown on VH-2 cells, primary chick embryo fibroblasts or primary foetal turtle cells. The isolates were preliminarily identified as PMVs based on their sensitivity to chloroform, HA activity with chicken erythrocytes and their morphology by EM.

Table 5. PMV isolates and field samples used in the phylogenetic comparison based on 3 (U-, HN- and L-) gene sequences

	Species	Origin	Id. Nr.	Case history	Reference
Squamata, Lacertilia	**emerald tree monitor** *Varanus prasinus*	Germany	**Var-GER95** (3319/1995)	healthy, kept with PMV positive snakes	Gravendyck et al., 1998
	flathead knob-scaled lizard *Xenosaurus platyceps*	USA	**Xeno-USA99** (28xpc/1999)	healthy, originally imported from Mexico	Marschang et al., 2002b
	green iguana *Iguana iguana*	Germany	**Igu-GER00** (324/2000)	pneumonia	Marschang et al., 2009
Squamata, Ophidia	**montane egg-eating snake** *Dasypeltis atra*	Germany	**Dasy-GER00** (936/2000)	died suddenly	Marschang et al., 2009
	beauty snake *Orthriophis taeniurus*	Germany	**Orth-GER05** (20/3/2005)	live animal with respiratory signs, several snakes affected	Marschang et al., 2009
	ball python *Python regius*	Germany	**Pyth-GER01** (368/2001)	unknown	Marschang et al., 2009
	timber rattlesnake *Crotalus horridus*	Germany	**Crot-GER03** (9/5/2003)	resp. signs, several viperid snakes affected	Marschang et al., 2009
	***corn snake** *Pantherophis guttatus*	Germany	***PanGut-GER09** (6/2009 & 14/2009)	24 corn snakes of a collection tested. Kidney and intestine of 1 dead and swabs of 7 living animals PMV positive, coinfection with reo- and AdV.	Abbas et al., 2011
	***masked water snake** *Homalopsis buccata*	Hungary	***HoBuc-HUN09** (80/2009)	snake floating on water, unable to dive, died with signs of pneumonia	Papp et al., 2012
Chelonia	**Hermann's tortoise** *Testudo hermanni*	Germany	**THer-GER99** (tPMV/1999)	pneumonia	Marschang et al., 2009

***Field samples, which could not be isolated in pure culture.

During a course of 18 months, a total of 203 clinical samples (organs, swabs and tracheal washes) originating from 102 snakes, representing 21 boid, viperid, pythonid and colubrid species, were screened in our laboratory (Table 6 and Suppl. Table 6 in Appendix) for the presence of PMV by RT-PCR and virus isolation. The animals originated from private collections mainly from Germany (in one case from Hungary) and samples were collected by veterinary practitioners.

Table 6. Field samples used in clinical survey for rPMV

		Species	Identifying Nr.	Sample type	Case history
Squamata, Ophidia	Boidae	**red tail boa** *(Boa constrictor)*	**Boa-1**[#] (1/2006)	*cloacal swab,* blood	CNS signs
		common boa *(Boa constr.)*	**Boa-2** (44/2007)	*lung*	proliferative pneumonia
		common boa *(Boa constr.)*	**Boa-3** (12/2008)	*swab*[§]	CNS signs
		2 common boas *(Boa constr.)*	**Boa-4a, b** (24/2008)	*2 swabs*[§]	PMV positive stock
		common boa *(Boa constr.)*	**Boa-5** 61/2008	*intestine, lung,* kidney	CNS signs
	Colubridae	**corn snake** *(Panth.guttatus)*	**Col-1** (13/2006)	oral & *cl. swab*	pneumonia
		leopard snake *(Elaphe situla)*	**Col-2a*** (32/1/2006)	*swab*[§]	PMV positive stock, samples tested from four living and one dead snake
		leopard snake *(Elaphe situla)*	**Col-2b*** (32/4/2006)	*swab*[§]	
		leopard snake *(Elaphe situla)*	**Col-2c*** (32/2006)	*kidney, lung, liver, intestine,* spleen	
		garter snake *(Thamnophis sirtalis)*	**Col-3**** (6/2007)	*lung, kidney, trachea,* intestine	died, no specific signs, stock PMV problem
		garter snake *(Thamn. sirtalis)*	**Col-4**** (40/2007)	*intestine, lung,* kidney	PMV pos. collection
		corn snake *(Panth. guttatus)*	**Col-5** (48/2007)	trachea, *kidney,* intestine	PMV stock problem
		Cox red bamboo snake *(Oreophis porphyraceus coxi)*	**Col-6** (58/2008)	*lung, kidney,* intestine	died suddenly
	Pythonidae	**ball python** *(Python regius)*	**Pyt-1** (36/2006)	*lung, liver, intestine,* brain, kidney	4 young pythons suddenly died, samples from one
		ball python *(Python regius)*	**Pyt-2** (66/2006)	*lung, liver, kidney, intestine*	pneumonia
		ball python *(Python regius)*	**Pyt-3** (2/2007)	*trachea, lung, intestine*	coordination disorders, convulsions
		reticulated python *Python reticulatus*	**Pyt-4** (4/2007)	*lung, kidney, intestine, oesophagus*	pneumonia
		ball python *(Python regius)*	**Pyt-5** (32/2/2007)	*intestine, kidney*	died suddenly
		Indian python *(P. molurus)*	**Pyt-6** (39/2007)	*intestine, kidney,* trachea	pneumonia
		Indian python *(P. molurus)*	**Pyt-7** (45/2007)	*swab*[§]	stock PMV problem, respiratory signs
		ball python *(Python regius)*	**Pyt-8a** (82/2007)	*swab*[§]	animal died with PMV earlier in stock
	Viperidae	**asp viper** *(Vipera aspis)*	**Vip-1***** (33/2006)	*intestine,* spleen, liver lung, kidney	from positive animal stock
		asp viper *(Vipera a. aspis)*	**Vip-2a*** (61/2006-A6)	*swab*[§]	9 asp vipers & 2 lineated rat snakes kept together in a PMV positive stock, one viper died
		asp viper *Vipera a. francisciredi*	**Vip-2b*** (61/2006-A8)	*swab*[§]	
		asp viper *Vipera a. francisciredi*	**Vip-2c*** (61/2006-A9)	*swab*[§]	
		common European viper *Vipera berus*	**Vip-3***** (69/2006)	*kidney,* lung, liver, intestine	from PMV positive stock
		mountain Mang pit viper *Trimeresurus mangshanensis*	**Vip-4*** (60/2008)	*lung, intestine, kidney*	from PMV positive stock
Chelonia	Testudinidae	**leopard tortoise** *Geochelone pardalis babcocki*	**Geo** (8/2008)	tongue, salivary glands, lungs, kidneys, bladder, testes, penis, spleen, stomach, *liver*, *heart*, *small-* & large *intestine, cloaca*	lethargy, respiratory distress, died

Field samples used in rPMV survey and phylogenetic comparison based on PMV L gene sequence. Only positive samples are listed (with positive organs italicised), for negatives see Appendix Suppl. Table 6. Abbreviations: *, **, and ***: indicate animals from the same owner; swab[§] = oral and cloacal swabs were collected together; [#]from Hungary, all other samples were from Germany.

5.2. Cell culture-based methods

5.2.1. Virus isolation

Isolation of viruses was attempted from samples on the iguana heart cell line (IgH-2, ATCC: CCL-108) and/or on Russell's viper heart cells (VH-2, ATCC: CCL-140) and/or Terrapene heart cells (TH-1, ATCC: CCL-50) depending on the origin of the sample. Cell types in closest phylogenetic relationship to the virus-host were preferred. Small pieces of tissues or the cotton heads of swabs were sonicated in 3 ml Dulbecco's modified Eagle's medium (DMEM) (Biochrom AG, Berlin, Germany) supplemented with antibiotics. The samples were centrifuged at low speed (1500 x g, 10 min) for the removal of cell-debris and bacteria, then 200 µl of the homogenate was inoculated onto approximately 70% confluent cell monolayers in 30 mm diameter Cellstar® tissue culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). In cases, when centrifugation of the samples was insufficient to avoid contamination with bacteria, samples were filtered with 0.45 µm filters FP 30/0.45 CA-S (Schleicher & Schuell MicroScience, Dassel, Germany). After incubating for 2 hours at 28ºC, 2 ml nutrient medium (DMEM supplemented with 2% foetal calf serum, 1% non-essential amino acids and antibiotics, AB) was added to each dish. Cells were examined for cytopathic effects (CPE) approximately every third day with an inverted light microscope (Wilovet, Wetzlar, Germany), and dishes were frozen when extended CPE was seen. Cultures showing no CPE were frozen after 2 weeks of incubation for blind passaging. Two additional passages were performed from each dish after a freeze and thaw cycle and low speed centrifugation.

5.2.2. Virus propagation, titration and purification

For the purpose of the animal infection studies or the antibody production or the partial genome sequencing experiments, in which higher virus yield or pure concentrated DNA was required, two lizard AdVs (Aps.7 & 10) and selected IIV isolates (Ir.iso.1 to 8) were further propagated in 175 cm^2 tissue culture flasks using the same cell line as described in the section on virus isolation (5.2.1.). For the sequencing experiments the purification and concentration was performed by ultracentrifuge pelleting (Beckman, Ti90 Rotor). After a freeze and thaw cycle, cell-debris was first removed from the cell culture supernatants with low speed centrifugation (1500 x g , 10 min, 4°C), and viruses were then concentrated by ultracentrifugation (120 000 x g, 3 hours, 4°C). Supernatants were decanted, and pellets were resuspended in PBS.

Infectivity titre of the isolates was determined on 96-well cell culture plates (Greiner Bio-One GmbH). Overnight cultures of the identical cell lines were infected at the confluence of approx. 70% with a 10-fold dilution row of the virus isolate in nutrient medium. Each well of the decanted plates was inoculated with 100 µl from each dilution step of the row, with 4 repetitions, as well as with virus free control medium. Plates were checked for the appearance of virus specific CPE every third day and finally evaluated after 2 weeks. $TCID_{50}$ titres were calculated using the Spearman-Karber estimation formula (Hierholzer & Killington, 1996).

For the bearded dragon infection studies the propagated virus was purified as follows. Cell-debris was separated by centrifugation and possible toxic or allergenic components in the supernatant were dispersed by 3 consecutive dialisations against PBS for 20 hours through 12 kDa cellulose membrane sacs (Spectrum Labs).

5.3. Animal infection studies

5.3.1. Cricket bioassay

An iridovirus negative colony of field crickets (*Gryllus bimaculatus*) was established in the Institute for Environmental and Animal Hygiene at the University of Hohenheim in Stuttgart. The first animals were obtained by the courtesy of Dr. Regina Kleespies, Biologische Bundesanstalt für Land- und Forstwirtschaft, Darmstadt, Germany, who had already tested them negative for IIV. The crickets had no contact with other insects, were fed with salad and dog food (Matzinger Hundeflocken, Nestlé Purina Pet Care, Euskirchen, Germany) and were regularly tested negative for IIV by nested PCR.

To study the virulence of IIVs from different hosts, a bearded dragon (*Pogona vitticeps*) “lizard” isolate (Ir.iso.3), a scorpion (*Pandinus imperator*) isolate (Ir.iso.8) and a cricket isolate (Ir.iso.7) were propagated on IgH-2 cell lines to an equal titre of $TCID_{50}$ = $10^{5.5\text{-}6}$ /ml. *Per os* transmission experiments were carried out modified from Kleespies et al. (1999) with subadult (last instar nymph) or young adult *Gryllus bimaculatus*. A total of 20 nymphs were completely immersed in virus suspension for approximately 30 seconds. Ten crickets were dipped into IgH-2 supernatant as negative controls. In view of the cannibalistic behaviour of this species, all animals were housed individually in beakers (diameter, 8.5 cm; height, 9.5 cm). The animals were kept at 20ºC and 30ºC in cooling-heating thermostat chambers with an adjusted 12/12h light and dark routine. Tests were run for 60 days and mortality was recorded every 1-2 days, fresh food was

provided as necessary (every 3-4 days). Fat body samples were collected from dead crickets and prepared for virology, EM investigations, half bodies of crickets were embedded in paraffin for later histology, ISH and IHC studies. The survived crickets at the end of each study were killed by snap freezing and decapitation and also tested.

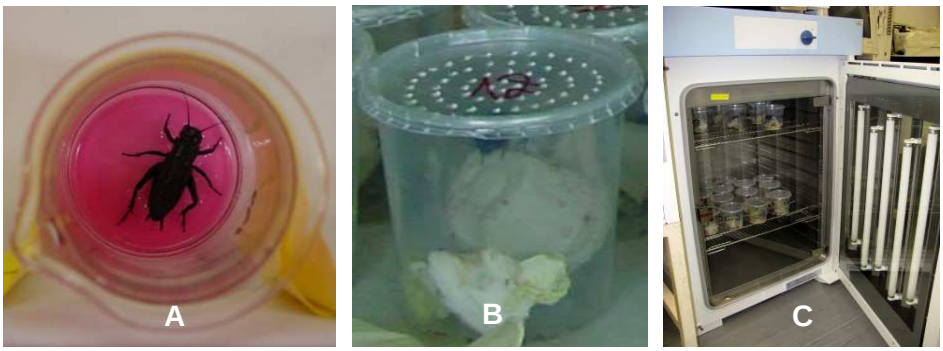

Figure 8 (A) Infection of a cricket by dipping into virus supernatant. **(B)** Individual beaker with a cricket used in the infection studies. **(C)** Beakers were kept in a thermoregulated incubator with lights.

5.3.2. Bearded dragon transmission study

As parents for the bearded dragons used in the transmission study, 6 bearded dragons (3 pairs) were bred. These animals were raised in separate facilities of the Institute for Environmental and Animal Hygiene at University of Hohenheim in Stuttgart from eggs. Their parents had tested negative for IIV in oral and cloacal swabs. All animals had also repeatedly tested negative for IIV in oral and cloacal swabs and had been previously fed IIV negative crickets only. Eggs were collected from these pairs and incubated at 28 ºC in a Jaeger-Kunstglucke FB 50M incubator in the laboratory. The hatched bearded dragons had no contact to other reptiles and were fed IIV negative crickets from our own colony before the start of the study. Organs of 5 young animals that died soon after hatching were negative for IIV by nPCR and virus isolation.

Fifteen young bearded dragons at the age of 5 months were put into individual terrariums. The lizards were divided into 3 groups. The terrariums for negative control animals (NK) and infected animals (A and B) were kept in separate facilities away from each other and necessary hygienic measures implemented to avoid contamination of negative lizards during the infection trial. Animals were infected using the lizard IIV isolate Ir.iso.3 from a bearded dragon lung, propagated on IgH-2 cells to a titre of $TCID_{50}= 10^{6.5}$ /ml. Supernatant was purified as described in section 5.2.2. The titre of purified suspension was checked again on IgH-2 cells and we found that it did not

change with this treatment. For negative controls, DMEM supplemented with 2% FCS, 1% NEA and AB was treated the same way.

Two months before the beginning of the bearded dragon study, a batch of crickets was infected with the same lizard virus. Dead crickets were frozen and stored at -80ºC, the rest were killed at the end of the 60 day period and stored similarly. In order to obtain a quasi homogenous food-portion for each lizard, thawed crickets were dissected and predominantly, the iridescent ones were used in small cut pieces to feed the lizards. For the negative control lizards, negative crickets were prepared similarly.

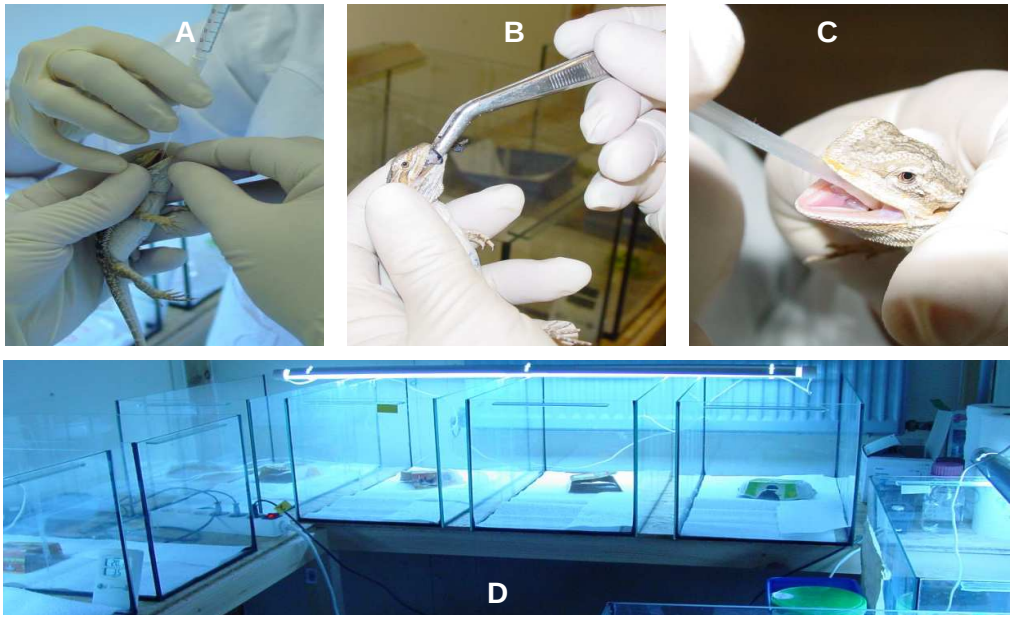

Figure 9. Transmission study of IIV with bearded dragons (*Pogona vitticeps*) **(A)** Administering virus preaparate with intra-gastric tube. **(B)** Force feeding with cricket-preparation. Note the bluish iridescence of the cricket preparate at the tip of the forceps. **(C)** Collecting oral swabs from the lizards. **(D)** Individual terrariums of the infected lizards in a separate room before the start of the study. Note that the terrariums do not have spot lights.

Bearded dragons of ***group A*** were infected with 1 ml of the virus containing solution administered through sterile cat catheters into the stomach (Fig. 9/A) and were force-fed with infected dead crickets (Fig 9/B): “single infected groups”. Lizards in ***group B*** were administered an additional 1 ml of virus suspension by intra-coelomic injection: “single infected” group. Negative control lizards received the same treatment with the mock-solutions.

Animals were kept at 21-26ºC and 28-38% relative humidity, using desert UVB fluorescent lamps without spot bulbs (in order to avoid an increase in body-temperature by basking which could adversely affect virus replication — see Fig. 9/D), and were checked daily. The lizards were fed with green salad and force-fed with the prepared crickets supplemented with mineral-vitamin powder (Korvimin ZVT+ Reptil) every 2-3

days. Two weeks before the end of the study no more crickets were fed. Oral and cloacal swabs were collected (Fig. 9/C) every week and changes in weight were also recorded (Suppl. Table 4 in Appendix). Swabs were examined for the presence of IIVs by cell-culture based and PCR methods. After 60 days, animals were euthanized by ketaminhydrochlorid injection i.m. (Ursotamin, Bernburg AG, ca. 1000 mg/kg) and dissected. Gross pathology was performed and 12 different organs were collected for further assays as described above (Fig. 10). The bearded dragon infection study received authorisation from the Regierungspräsidium Stuttgart and was controlled by the Animal Wellfare Committiee of the Hohenheim University.

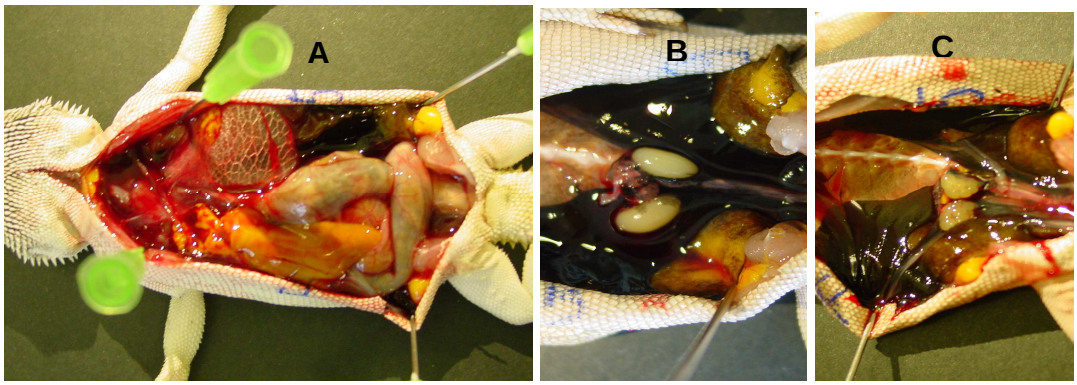

Figure 10. **(A)** Dissection of a bearded dragon. **(B, C)** Male and female gonads.

5.4. Microscopic techniques

5.4.1. Light microscopy based techniques

Following *post mortem* examination, organs from the bearded dragons as well as longitudinally dissected body halves of crickets in the transmission study were fixed for 48 hours in Bouin´s solution of 714.3 ml/l picrin acid, 238.1 ml/l formalin (36%) and 47.6 ml/l acetate acid (all Merck, Darmstadt, Germany), and/or in 4% buffered paraformaldehyde solution and routinely processed for histology, cut at 3 µm and stained with haematoxilin-eosin (HE).

Two different digoxigenin (DIG) labelled probes were made for the ISH studies following a modified protocol originally described by Emanuel (1991) using the primers MCP-F3 & R2 in the PCR described in section 5.5.3. The dNTP mix was substituted with the prescribed amount of DIG DNA labelling mix (Roche Diagnostics GmbH, Mannheim, Germany). The probes were tested for specificity by Southern blot hybridization against various concentrations of four different IIV isolate's partial MCP gene PCR amplicons and λ-phage DNA as a negative control.

Sections were dewaxed with xylene and rehydrated before proteolysis by proteinase K. After refixation in absolute ethanol, they were air-dried. Hybridization was carried out in a humid chamber at 40ºC overnight in the following solution: 2.5 ml herring sperm-DNA, 1 ml probe, and 3.5 ml formamide were mixed and denatured for 5 min at 95ºC. On ice, an additional 21.5 ml formamide, 10 ml 20 x SSC solution (3 M NaCl, 0.3 M Na-citrate, pH 7), 1 ml 50xDenhardt solution (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) and 5 ml of 50% dextran sulfate and bidistilled water were added to a final volume of 50 ml. After further washing steps, the specific colour reaction was performed during 60 min, and tissues were sealed with aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany.)

Iridovirus isolate from a bearded dragon (Ir.iso.3) was used for the production of rabbit antibodies. After propagation on VH-2 to a titre of $TCID_{50}=10^{5.5}$/ml, the virus was concentrated and purified as described in section 5.2.2. The titre after ultracentrifuge pelleting had increased to $TCID_{50}=10^{6.5}$/ml. Inactivation of the virus with 0.1% formaldehyde over two weeks at 4°C was successful as proven on titration plates. Two immunization boosts (at 20 day intervals) were done on two rabbits at the Biolux GmbH (Stuttgart, Germany) in cooperation with Genosphere Biotechnologies (Paris, France). 50 ml of antiserum per rabbit was used for total IgG purification (29 ml in PBS from each). ELISA readings indicated good titres for both rabbits (Table 8). The IgG solution was aliquoted and stored at -20ºC for later use in the ICH tests.

Table 7. Pre- and post-immune ELISA readings of rabbits used in antibody production.

Antibody dilution:	**Rabbit No.1**		**Rabbit No. 2**	
	Pre-immune	Bleed 1	Pre-immune	Bleed 1
1:1 000	0,137	1,94	0,139	1,89
1:10 000	0,034	1,56	0,037	1,52
1:10 000	0,035	0,87	0,039	0,80

5.4.2. Electron microscopy

In the case of the AdV isolates, cell culture supernatant was negative-stained with 2% potassium phosphotungstate (pH 7.3) on 3.05 mm copper grids (Plano GmbH, Wetzlar, Germany), and examined using a JEM-1011 transmission electron microscope (Jeol LTD, Tokyo, Japan) for the presence of viral particles. Electron microscopic examinations were carried out by Valerij Akimkin at the Chemische und Veterinäruntersuchungsamt (CVUA) Stuttgart, Germany.

From animals in the transmission studies: examined organs of bearded dragons and fat body samples from selected crickets were fixed in 2.5% glutaraldehyde (Plano, Wetzlar, Germany) in cacodylate buffer (0.1M sodium-cacodylate, pH=7.4). After fixation the samples were washed three times in sodiumcacodylate buffer, and fixed in a 1% solution of osmiumtetroxide in 0.1 M sodiumcacodylate buffer for 1 h at 4°C. The samples were washed three times with 0.1 M acetic acetate buffer, followed by contrast staining with a saturated uranylacetate solution (5%) in 70% ethanol for 2 h at room temperature. Dehydration in an ascending row with alcohol followed. Propylenoxide (Serva, Heidelberg, Germany) was used as an intermediate matrix. The samples were then embedded in an epoxid resin combination (Plano, Wetzlar). After 48 h in an incubator at 60°C, the blocks were polymerised, routinely cut, adsorbed onto 300 mesh copper grids and examined in a JEM-100SX electron microscope (Jeol LTD, Tokyo, Japan) at 80 kV by Prof. Dr. Alves de Matos in the Curry Cabral Hospital, Lisbon, Portugal.

5.5. Molecular biological techniques

5.5.1. DNA/RNA extraction

DNA was extracted from sample homogenates and virus suspension with the DNeasy® kit (Qiagen GmbH, Hilden, Germany) following the manufacturers protocols and using 100 µl of final elution volume. RNA was prepared from sample homogenates and cell culture supernatants using the guanidinium isothiocyanate method described by Boom et al. (1990) and resuspended in 75 µl of RNAse free water.

Concentration and purity of the DNA extracts (also of purified plasmids; see in next section) was determined with the spectrophotometric method (Ultrospec 2100 pro, Amersham Biosciences Europe GmBH, Freiburg, Germany) at 230, 260 and 280 nm absorbance. Copy number was calculated using the ca. 660 g/mol average molecular weight for a single nucleotide-pair.

5.5.2. Molecular cloning

DNA extracts of concentrated AdV and IIV isolates were subjected to restriction endonuclease digestion using BamHI, EcoRI, HindIII and PstI (Fermentas, St Leon-Rot, Germany) enzymes according to the manufacturers recommendation. Phagemid pBluescript® II KS(+/-) (Stratagene Ltd., Santa Clara, CA, USA) was digested with

HindIII and PstI restriction endonucleases for the random molecular cloning experiments.

Random cloning of the HindIII and PstI digested lizard AdV isolates was performed with the contribution of Inna Ball [Romanova] following the guidelines of Sambrook et al. (1989). Various insert:vector rates (ranging from 4:1 to 1:1) were tested. Ligation was performed in 15 µl end-volume using T4 DNA ligase enzyme with buffer (Fermentas, St Leon-Rot, Germany) at 16ºC overnight. 5 µl of the ligated mixture was heat-shock transformed at 42ºC for 40 seconds into chemically competent *E. coli* Top10F strain (Invitrogen, Life Technologies GmbH, Darmstadt, Germany). Transformed bacteria were plated on standard selective LB agar, containing ampicillin (100 µg/ml)and incubated at 37ºC. Agar plates were supplemented with X-gal (20 mg/ml) and IPTG (200 mg/ml) (both Sigma, St. Louis, USA) to detect loss of β-galactosidase activity. Selected white colonies were propagated in selective LB broth and mini-preparations of the plasmid DNA were obtained by the alkaline lysis method (Sambrook et al., 1989) and with Plasmid Mini Kit (Qiagen GmbH, Hilden, Germany).

If direct sequencing of PCR products was unsuccessful or gave dubious results, then purified PCR amplicons were blunt-end cloned into pJET 1.2 vector (Fermentas, St Leon-Rot, Germany) following the manufacturers recommendations. Heat-shock competent *E. coli* cells (strain Top10F) were heat-shock transformed with the ligate and then plated onto selective agar plates. Propagation and plasmid preparation was identical to that described above.

5.5.3. Conventional PCRs (nPCR)

For the RT-PCR targeting RNA (the L-, HN- and U-genes of PMV), the first round single tube reverse-transcription polymerase chain reaction was carried out in 25 µl reaction mixtures containing 2.5 µl of prepared RNA, 1 µM of forward and reverse primers, 1x Taq buffer with KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 70 units reverse transcriptase, 6 units ribonuclease inhibitor, and 1.25 units Taq polymerase (all Fermentas, St Leon-Rot, Germany). After the 60-min RT reaction at 40°C, cycling conditions were similar to those described below.

All other PCRs targeting viral DNA or copy DNA were performed in 25 µl reaction mixtures, containing 1x concentration of Taq buffer with $(NH_4)_2SO_4$, 1-3 mM of $MgCl_2$, 200 µM of each dNTP, 1 µM from both forward and reverse primers and 0.5-1.25 U of Taq polymerase (all from MBI Fermantas, St Leon-Rot, Germany). The mixtures were amplified with an initial denaturation at 95°C for 5 min followed by 35-45 cycles at 95°C for 30 s, 45-60°C for 30-60 s, and 72°C for 1-4 min. There was a final

extension at 72°C for 7 min. In the amplification cycles, the annealing temperatures for specific primers were set to T_m -4ºC, but not lower than the standard for the degenerated primers at 45ºC. Elongation times were set according to the expected product size, calculating 1 minute for 1 kb In the first rounds, 1 µl DNA extract, in the second round 1-2.5 µl first round amplicon served as a template. Products were separated on 1-1.5% agarose gels (Bioenzym, Oldendorf, Germany) in TAE puffer containing 0.5 µg/ml ethidium-bromide and visualised under 320 nm UV light.

For the AdV studies, six primers were previously described in the literature (Wellehan et al., 2004; Parkin et al., 2009), whereas thirty-eight others were newly designed and synthesised (Biomers, Ulm, Germany). The primer sequences are listed in Supplementary Table 1 in Appendix.

For IIV studies, fourteen primers targeting six different genes were taken from the literature (Jakob et al., 2002b). Further fifteen primers targeting CIV-VAB (160L) and flanking genes as well as six primers targeting the MCP gene and flanking region were newly designed. Another thirteen primers targeting the DNA polymerase (037L) gene and twenty-three targeting the exonuclease (012L) gene were newly designed by undergraduate student Dirk Spann under our supervision. All these primer sequences are also listed in Supplementary Table 2 in Appendix.

For PMV studies, ten primers targeting three genes were taken from the literature (Ahne et al., 1999; Kurath et al., 2004). Further twelve primers to detect two of these genes (U, HN) were designed (Marschang et al., 2009). All PMV primers are also listed in Supplementary Table 3 in Appendix.

5.5.4. Sequencing

Gel purified PCR amplicons (Invisorb Spin DNA Extraction Kit; Invitek GmbH, Berlin, Germany) and kit purified plasmids were sequenced using a BigDye Terminator Cycle Sequencing Kit v.1.1 applying the PCR primers, pJET-F & -R primers and T3 & T7 primers for the PCR amplicons, cloned amplicons and random cloned genome fragments, respectively. Sequencing reactions were analysed on an ABI prism 310 automated DNA sequencer (both Applied Biosystems, Foster City, CA, USA) at the Institute for Environmental and Animal Hygiene of Hohenheim University. Occasionally commercial service providers (Biolux GmBH, Stuttgart; Eurofins, MWG Operon, Ebersberg, Germany; Biological Research Centre, Szeged, Hungary) were hired.

5.5.5. Analysis of sequences

Raw sequences were processed by the ABI Sequence Analysis Programme 5.1.1 (Applied Biosystems, Foster City, USA) then edited, assembled and compared using the STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield et al., 1995). The sequences were compared to the data in GenBank (National Center for Biotechnology Information, Bethesda, USA) online (www.ncbi.nih.gov) using the BLASTN and BLASTX homology search programs. Homologous sequences were retrieved from GenBank (for AdV, from the non-redundant database of the Molecular Virology Group at the Veterinary Medical Research Institute, Budapest www.vmri.hu/~harrach/ADENOSEQ.HTM). Multiple alignments of sequences were performed with ClustalW algorithm of the BioEdit Sequence Alignment Editor programme (Hall, 1999) using default settings. The alignments were used for phylogenetic calculations in the PHYLIP program Package version 3.6. (Felsenstein, 1989) applying various methods (distance based & maximum likelihood) to obtain an optimal tree. Bootstrap analysis of 100 replicates was performed to estimate robustness of trees. Bayesian trees were constructed using the MrBayes on the TOPALi v2.5 platform from the nucleotide (nt) and deduced amino acid (aa) alignments, selecting Hasegawa-Kishino-Yano (HKY) and Whelan & Goldman (WAG) models, respectively (Huelsenbeck & Ronquist, 2001). Two independent runs were performed for 10^5 generations. Every tenth tree was sampled out of which 40% was discarded as burn-in. SimPlot and BootScan analyses of the sequenced IIV and PMV genes with their homologues retrieved from GenBank were performed using the SimPlot for Windows v.3.5.1 programme.

Structural analysis of the predicted IIV exonuclease and the DNA polymerase proteins was done using the GENtle program v. 1.9.4. and SWISS-MODEL automated protein structure homology-modeling server (http://swissmodel.expasy.org) by my consultant Prof. Dr. Arthur Pfitzner (Institute of Genetics at the University of Hohenheim in Stuttgart).

5.5.6. Real-time PCR (qPCR)

Sequences of the MCP genes of IIV isolates (Table 3) were determined and they were found to be identical to one another and to the earlier described cricket iridovirus sequence (Jakob et al., 2002b) with 97.9% similarity to that of CIV, the type species of the genus. These sequences were used for primer and probe design using the Primer Express Version 3.0 (Applied Biosystems, Darmstadt, Germany) program.

The following primers: IIV-MCP fwd 130 5'-TGGTTYACCCAAGTACCKGTTAG-3' and IIV-MCP rev 182 5'-ATGCKGACCATTCGCTTC-3' (both from biomers.net, Ulm, Germany), and probe: 5'-6FAM-TTAACTAGAGCAAACGGATCA-MGBNFQ-3' (Applied Biosystems, Darmstadt, Germany) were chosen.

The qPCR assays contained a total volume of 25 µl, including 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 900 nM forward and reverse primers, 250 nM probe and 2.5 µl of template DNA. Reactions were carried out in a StepOne™ PCR Real-Time PCR System, using MicroAmp™ 48-well reaction plates and the StepOne™ Software version 2.0 (all Applied Biosystems, Darmstadt, Germany). The thermal cycling conditions were as follows: holding stage at 50ºC for 2 min followed by 95ºC for 10 min and a cycling stage of 40 repetitions of 95ºC for 15 s followed by 60ºC for 1 min, during which fluorescent data were collected. Purified PCR products of the house cricket isolate (Ir.iso.7) and the clone of pJET plasmid inserted 1st round MCP products (primers F1 & R4) of a spiny tailed lizard IIV isolate (Ir.iso.4) were used as references in different dilutions for the quantification.

The specificity of the qPCR was tested using DNA of previously isolated iridoviruses as well as DNA prepared from uninfected IgH-2, VH-2, and TH-1 cell-lines, uninfected crickets (*Gryllus bimaculatus),* uninfected bearded dragon tissues and a number of DNA viruses from reptiles. The sensitivity of the assay was tested with contribution of Dirk Spann using a purified IIV-MCP conventional PCR product and a plasmid-inserted PCR product. Intra-assay (3 replicates) and inter-assay precision were estimated by analysis of aliquoted conventional PCR-amplicon and plasmid-construct DNA dilution steps on the same or on separate plates. Coefficients of variation were calculated for copy number and C_t at high (10^6 copies) and low (10^2 copies) plasmid or purified PCR product concentration.

Diagnostic samples arriving in the laboratory with a suspected IIV infection were parallel examined in 3 tests: the nPCR, virus isolation in cell culture (IgH-2) and qPCR. These three methods were also compared directly, using an IIV isolated from a chameleon (Table 3; Ir.iso.1). The virus was propagated on IgH-2 cells until CPE was observed over ca. 80% of the monolayer. Virus was then harvested by freezing and thawing. Cell-debris was removed by low speed centrifugation and supernatant was serial 10-fold diluted in DMEM and inoculated onto IgH-2. DNA was also prepared from each dilution step and tested for the presence of IIVs by nested PCR and qPCR.

6. Results

6.1. Adenovirus survey

6.1.1. Screening with consensus nested PCR

We found AdVs in a total of 25 diagnostic samples during the course of two years (Table 2 & 8 – AdV positive samples (Aps)). AdVs were detected from 6 bearded dragons. In three cases (Aps.1, Aps.2 and Aps.6) virus was detected from both oral and cloacal swabs, in one case (Aps.5) from a cloacal swab only, in one case (Aps.3) from mixed tissue and faeces, and in one case (Aps.4) from several different tissues (lung, liver and kidney) (Table 8). In the case of one dead animal (Aps.4) and a chronically sick one (Aps.5) we also tested oral and cloacal swabs of the partner dragons from the same enclosure, but they were all negative.

During this study and the follow-up investigation at a Danish zoo, a total of 34 *Heloderma* spp. specimens (10 Mexican beaded lizards and 24 Gila monsters) were tested for AdV. At first, organs from a dead Mexican beaded lizard (Aps.7) tested positive. Oral swabs as solitary probes taken two weeks later from a Gila monster and two Mexican beaded lizards from the same enclosure were also positive (Aps.8 & Aps.9). However, the companion beaded lizard and two leopard tortoises from the same enclosure tested two months after the original detection gave negative PCR results (data not shown). The latest sampling of 28 new quarantined *Heloderma* spp. specimens 1 year after the outbreak, detected 12 positives among the Gila monsters (Aps.11).

Kidney and spleen tissue samples from an emerald monitor (*Varanus prasinus*) that died with hepatitis were PCR positives, although the liver was negative (Aps.12). AdVs were also detected in a snake collection with no specific signs indicative of AdV infection. Aps.13 was a batch of samples from snakes at a collection containing nine specimens from two asp viper subspecies (*Vipera aspis aspis*, *Vipera a. francisciredi*) and two striped Aesculapian rat snakes (*Zamenis [Elaphe] lineatus*). The solitary AdV positive sample from this batch was an asp viper which died suddenly. PMV was detected in three of these survivors but not in the tissues of the dead snake (see section 6.3.2. for detailed results). However, a pooled organ sample from this animal was positive in the AdV consensus nested PCR.

All of the listed AdV positive samples were tested using a single round PCR targeting the hexon gene (Parkin et al., 2009), but only those from the helodermatid samples (Aps.7, 10, 11) yielded products.

Table 8. Adenovirus **p**ositive **s**amples (Aps)

	Species	Id. No.	PCR positive sample	Isolation	AdV type	Acces. No.	Comments
Lacertilia	bearded dragon	**Aps.1**	o. & cl. swabs	–	AgAdV-1	EU914202	coccidiosis
		Aps.2	o. & cl. swabs	–	AgAdV-1	EU914203	
		Aps.3	organ pool, feaces	–	AgAdV-1	EU914203	
		Aps.4	liver, kidney, lung	–	AgAdV-1	EU914204	partners lung was IIV pos.
		Aps.5	cloacal swab	–	AgAdV-1	EU914205	
		Aps.6	o. & cl. swabs	–	AgAdV-1	EU914206	
	Mexican beaded lizard	**Aps.7***	intestine, liver, heart	all +	LAdV-2	EU914207	
		Aps.8*	o. & cl. swabs	–	LAdV-2	EU914207	
	Gila monster	**Aps.9***	oral swab	–	LAdV-1	AY576680	
		Aps.10*	2 oral swabs	1 swab +	LAdV-1	AY576680	
		Aps.11*	12 oral swabs	1 swab +	LAdV-1	AY576680	
	emerald monitor	**Aps.12**	spleen, kidney	–	VaAdV-1	EU914208	
Ophidia	asp viper	**Aps.13**	pooled organs	–	SnAdV-2	EU914209	3 PMV pos. survivors in the collection
	common boa	**Aps.14**	VH-2 cell passage*	+	SnAdV-1	NC_009989	*served as control

6.1.2. Isolation of reptilian adenoviruses

Viruses were isolated from three animals out of the total of 25 that had been tested adenovirus positive by PCR (Table 2). All three of these were *Heloderma* spp. (one Mexican beaded lizard and two Gila monsters). The isolated viruses caused a CPE with rounding and detachment of the cells. The samples from the dead lizard (Aps.7) were strongly positive on IgH-2 cells. Liver, intestine, liver-swab and heart samples showed extensive CPE after different periods: 4, 14, 24 and 30 days post inoculation, respectively (Fig. 11). Oral and clocal swabs of a Gila monster tested 6 months later (Aps.10) and one year later (Aps.11) from the same collection showed a similar CPE on IgH-2 cells. All of the isolates could be passaged. The isolated viruses were identified as AdVs by PCR amplification of viral genes from the cell culture supernatant as well as by EM examination. Non-enveloped icosahedral particles of approximately 80 nm were detected by negative staining in both the Gila monster and

the Mexican beaded lizard isolates (Fig. 12). In the dissertation hereafter, they be referred to as lizard AdV type 1 (LAdV-1) and LAdV-2, respectively.

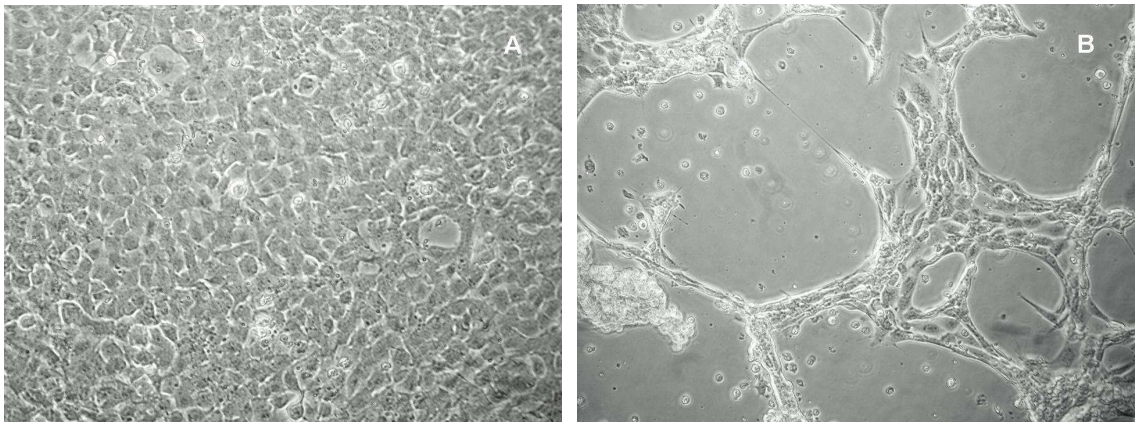

Figure 11. Adenovirus isolate (LAdV-1) from a Gila monster (*Heloderma suspectum*). Cytopathic effect (CPE) five days after inoculation. **(A)** Negative control IgH-2. **(B)** 3rd passage of an isolate from an oral swab from case Aps.11 on IgH-2 cells 5 days post inoculation. (Microscopic magnification: 400x) (Papp et al., 2009)

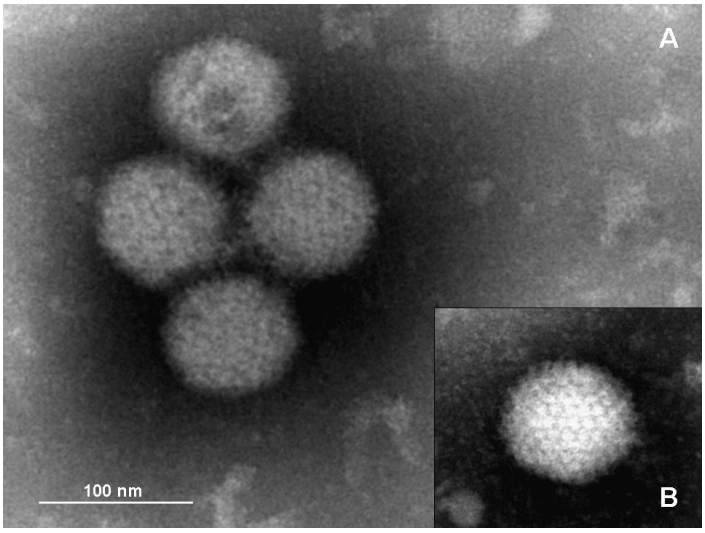

Figure 12. Electron micrographs of icosahedral non-enveloped virus particles in cell culture supernatant from two helodermatid isolates. (Both 3rd passage on IgH-2 cells 5 days post inoculation). **(A)** Mexican beaded lizard isolate with identification Aps.7 **(B)** Isolate from an oral swab from Gila monster with identification number Aps.10. (Papp et al., 2009)

6.1.3. Sequence analysis

The degenerate nested PCR targeting the DNA polymerase of AdVs (Wellehan et al., 2004) yielded nucleotide data of 272 nt length for all samples after editing out the primer sequences. These data were submitted to GenBank and have been assigned the accession numbers: EU914202–EU914209. G+C contents of these gene fragments were found 42.5-58.3%.

The six agamid AdVs that we have detected were most similar to one another and to agamid AdV sequences available in GenBank. There was a maximum of 6 nt differences between our agamid AdV sequences, with at most 2 non-silent mutations. In the data set of the follow-up study from the *Heloderma* spp., the sequences from the Mexican beaded lizards (LAdV-2) were always distinctly different from those of the Gila monsters (LAdV-1). All Gila sequences in the batches Aps.10 and Aps.11 were identical with the corresponding GenBank helodermatid AdV sequence, which was described from a Gila monster in the USA. Similarly, the sequences of all Mexican beaded lizard AdVs were identical with one another, but showed 24 nt differences compared to the Gila sequences, although 3 of these were non-silent mutations only. The partial sequence obtained from the emerald monitor AdV (Aps.12) had the lowest similarity values (ranging 54-61% and 59-64% for nt/aa) with any other reptilian AtAdV. The asp viper AdV (Aps.13) had the greatest similarity to the eublepharid AdV and the helodermatid AdV. This virus was simultaneously reported in the USA and named snake AdV type 2 (SnAdV-2). The partial DNA polymerase sequence from the AdV isolate from a *Boa constrictor* used as a positive control in all PCRs (Aps.14) was identical to the corresponding region of the SnAdV-1 (Fig. 13).

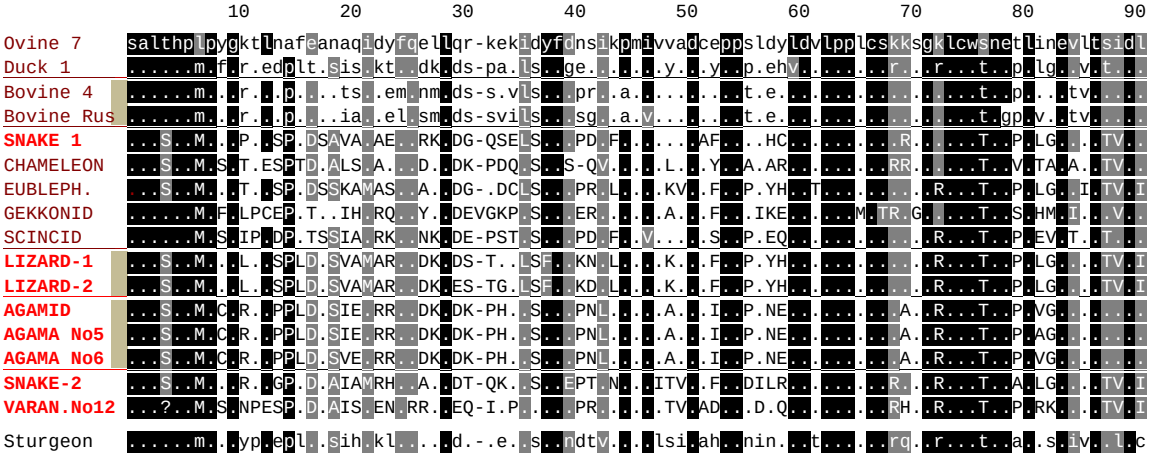

Figure 13. Alignment of predicted partial DNA polymerase sequences of atadenoviruses and the sturgeon adenovirus. (Identical sequences are represented by one selected virus. Start of alignment corresponds to the codon at position 6458 of HAdV-1 [AF534906]). Sequences of reptilian viruses are printed with capital letters, those from our study are highlighted in light red. OAdV-7 type species was taken as a reference sequence, identical amino acids are marked with dots. Virus species with more than one serotype are separated with lines and indicated with khaki columns. Background shading of sequences in black and grey refers to 80% identity and similarity of conserved amino acid positions respectively, regarding the alignment of all available sequences for this region. For abbreviations see legend of Fig. 14.

In phylogenetic analysis, the partial DNA polymerase sequences each newly detected squamatid AdV clustered to the genus *Atadenovirus* (Fig. 14). Analysis of the hexon gene loop-2 portion sequences could be performed with less squamatid AdV representatives only, and the result was consonant with that of the DNA polymerase sequences (Fig. 15).

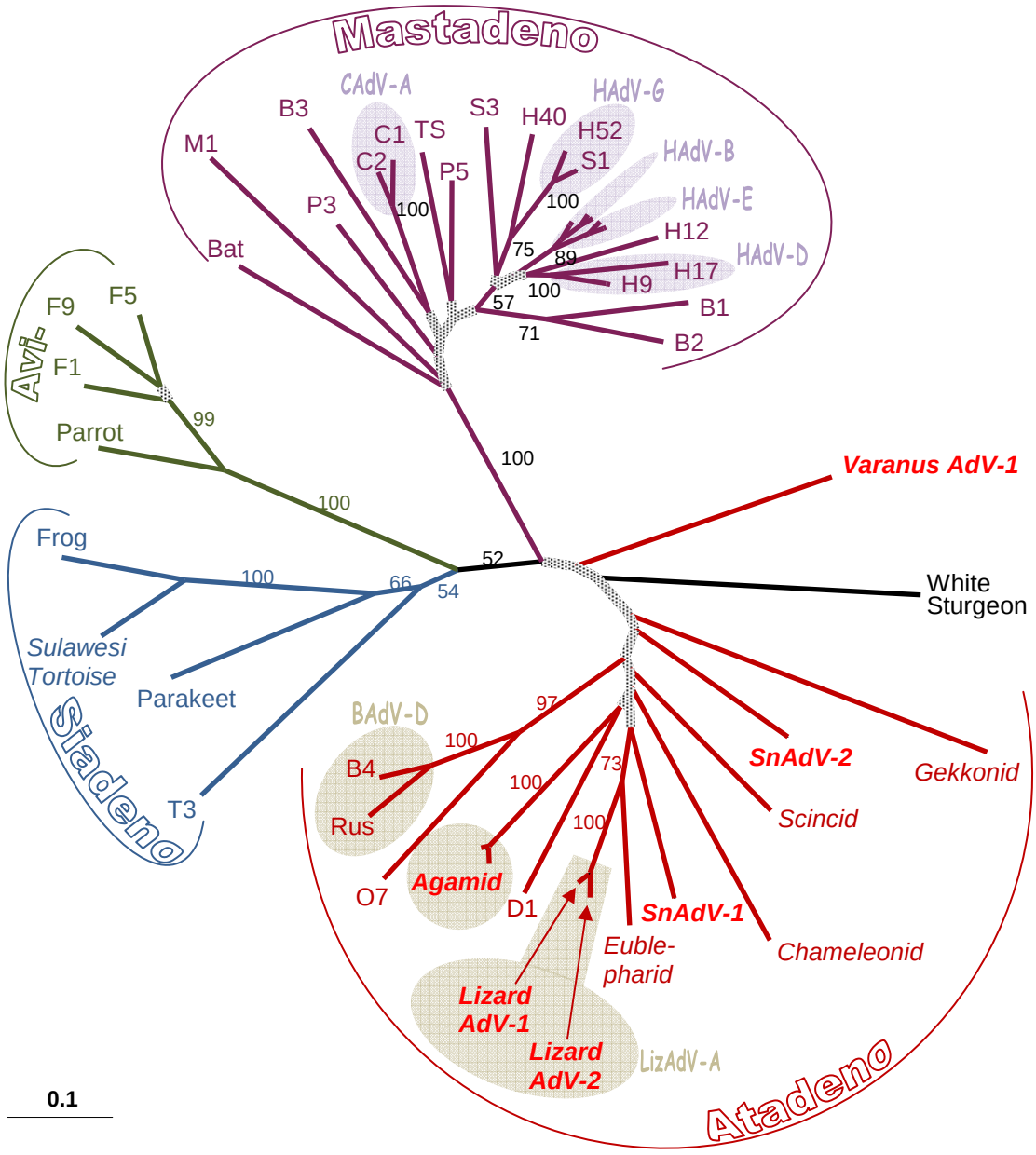

Figure 14. Phylogenetic distance tree of partial AdV DNA polymerase amino acid sequences. The tree was generated using the ProtDist program with Dayhoff Matrix, followed by Fitch program with global rearrangements. Bootstrap values above 50 (for 100 replications) are shown beside the branches, lower value branchings are shown with dotted lines. Virus species are indicated with shaded circles, genera with half circles. Names of reptilian viruses are printed in italics, and those representing sequences from our study (too), are in red. Abbreviations: B=Bovine, C=Canine, D=Duck, F=Fowl, H=Human, M=Murine, O=Ovine, P=Porcine, Rus=BadV-4 strain Rus, S=Simian, T=Turkey, TS=Tree shrew
For GenBank accession numbers see section 12.2.1 in Appendix.

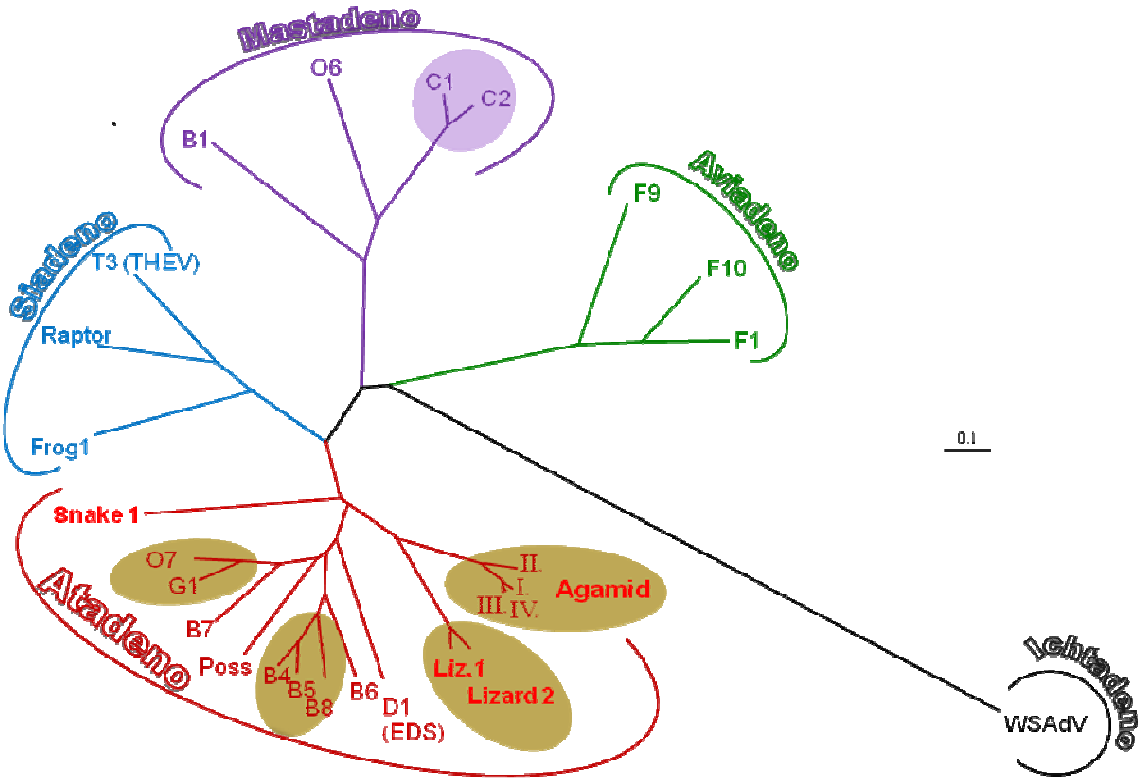

Figure 15. Maximum likelihood tree of partial AdV hexon gene sequences (250 bp). Genogroups of agamid AdVs (Parkin et al., 2009) are listed with Roman numbers. Abbreviations (other than in Fig. 14): EDS= Egg drop syndrome virus, THEV=Turkey haemorrhagic enteritis virus, WS=White sturgeon. See also legend of Fig. 14.

The two LAdV isolates were propagated, viral DNA was extracted and randomly cloned. Several LAdV fragments were amplified by newly designed consensus and specific primers in order to determine further portions of their genomes. For the LAdV-2, several randomly gained clones could be sequenced and joined with each other and with the short DNA polymerase or hexon PCR amplicons. Longer sequences were determined by primer walking. For isolate LAdV-2 two genome fragments could be assembled (Fig. 16) with a summarised length of 17.4 kb, and G+C content of 43.82%. For the LAdV-1 genome, the PCR based methods yielded results only, and 3 genome portions could be assembled with a summed length of 13.4 kb and G+C content of 43.31% (Fig. 16, Table 9).

The two LAdV partial genomes revealed high degree of similarity to each other. Nucleotide and deduced aa identity between the homologous genes were found to be 81-99% and 77-99% respectively. Lengths of completely determined genes, as well as lengths of intergene regions (IGR) were found to be identical in most cases (Table 9). These data suggest a most probable colinear arrangement of the two genomes, and enables further discussion of the two in a joint form. Specificities for either one will be marked.

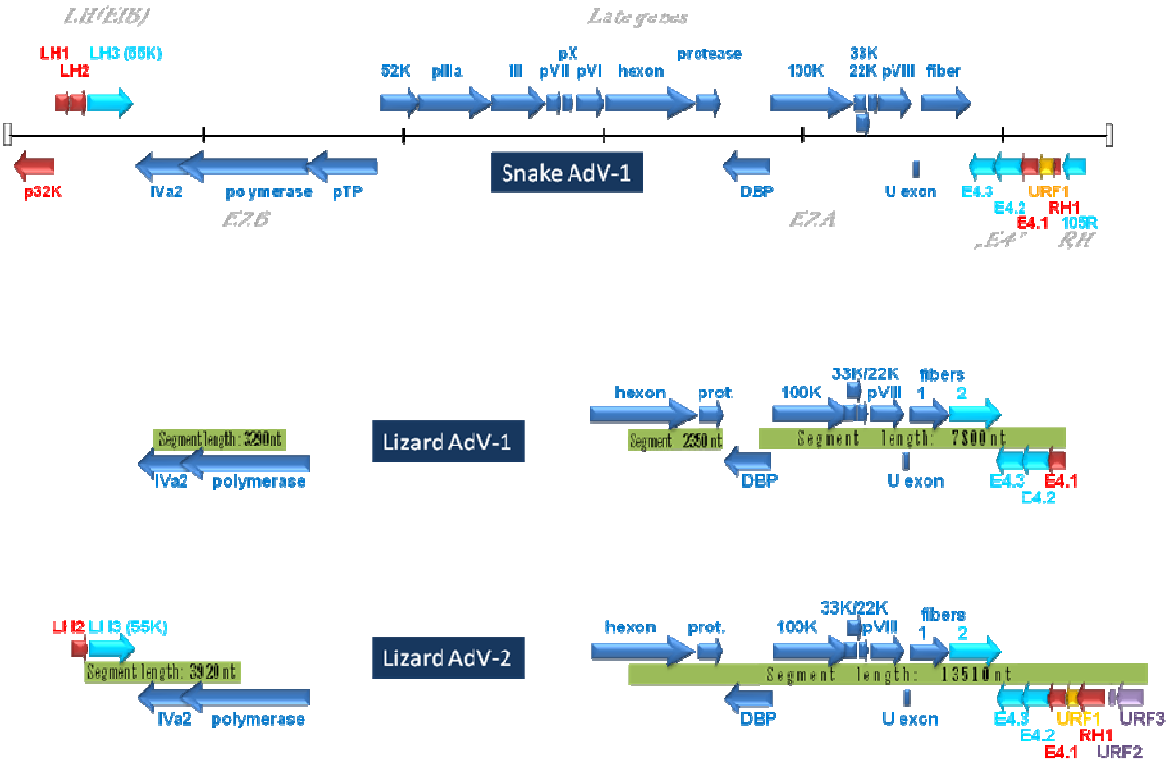

Figure 16. Presumed genome organization of LAdV-1 & -2 in comparison to SnAdV-1. Vertical bars on SnAdV-1 genome mark 5000 bp. Sequenced LAdV segments are indicated with green rectangles in proportional length. The genes (arrows) are coloured according to their occurrence in known AdV types, as follows: blue (dark)= conserved in each genus, turquoise= occurs in members of more than one genus, red= specific for members of genus *Atadenovirus*, yellow= found in analysed squamatid AdVs only, purple (light)= unique in analysed lizard AdV.

The lizard AdVs seem to have SnAdV-1 as the closest relative of the types completely sequenced so far. Of the conserved late genes, which are found in the middle part of every AdV, the BLAST identity values were always highest with SnAdV-1 and ranged between 57-78% & 51-89% for nt & aa sequences respectively. Both types were proven to possess a second fiber gene downstream of the surprisingly short SnAdV-1 homologue first fiber gene. The deduced protein sequence of fiber-2 was found most similar to fibers of unrelated AdVs: Odocoileus AdV-1 [AAK32726] and Bat AdV TJM [YP_005271200] with 34/46% and 38/54% identity/similarity values for LAdV-1 and LAdV-2 respectively. Right from this gene the E4 region genes are located on the leftward transcribing strand. Both types possess 3 so called E4 genes (E4.1, E4.2 and E4.3), homologous to those of SnAdV-1. In LAdV-2, upstream of this region URF1 and RH1 genes were detected collinear to those in SnAdV-1. The latter gene was more than tripled in size. Further right two ORFs (putative genes) were identified with no homology to any sequence available in GenBank. In LAdV-2, analysis of the short assembled genome portion revealed the presence of LH genes, another genus specific trait of AtAdVs.

Table 9. Predicted genes and encoded proteins of LAdV-1 and LAdV-2*

		Snake adenovirus 1 (SnAdV-1)			Lizard adenovirus 1 (LAdV-1) from Gila monster				Lizard adenovirus 2 (LAdV-2) from Mexican beaded lizard				LAdV-1 LAdV-2
Gene	**Coding strand**	protein length (aa)	G+C cont. (%)	IGR length (nt)	protein length (aa)	G+C cont. (%)	[a]Id. to SnAdV (%)	IGR length (nt)	protein length (aa)	G+C cont. (%)	[a]Id. to SnAdV (%)	IGR length (nt)	[a]Identity between two (%)
ITR	R	NA	51.7	215									
p32K	L	343	55.8	15									
LH1	R	118	50.4	-4									
LH2	R	132	48.6	36					NC	41.4	54/ 60	41	NA
LH3-55K	R	373	52.2	14					370	44.1	60/ 58	7	NA
IVa2[b]	L	443	47.2	32	NC	43.1	75/ 79	-190 ?	417	40.5	77/ 85	-190	94/ 98
DNS-pol.	L	1085	48.3	-25	NC	41.3	72/ 75	NA	NC	43.2	74/ 77	NA	95/ 97
pTP[b]	L	610	49.7	29									
52K	R	322	50.2	-16									
pIIIa	R	615	50.2	44									
III	R	450	49.6	40									
pVII	R	115	54.3	19									
pX	R	84	56.5	66									
pVI	R	223	56.3	22									
hexon	R	909	51.8	-4	NC	45.9	76/ 88	-4	NC	46.1	76/ 89	-4	94/ 99
protease	R	201	49.8	7	**179**	42.8	76/ 87	NA	**178**	43.8	78/ 86	75	96/ 94
DBP	L	397	49.8	36	NC	40.6	60/ 57	35	389	44.8	68/ 70	35	99/ 98
100K	R	679	50.8	-2	675	46.8	73/ 77	-299	675	46.8	74/ 77	-299	97/ 99
33K[b,c]	R	180	51.9	24	179	50.9	57/ 57	16	179	50.3	57/ 58	16	98/ 96
22K	R	104	56.8	360	110	52.3	61/ 59	312	110	52.6	62/ 60	312	96/ 94
pVIII	R	278	53.8	22	278	48.9	72/ 74	8	278	50.3	71/ 73	8	92/ 93
U exon[b]	L	58	48.0	17	58	41.2	69/ 67	**-6**	58	41.2	68/ 69	**14**	90/ 90
fiber-1	R	415	51.2	-115	331	48.8	58/ 52	18	331	48.1	57/ 51	18	95/ 95
fiber-2	R	NP			**431**	44.9	**38/ 16**[e]	**-50**	**433**	44.6	**41/ 16**[e]	**-23**	81/ 87
E4.3	L	217	48.3	-16	**235**	40.8	67/ 61	-17	**225**	41.2	67/ 64	-17	82/ 83
E4.2	L	216	43.9	-10	212	41.3	58/ 49	-10	212	38.0	59/ 48	-10	81/ 77
E4.1	L	149	44.7	42	148	39.2	56/ 46	NA	148	38.2	57/ 46	16	86/ 84
URF1	L	112	54.3	-14					101	49.2	52/ 51	-8	
RH1	L	68	47.3	83					243	39.3	**47/ 37**	96	
URF2[d]	L	NP							40	37.4	**NA**	47	
URF3[d]	L	NP							289	42.0	**NA**	NA	
105R	L	178	52.7	52									

*compared to those of SnAdV-1. Names of novel genes found in LAdV are printed bold. Prominent values are highlighted in boldface. Abbreviations and markings (for gene names see section 3.2.1 of dissertation): Id.= identity; IGR= length of the following intergene region (negative values mean overlap); L= left; NA= not applicable, NC= not completed, NP= not present, R= right; [a]Identity values refer to nt/aa sequences respectively. [b]Splicing is supposed. [c]Without splicing a 22K protein homologue is presumed. [d]Open reading frame with unidentified relatedness, [e]Identity calculated to fiber-1.

6.2. Iridovirus studies

6.2.1. Real-time PCR sensitivity and specificity

None of the DNA from the tested reptilian rana-, herpes-, and adenoviruses produced a signal in the assay. No signal was detected when DNA prepared from IIV negative crickets, lizards, or cell cultures was used as a template. The fluorescent curves generated using serial dilutions are shown in Figure 17. The reaction sensitivity and efficiency were very good, consistently detecting 1 copy of the MCP gene. In some cases lower than calculated 1 copy also produced low peaks with very bad intra- and inter-assay reproducibility, a cut-off was therefore set at over threshold cycle 38 to omit false positive results. Intra- and inter-assay reproducibilities were good (Table 10); no significant difference was measured in Ct values of the same aliquot. For the qPCR standard curve, Ct values ranged from 13.5 to 33.8.

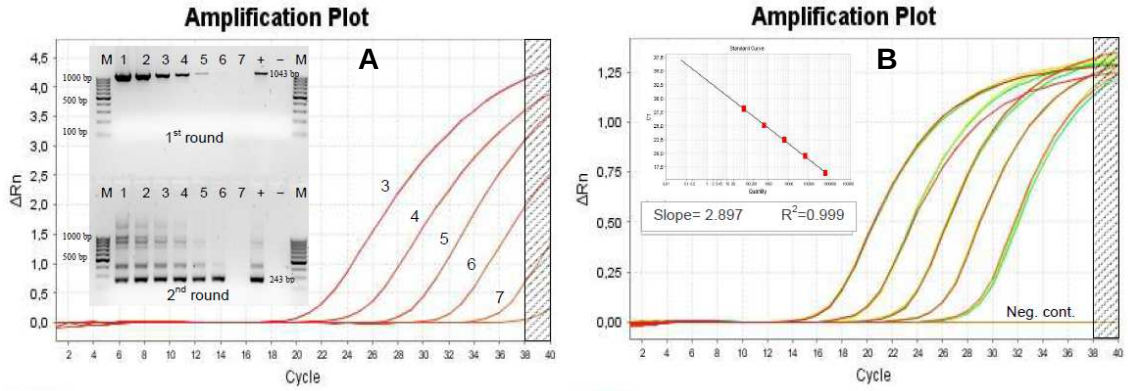

Figure 17. Comparison of sensitivities with decimal dilutions of DNA constructs, **(A)** MCP-gene amplicon purified and **(B)** cloned into plasmid. Set cut-off region is indicated on both plots with shading. **(A)** The analytical sensitivity of the qPCR was approx. 1 copy equivalent, in this case one fold higher as that of the conventional nested-PCR. M = marker (GeneRuler™ 100 bp). **(B)** Dynamic range of qPCR (partly shown) with intra-assay repeats for each dilution step.

A comparison of the sensitivity of the nested PCR and the real-time PCR with DNA preparations of serial dilutions of two lizard IIV isolates (Figure 17) showed that both assays were capable to detect the same low number of copies. Cell culture based virus titration was 10^2-10^3 less sensitive than the PCR methods (data not shown).

Table 10. Coefficients of variation of the qPCR assay

		copy number	threshold cycle
Inter-assay	low concentration	18.4 %	1.84 %
	high concentration	5.6 %	4.87 %
Intra assay	low concentration	25.6 %	0.54 %
	high concentration	9.5 %	2.01 %

The results of the comparison of all three methods using diagnostic samples from lizards and crickets as template are shown in Table 11. Nineteen different swab and tissue samples from twelve lizards, and ten out of eleven crickets were found positive by the real-time PCR (qPCR) and the conventional nested PCR (nPCR), whereas in the cell-culture assay seven and six of these were found positive, respectively (Table 11).

Table 11. Comparison of virus isolation, nPCR and qPCR methods for diagnostics of IIVs

	Id. Nr. / sample type		cells	nPCR*	qPCR**	Comments
Lizards	**Ips.1**	fibrome biopsy	–	+	++	
	Ips.2	growth r. shoulder	–	+	+	
		growth r. carpus	–	+	++	
		growth r. hind leg	–	+	++	
	Ips.3	cloacal swab	+	+	(+)	
	Ips.4	oral swab	–	+	++	
		cloacal swab	+	+	+++	AdV +
	Ips.5	oral swab	+	+	++	AdV +
		cloacal swab	+	+	++	AdV +
	Ips.6	oral swab	+	+	++	
		cloacal swab	+	+	++	AdV +
	Ips.7	fem. pore biopsy	–	+	+	
	Ips.8	skin-crust	–	+	(+)	
	Ips.9	skin	–	+	+	
	Ips.10	fem. pore biopsy	–	+	+	
	Ips.11	skin	+/-	(+)	(+)	
		oral swab	–	(+)	(+)	
		cloacal swab	–	+	+	
	Ips.12	swab	–	+	++	
Crickets	**Ips.13**	**a** whole body	+	+	++++	
		b whole body	+	+	++	
		c whole body	+	+	++	
		d whole body	–	+	++	
		e whole body	–	+	++	
	Ips.14	**a** whole body	+	+	(+)	
		b whole body	–	–	–	
		c whole body	–	(+)	(+)	
		d whole body	+	+	+	
		e whole body	+	+	+	
		f whole body	–	+	++	

*nPCR results were interpreted on intensity of the second round MCP PCR products by electrophoresis, (+)= faint, += normal bands. **qPCR values for calculated copy numbers per µl template DNA were coded as follows: (+)= 1- 5 copies/µl, += 5-100 copies/µl. ++= 1xE2-1xE4 copies/µl, +++= 1xE4-1xE6 copies/µl, ++++= over 1xE7 copies/µl. Abbreviations: Id. Nr.= identification number, fem.= femoral, r.= right.

6.2.2. Sequence comparison of isolates, similarities to CIV

We characterised IIVs isolated over the course of six years from crickets and insectivorous pets fed with crickets (Table 3) based on complete and partial gene sequences. Full CDS were obtained from eight genes: the exonuclease II gene (ORF012L), the DNA polymerase gene (ORF037L), ORF155L, ORF157L, ORF159L, ORF160L (viral antibiotic peptide, VAB gene), the major capsid protein gene (MCP,

ORF274L) and the upstream flanking ORF281R (Fig. 18). Partial CDS were determined from seven other genes: ATPase (ORF075L), helicase (ORF161L), DNA-ligase (ORF205R), thymidilate synthase (ORF225R), an apparently noncoding region (WIV ORF011 homologue) downstream of the MCP, ORF282R and immediate early protein gene (IE, ORF 393L) (Fig. 18). Identity values compared to corresponding CIV genes are shown in Table 12. The overall summarised length of the determined portions for the different isolates exceeded 14 kb.

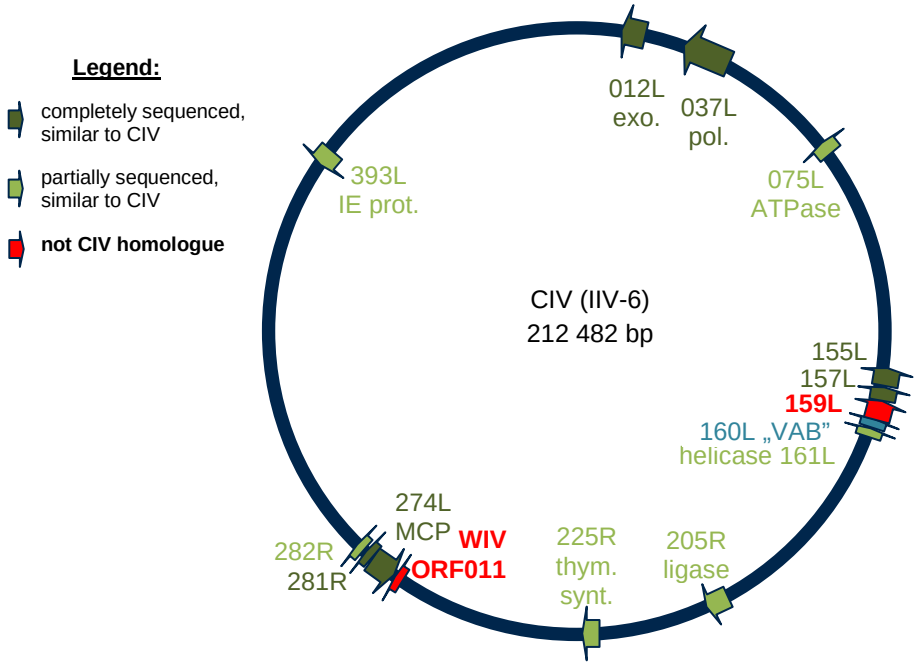

Figure 18. Sequenced gene(portion)s of IIV isolates. Genes are drawn according to their presumed layout projected on the circularly illustrated map of the CIV genome.

The isolates included in this study were all identical to GenBank cricket iridoviruses: GbIV (Just & Essbauer, 2001) and CrIV (Jakob et al., 2002b) based on the available partial MCP gene data. The studied isolates were found to be identical by using four different endonucleases (PstI, EcoRI, BamHI, HindIII) and by comparing sequences of several genes (075L, 160L and flanking genes, 205L, 225L, MCP and flanking genes, 393L). However, a very low inter-isolate variance (up to 0.4%) was detected in genes coding for DNA synthesis and -degrading enzymes (DNA polymerase, exonuclease). In the DNA polymerase gene (037L) chameleon isolates Ir.iso.1 and Ir.iso.5 and bearded dragon isolate Ir.iso.2 had one or two silent (at nt position: 831 or 855 & 1110) and a conservative non-silent mutation (nt pos.: 1019), respectively, while in the exonuclease gene (012L) chameleon isolate Ir.iso.1 had a single silent nucleotide exchange (nt pos.: 1695). The studied reptilian and invertebrate IIVs are therefore considered a single virus type and are hereafter referred to as cricket IV variants (GbIV variants).

Table 12. Identity values (%) between GbIV-variants and CIV

Region:	Exonuc.	Polym.	ATPase	**V**iral **A**ntibiotic **P**eptide and flanking genes					Ligase	Thym. synt.	**M**ajor **C**apsid **P**rotein and flanking genes				I.E. prot.
ORF (CIV)	012L	037L	075L	155L	157L	159L	160L	161L	205L	225R	WIV orf011	274L	281R	282R	393L
Length (nt)	1959	3831	650	1267	459	930	198	150	1293	507	549	1428	144	377	672
full/partial	full	full	part	full	full	full	full	part.	part.	part.	na	full	full	part.	part.
DNA	91.2	97.0	97.8	97.4	95.4	**54.5***	**73.6**	99.3	95.8	95.7	**43.8***	94.5	84.9	98.1	97.8
PROTEIN	91.6	96.7	100	99.2	98.0	**46.0***	**39.6**	100	97.2	92.4	**NA**	95.5	78.8	97.6	96.3

Lowest values are highlighted. *Long gaps were omitted in the comparison to maximalise identity values. NA= not applicable

The MCP gene of GbIV-variants was completely determined with approx. 500 nt flanking regions on both sides (total of 2543 nt). The length of the MCP coding sequence is 1428 nt (475 aa), the second longest reported in the family after IIV-9 (WIV). An approx. 500 nt long region downstream of the MCP has no BLAST similarity to any parts of the CIV genome, but does correlate with group III invertebrate iridovirus MCP flanking sequences (IIV-1, -9, -22; Acc.Nos: M39542, GQ918152, M32799) (Fig. 19).

We studied our IIV isolates for the presence of the sillucin homologue gene, since the Chilo iridescent virus genome was the first in which a viral antibiotic peptide (VAB) was detected. The VAB gene was detected and sequenced in all 8 isolates and all were found to be identical to one another. The putative protein product is 65 aa long (53 aa in CIV). The N-terminal signal peptide sequence has 3 point mutations and a 7 nt long deletion near the cleavage site compared to CIV-VAB (Fig. 20). This latter results in a frame shift and a longer (42/30 aa) non-homologous active peptide, with no BLAST homology to any GenBank entry.

The adjacent ORF downstream showed even larger dissimilarity to its CIV homologue (ORF159). In the GbIV-variants this ORF is shorter (930 aa/ 1428 aa in CIV) and only its first trisect has high similarity to CIV ORF159 (80%), whereas for its middle part counterparts were found in the IIV-9 (WIV) genome (Fig. 20). The second half of the gene has very limited (up to 30%) similarity to any known iridovirus sequence. This second and third trisect of ORF159L in the GbIV-variants is another hypothetical recombination site.

The following genes downstream are again highly similar to their corresponding ones in CIV, but one of them is formed as a fusion of two CIV ORFs (155L & 149L) due to a point mutation and loss of a stop codon.

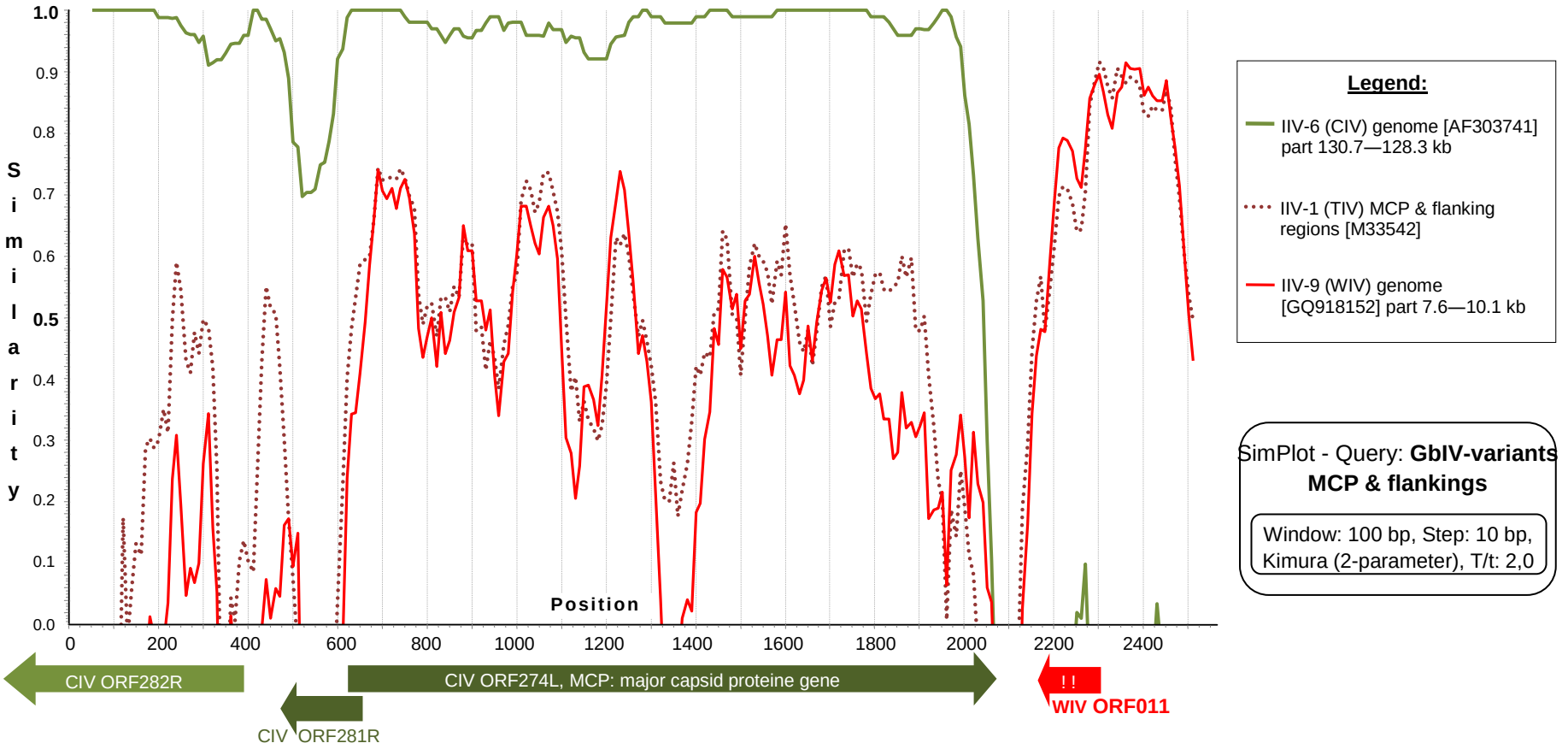

Figure 19. SimPlot analysis of the MCP gene with its flanking regions from our isolates. Three further members of the genus *Iridovirus* were available for the analysis in GenBank (IIV-22 is highly similar to IIV-1 and thus was omitted from the figure, for a better overview). Gene homologs with the highest BLAST values are drawn under the diagram. The region downstream of the MCP gene is a non-functional pseudogene homolog of WIV ORF011. Exclamation marks indicate stop codons in this pseudogene.

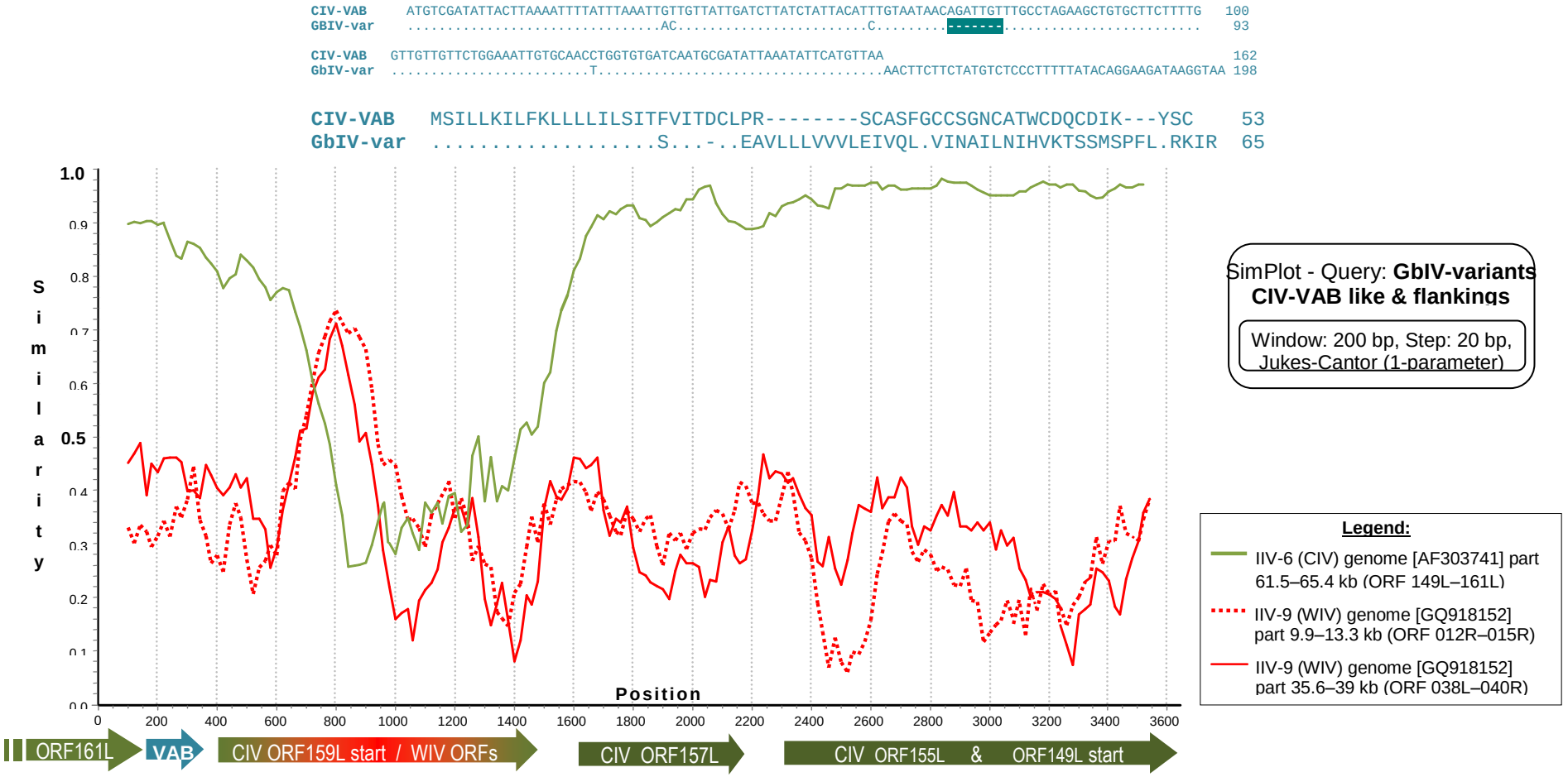

Figure 20. SimPlot analysis of the VAB homologue gene and its flanking region from our IIV isolates. The corresponding region of CIV along with two partially homologeous sections of IIV-9 (WIV) were included in the alignment. Gene homologs with the highest BLAST values are drawn under the diagram. The third ORF (910 nt) shows a suspected recombination site. Alignment of the CIV-VAB with that of these isolates is shown above the graph.

In two further completely sequenced genes (exonuclease & DNA polymerase), the nucleotide changes are clustered resulting in distinct insertions, which are significant. In the exonuclease, four insertions were found (aa 345-350, 470-474, 499-519 and 537-540) with a summed length of 33 aa, while the DNA polymerase of GbIV-variants contains one insertion of 3 amino acids (aa 287-289). This latter insertion together with the further 8 aa exchanges in the flanking region are found in the active centre “DEDDY domain” of the DNA polymerase. These structural alterations were confirmed by the protein structure analysis (Fig. 21).

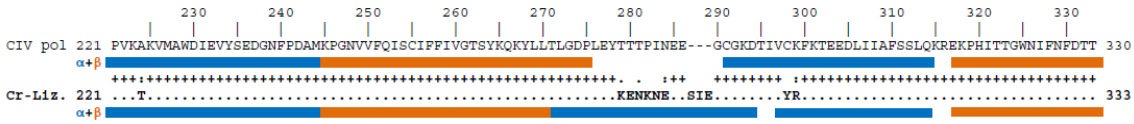

Figure 21. Predicted domain structure in the DNA polymerase enzymes of CIV and GbIV-variants. The active centre DEDDY 3´-5´exonuclease lies between positions 230 to 320 of the protein and has a putative extra α-helix in the GbIV variants. Predicted α-helices are indicated with blue bars, β-sheets with orange bars below sequences. Dots of CrIV sequence represent amino acids identical to CIV. Between sequence lines: + signs indicate identical amino acids, whereas single and double dots weakly or well conserved positions respectively. Analysis was performed by the GENtle program and SWISS-MODEL.

6.2.3. Comparing isolates in cricket infection study

The results of the cricket bioassay are shown summarized in Table 13 and in detail in Suppl. Figure 1 in the Appendix. We found rates of mortality varying between 15 and 60% in the infected groups and 0 to 40% mortality in the negative groups (data not shown). In the case of the negative control animals, however, the death of the crickets could never be associated with virus propagation. All negative control animals were negative by cell-culture, and by nPCR methods.

In the infected groups, apparent signs of infection were increased activity, swollen abdomen, and moulting abnormalities (Fig. 22). The so called “patently” infected crickets with very high virus loads and most often showing blue iridescence proved to have high virus copy numbers: 10^7 to 10^{10} according to qPCR, while the so-called covertly infected animals had considerably lower (10^2-10^4) copy numbers in their fat bodies. In selected patently infected crickets, the viral DNA could be detected by ISH as well (Weinmann et al., 2007).

Table 13. Comparison of cricket bioassay results with those of an earlier study in our laboratory (Weinmann et al., 2007) with non-controlled temperatures

	lizard isolate			scorpion isolate				cricket isolate		
	20ºC	30ºC pre[#]	30ºC	20ºC I.	20ºC II.	30ºC I.	30ºC II.	20ºC I.	20ºC II.	30ºC [##]
mortality (%)	40	60	35	45	40	60	60	15	50	100 [##]
patent inf. (%)	15	25	10	35	30	25	30	10	30	20
MST* (days)	42	38.6	36.5	34	24.2	24.4	34.2	35	42	19.3
qPCR (%)	30 (15)	85 (15)	25 (5)	75 (10)	70 (5)	65 (15)	80 (20)	40 (40)	45 (20)	55 (10)
nPCR (%)	30 (15)	55 (15)	30	75 (5)	65 (5)	55 (25)	55 (25)	50 (10)	55 (10)	60 (5)
isolation (%)	15	20 (5)	15	35	30	35	35	10 (5)	25	15 (5)
iridescence(%)	15	20	10	25	20	10	20	10	15	10

	Weinmann et al., 2007		
	I.	II.	III.
mortality (%)	35	20	20
patent inf. (%)	NA	NA	NA
MST (days)	NA	NA	NA
qPCR (%)	NA	NA	NA
nPCR (%)	75	15	30
isolation (%)	45	5	25
iridescence(%)	25	5	10

*MST was calculated for the proven patently infected animals

pre[#]: preliminary study with not uniform sex/age animals

##: high mortality occured to technical reasons (overheating)

Data in brackets refer to samples with dubious results in the different tests.
qPCR= 1-5 copies/µl detected,
nPCR= faint bands on agarose gel,
isolation= not consistent results in the repeats

NA= not analysed

When comparing the ratio of patently infected crickets and their mean survival time (MST) with the different virus isolates used, considerable differences were found. ***At 30ºC:*** the crickets infected with the *lizard* IIV isolate 10 (& 25)% were patently infected with a MST of 36.5 (& 38.6) days. (Preliminary study data in brackets.) The values with the *scorpion* isolate are: 25 & 30% patently infected with MST=24.4 & 34.2 days, while with the *cricket* isolate: 20% patently infected with MST=19.3 days.

The same data for the *lizard* isolate ***at 20ºC***: 15% and 42 days, for the *scorpion* isolate 30 & 35% and 24.2 & 34 days, while for the *cricket* isolate 10 & 30% with 35 & 42 days. The ratio of covert infection with middle range IIV load (isolation on reptilian cell-lines is possible, qPCR measured copy numbers >100) was surprisingly low (0-10%), and did not vary between temperatures and isolates.

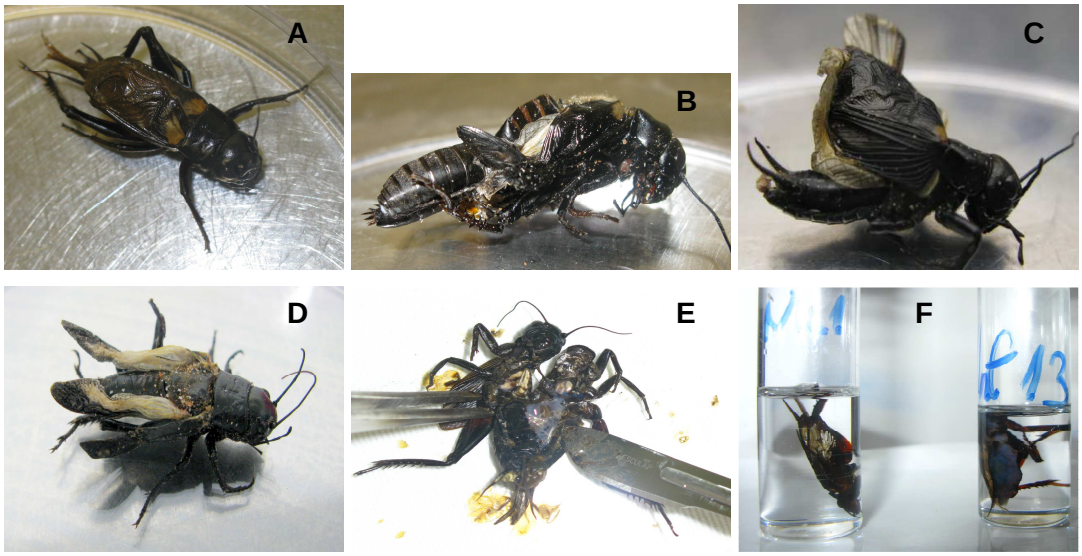

Figure 22. Malformations associated with IIV infection in crickets. **(A)** Negative control cricket. **(B)** Inability to complete ecdysis. **(C, D**) Distorted development of the wings in patently infected crickets. **(E, F)** Bluish iridescence in the fat body of patently infected crickets (on picture **F** right side tube is infected, left is negative control).

6.2.4. Transmission study with bearded dragons

None of the bearded dragons included in the study showed any signs of clinical disease during the course of the study. They continued eating normally and gaining weight during the entire 60-day period (see Suppl. Table 4 in Appendix). There were, however, drops in body weight up to 12% recorded from one week to the other due to shedding. “Single infected” animal A5 started to loose weight (with 15% loss) during the second week and showed no appetite. It died emaciated on day 16 post infection (dpi) and was dissected. Gross pathology did not reveal remarkable alterations, except for a yellowish friable liver with glycogen depositing and lipidosis in the histopathology.

Virus was detectable by isolation and PCRs from the oral and cloacal swab of the infected animals from the 1st week on after the beginning of infection, while the negative control lizards remained negative in these tests. The fourteen surviving animals were dissected at the end of the study. No changes were noted on gross pathology. A1 and A4 showed similar liver changes to those seen in A5.

No virus was detected in any of the negative control animals. In the infected animals, however, virus isolation and PCRs were positive from several organs (for details see Suppl. Fig. 2 in Appendix).

However, no specific macroscopic or histological changes could be detected in any of these organs, and the other detection methods (ISH, IHC, EM) failed to detect the traces of replication of IIV in the lizard tissues unequivocally. Comparing the virus

loads (according to qPCR data) of these lizard tissues to those found in patently infected crickets (section 6.2.3), these values are in a 3 to 6 fold lower range.

6.3. Paramyxovirus studies

6.3.1. Analyses of three genes of rPMV from snakes, lizards and a tortoise

Optimised nested RT-PCRs targeting part of the L gene (627/566 bp), part of HN gene (572/537 bp) and complete U gene (684/648 bp) yielded products ($1^{st}/2^{nd}$ round) from almost all of the isolates and field samples (hereafter: isolates; listed in Table 4). The single exception was the Hermann's tortoise isolate (THer-GER99) which could not be amplified with any of the tested HN gene specific PCR primers and also yielded an unexpectedly large amplicon (ca. 1800 bp) with the consensus U gene primer set. Sequencing revealed that this product was amplified from the viral F gene and not from U gene (data not shown).

During the PCR optimisation trials, the following combination of primers provided strongest reaction (Suppl. Table 3 in Appendix): for L gene first round 5F/6R followed by 7F/8R; for the HN gene if 1^{st} round 1F/2R products (Ahne et al., 1999) were further amplified by new degenerate primers HN-cons F-out and R-out. The nested PCR targeting the U gene described by Kurath et al. (2004) resulted in amplicons from three of the ten tested isolates (Xeno-USA99; Var-GER95, Dasy-GER00), while the newly designed consensus primers (U-cons Fwd-out / Rev-out, Fwd-in / Rev-in) used in a nested system gave products from each of them.

Two lizard isolates (Xeno-USA99; Var-GER95) had identical L, HN and U gene sequences (indicated on the same external nodes of Fig. 23). The third lizard isolate (Igu-GER00) had an HN gene sequence identical to that of two snake isolates from this study (Pyth-GER01, Crot-GER03) and of three other isolates from previous studies. The sequences obtained in this study have been submitted to GenBank (accession nos. GQ277611–GQ277627, HQ148084–HQ148087 & JX186193–JX186195).

The phylogenetic analysis showed that all of our isolates were related to snake PMVs available in GenBank and formed together the recently established genus *Ferlavirus*. Within the genus, the Hermann's tortoise (THer-GER99) isolate branched off as an ancient sister for a monophyletic squamatid PMV cluster. This squamatid PMV cluster could be further subdivided into groups A, B and C. Phylogenetic trees calculated with various methods (distance matrix, maximum likelihood, neighbourhood-

joining, Bayesian) from the different gene portions resulted in trees with similar topologies and probability values. However the two recent field isolates (PanGut-GER09, HoBuc-HUN09), one from a German corn snake population with concurrent infection of reo- and AdVs (Abbas et al., 2011), and the other from a Hungarian population of masked water snakes (Papp et al., 2012a), clustered inconstantly with the other squamatid isolates. These two isolates have shown the largest dissimilarity among the squamatid PMV. Identity values for the U, HN and L gene sequences were 80, 82 and 83% respectively between these two isolates (Table 14). When constructing phylogenetic trees from the HN and L gene sequences, the relationship of these two isolates with previously described ferlaviruses could not be resolved properly. The HN gene tree indicated common ancestry with “group A” squamatid ferlaviruses whereas the L gene data rather suggested monophyly with “group B” members (Fig. 23/B); in most cases (depending on the method applied) with insufficient probability values. SimPlot analysis of concatenated sequences of U, partial HN, and partial L genes showed similarity ranging between 50 and 87% in the different windows and the HN and L region had varying ferlavirus strains at the highest similarity position with a slight advance (at most 3%) compared to opposite group members, but no sign of recombination could be detected. Thus the SimPlot analysis and phylogenetic tree reconstruction based on the U gene data, as well as the BootScan analysis or Bayesian phylogeny inferred from the concatenated sequences have agree on the “new group C” formed by these two isolates being equally separated from the group A and B viruses.

Isolates from lizards could be found among both group A and B members mixed with snake ones, and there was no sign of host species specificity or host-virus coevolution among these squamatid isolates at all. However, the L gene sequence obtained from the tortoise isolate differed significantly from the squamatid ones, thus indicating possible coevolution.

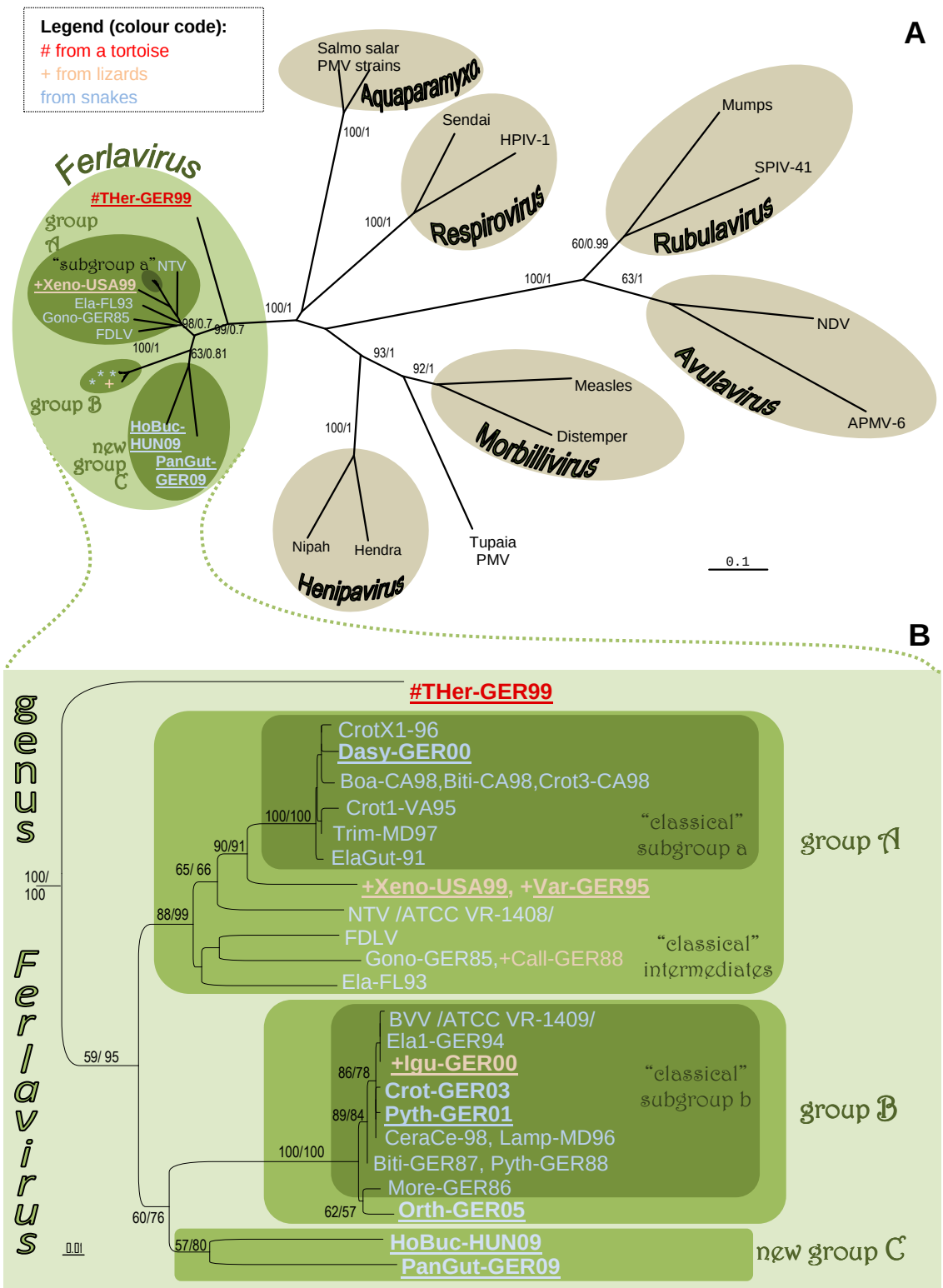

Figure 23. Phylogenetic tree reconstructions to resolve relationship of the reptilian PMVs. Own sequences are underlined and in bold face. Hash sign and red letters indicate the tortoise PMV isolate, cross sign and peach colour letters show lizard isolates, whereas the rest (printed in light blue) indicate PMV from snakes.
(A) Distance matrix tree of the partial L gene sequences for the *Paramyxovirinae* subfamily. Bootstrap values and posterior probabilities of the Bayesian analysis are indicated on the branches. In group B, names of isolates could not be shown. **(B)** Distance tree based on the partial L gene sequences, the genus *Ferlavirus* is enlarged for details. Bootstrap values of the DNAdist-Fitch and maximum likelihood analyses are on the branches.

Table 14. Variability table, showing the maximum nucleotide and amino acid (in bold face) sequence dissimilarity (%) values between any two members of the proposed ferlavirus taxons

	U gene (**ORF1/ORF2**)	HN gene partial	L gene partial	concatenated sequences
tortoise PMV and squamatid PMVs	NA	NN	24.6-28 / **20.6-24**	NA
max. in group A squamatid PMVs	17.5 / **28.1 / 13.3**	15.1 / **6.9**	16 / **6.9**	16.1
max. in group B squamatid PMVs	4.6 / **9.8 / 2.9**	2.9 / **1.8**	3.5 / **2.8**	3.3
max. in group C squamatid PMVs	20.1 / **25.7 / 11.2**	18.6 / **6.1**	16.9 / **6.2**	19.1
between squam. PMV groups	24.6-31.3 / **24.4-39.1 / 16-27.3**	16.9-22.6 / **4.4-10.4**	19.1-27.5 / **4.2-10.3**	21.3-25.7

NA= not applicable, NN= not known

The complete U gene nucleotide sequence alignment is shown in Fig. 24. There are two consensus ORFs starting at positions 68 and 87 and coding for 82 and 142/143/144 aa long polypeptides respectively. The amino acid divergence between the different virus isolates is higher in the case of the shorter polypeptide (ORF-1) as in the case of the second one (ORF-2) and both are higher than the corresponding values for the partial L and HN proteins (Table 14).

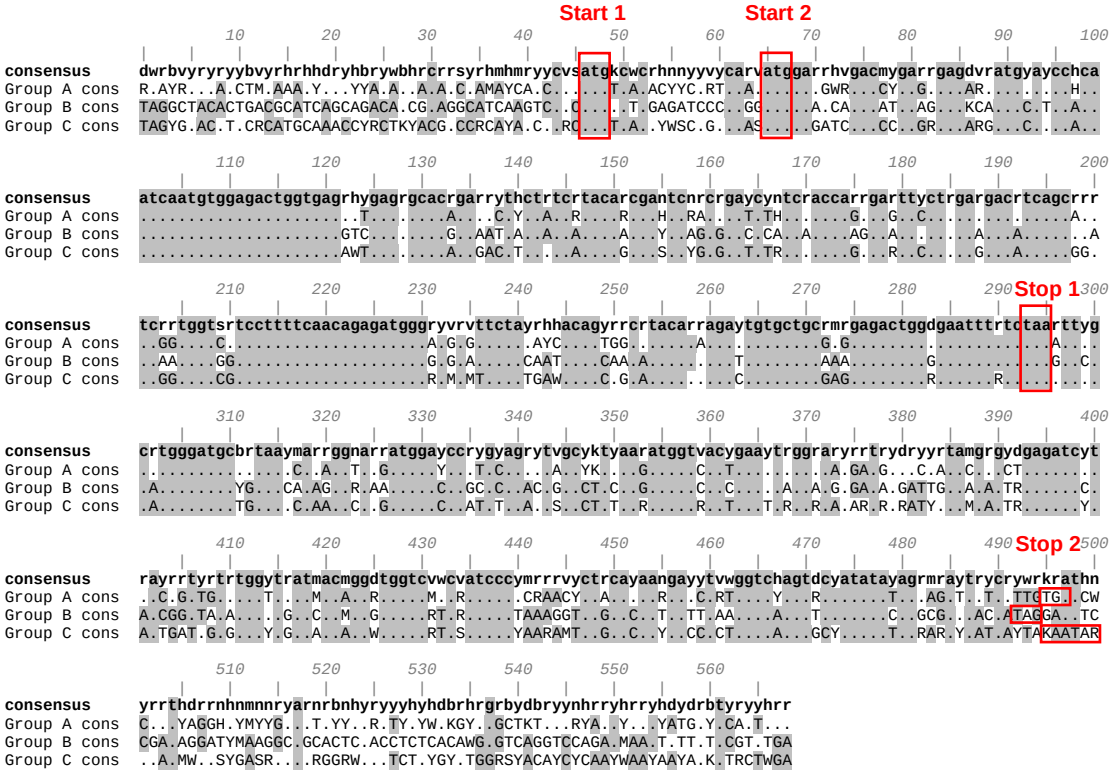

Figure 24. Nucleotide alignment for U gene sequences of the proposed groups of squamatid ferlaviruses. Dots indicate identical nucleotides with consensus. Hypothetical start and stop codons are marked with rectangles. Please note that ORF-2 has a consistent length of 143 and 142 aa for the group “A” and “B” members respectively, however the two members of group “C” are dissimilar in size (143/144 aa).

6.3.2. PMV survey in snakes and a tortoise

RT-PCR PMV screening of snakes was positive for a total of 50 samples (24.6% of the 203 tested organs/swabs) from 28 (27.5%) different animals (Table 15). Predominantly, CNS signs and pneumonia were in the anamnesis of these cases. Thirty-eight amplicons were sequenced directly, originating from 25 different individuals (with altogether 47 PMV positive organs/swabs) representing 11 different snake species. PMV was detected in swabs from twelve live animals (26.1%) out of 46 tested (Table 15).

Summarizing the PCR results from the dead, PMV positive snakes, intestine (11 pos./15 tested), lung (11 /13), kidney (10/14), liver (3/5), trachea (2/4), and oesophagus (1/1) were found positive, while the few spleen (0/2), blood (0/1) and brain (0/1) samples were all negative. Among the PMV positive animals lung, intestine and kidney were simultaneously examined 11 times and found all positive in four cases (36.4%). In three cases (27.3%) no amplicon was obtained from the kidney, while the other two organs were positive and the intestine was negative twice (18.2%), when lung and kidney were positive. Only once each, was the kidney (Vip-3) (9.1%) or the intestine (Vip-1) the single positive of the three organs. There was not a single case in our study when these tissues were all negative and another organ was positive in the L gene RT-PCR (Table 15 & Suppl. Table 6).

Virus isolation was attempted with snake samples, but gave positive results in three animals only (10.7% of PMV positive animals). Nested RT-PCR for the HN gene (Ahne et al., 1999) was also carried out with 47 of the L gene PCR positive diagnostic samples, but gave products of specific size in 16 (34%) cases only.

From the 38 selected amplicons, thirteen different partial L gene sequences (“sequence variants”) were obtained, of which six clustered to “group A”, and the rest “group B”. Eight sequences were identical to earlier characterised isolates (Ahne et al., 1999; Franke et al., 2001; Marschang et al., 2009). Five new sequence variants have been submitted to GenBank (accession numbers GU393344 to GU393348). In some cases, identical sequences were obtained from different animals in the same collection (Vip-2a & -2b & -2c) and different organs from the same snake (Col-3 lung & kidney; Col-6 lung & kidney; Pyt-2 lung & liver; Pyt-4 lung, kidney & intestine; Vip-4 lung & intestine). However, in several cases, amplicons from different tissues of the same animal (or amplicons from different animals in the same collection) indicated that snakes can be infected with more than one PMV during an outbreak. In a group of leopard snakes (*Elaphe situla*, Col-2), five organs of a dead snake (Col-2c) and oral

and cloacal swabs from six surviving animals were tested (Col-2a & -2b). The sequence of the PCR products from the lung of the dead snake ("variant A I") differed highly (23.7% nt, 6.9% aa) from that gained from the kidney of the same snake ("variant B I"), while it was identical to that from one of the swabs (Col-2b). The kidney originated viral sequence was rather similar (5.2/ 1.9% difference) to that from the other positive swab (Col-2a, "variant B VIII"). In another case of a young ball python (*Python regius*; Pyt-1), the virus detected in the liver differed (9/ 3.2%) from that detected in the intestine.

The multiple infection was proven also by cloning PCR-amplicons in two cases, where the direct sequencing results resembled a mixed sequence pattern. In the lung of a garter snake (*Thamnophis sirtalis*; Col-4), two distinct sequences ("variant A III" and "B IV") could be identified (with 20.5/ 5.6% difference), while in a ball python (*Python regius*; Pyt-2) five different sequences were found in the kidney, belonging to two distinct cohorts of sequence variants (A V-VII & B VI-VII) (Table 15, Fig. 25).

In the case of the leopard tortoise (*Geochelone pardalis*, Geo) which died with severe respiratory distress, 14 different organs were tested for the presence of virus by PCR and virus isolation. The PCRs for herpes-, rana-, and adenoviruses were all negative, however the RT-PCR targeting the L gene of ferlaviruses resulted in specific amplicons from the liver, heart, small intestine and cloacal samples. The phylogenetic analysis of the obtained sequences showed interestingly that these were all closely related to snake PMVs and not to the Hermann´s tortoise isolate (THer-GER99). Although the sequences were closely related to one another, each tissue contained a different "variant". Partial viral nucleotide data detected in the liver and small intestine were most alike to each other with one silent and one non-silent nucleotide change (0.5/ 0.7%). The sequence obtained from the heart differed from these two in 12 nt positions (2.8%) with 1 & 2 non-silent positions (0.7-1.4% aa), respectively. The partial PMV sequence from the cloaca differed the most from the other three (19.2-20.4% nt) with 9-10 non-silent changes (6.2-6.9% aa). All partial PMV sequences obtained from leopard tortoise tissues were distinctly different from the Hermann's tortoise virus (THer-GER99) sequence (25.3-27.1/ 19.1-21.1%). But they were either identical (liver, small intestine) or very similar (cloaca, heart) (0.3/ 0.7%) to earlier described snake PMV isolate sequences. These new sequences were submitted to GenBank (accession numbers: GU726898 & GU726899).

Table 15. PMV positive diagnostic samples (for further details see Table 6)

		Id. Nr.	Sample type	PMV RT-PCR results: L gene PCR	PMV RT-PCR results: partial L gene sequence	PMV RT-PCR results: HN gene PCR	Comments, other findings (virus isolation, other PCRs)
Squamata, Ophidia	Boidae	**Boa-1**[#]	cl. swab	+	A I	+	from Hungary[#]
		Boa-2	lung	+	B VI	–	reovirus isolated
		Boa-3	swab[§]	+	NT	NT	
		Boa-4a, & -4b	2 swabs[§]	+, +	NT	NT	4 snakes in group (Boa-4c to -4f) tested negative
		Boa-5	intestine, lung	+, +	B VII	+	PMV isolated from intestine
	Colubridae	**Col-1**	cl. swab	+	B II	–	
		Col-2a*	swab[§]	+	B VIII	–	4 living companions were tested negative *Animals have the same owner as Vip-2 and Vip-4
		Col-2b*	swab[§]	+	A I	–	
		Col-2c*	*lung,* liver, *kidney,* intestine	+,+ +, +	*A I,* NT *B I,* NT	+, – +, –	
		Col-3**	*trachea, lung, kidney*	+, +, +	*B VI B VIII*	– +, +	**same owner as Col-4
		Col-4**	*lung,* intestine	+ +/-	*A III, B IV* NT	– –	lung & intest.: reovirus isolated **same owner as Col-3
		Col-5	kidney	+	B IV	+	lung & kidney: reovirus isolated
		Col-6	lung, kidney	+, +	B VII	+, +	
	Pythonidae	**Pyt-1**	lung, *liver, intestine*	+, + +	NT, *A I A II*	–, – –	
		Pyt-2	*lung, liver, kidney,* intestine	+, + + +	*B VI A V-VII, B VI-VII* NT	– – –	
		Pyt-3	trachea, lung, intestine	+, + +/-	B VIII NT	–, – –	
		Pyt-4	oesophagus, lung, kidney, intestine	+/-, +, + +	NT B VIII B VIII	– +, + +	
		Pyt-5	kidney, intestine	+, +	NT, B VI	–, +	intestine: PMV isolated, partner animal (Pyt-16) neg.
		Pyt-6	*kidney, intestine*	+, +	*B VI, B V*	–, –	reovirus isolated from the kidney
		Pyt-7	swab[§]	+	A II	–	
		Pyt-8a	swab[§]	+	A I	–	12 animals were tested negative in the stock
	Viperidae	**Vip-1*****	intestine	+	A I	+	PMV isolated from intestine, *** same owner as Vip-3
		Vip-2a*	swab[§]	+	B VI	–	*Col-2 and Vip-4: same owner, dead viper was AdV positive but PMV negative (see Aps.13 of Table 2 & Table 8)
		Vip-2b*	swab[§]	+	B VI	–	
		Vip-2c*	swab[§]	+	B VI	–	
		Vip-3***	kidney	+	B VII	–	***same owner as Vip-1
		Vip-4*	lung, kidney, intestine	+, +/- +	B VII , NT B VII	+, – +	*same owner as Col-2 & Vip-2
Chel.	Tes.	**Geo**	*heart, liver, small intestine, cloaca*	+, + + +	*B III, B VII B VI A IV*	NT	The PCRs for herpes-, rana-, and adenoviruses were negative for all organs.

Denomination of partial L gene sequence data refers to the ''variants'' on the phylogenetic tree (Fig. 25). Sample types revealing multiple PMV variants in one animal are printed in italics.
Abbreviations: *, **, and ***: indicate animals from the same owner; swab[§] = oral and cloacal swabs were collected together; [#]all other samples are from Germany.
NT= not tested, += positive, +/- = weak pos. (faint band), – = negative

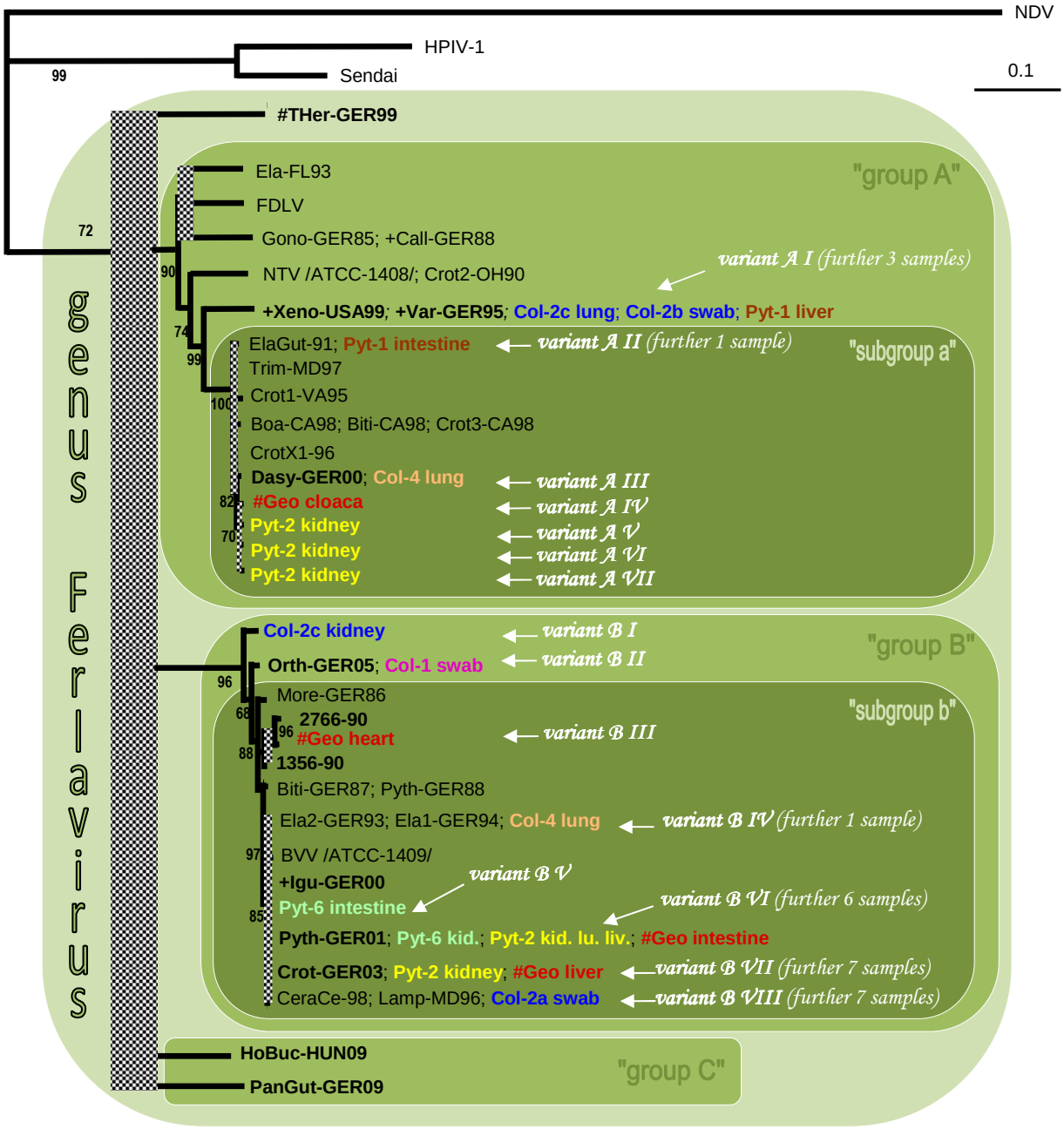

Figure 25. Distribution of diagnostic sample PMV L gene sequences on a phylogenetic distance tree. Bootstrap values above 70 (from 100 replications) are indicated beside the nodes, branches with lower values are indicated with checkerboard lines. Sequences from our studies are printed in bold, those retrieved from GenBank are normal. Diagnostic samples are coloured, identical colours denote identical animals and/or identical collections, but not all are listed on the tree for an easier overview. The ''[sequence] variant'' labels in white indicate the number of further samples (if any) that had identical sequences (for details see Table 15). *In sensu stricto* (Ahne et al., 1999) "a" & "b" as well as our proposed (Marschang et al., 2009; Abbas et al., 2011; Papp et al., 2010, 2012) *in sensu lato* "A", "B" & "C" groups are both shown. Dashes (#) indicate PMV found in tortoises, crosses (+) indicate PMV from lizards.
Abbreviations: kid. = kidney, liv. = liver, lu. = lung.

7. Discussion

7.1. Adenovirus studies

7.1.1. Novel reptilian adenoviruses

During our two year AdV survey, we successfully detected AdVs in a total of 25 squamatid reptiles, representing four lizard and one snake species. (Papp et al., 2009). Phylogenetic analysis of the partial DNA polymerase gene sequences revealed 5 different types of atadenoviruses, in all cases an AdV type specific for the host. Each of the viruses had a more or less balanced G+C content (42.5%-58.3%) in the sequenced region which might indicate that they have coevolved together with their hosts as hypothesized earlier by Benkő & Harrach (2003). Similar values have also been found so far in other studies with reptilian AtAdV sequences (Wellehan et al., 2004; Farkas et al., 2008, Parkin et al., 2009; Hyndman & Shilton, 2011; Pénzes & Doszpoly, 2011) in contrast with the unbalanced values found in non-squamate AtAdVs (e.g. Vrati et al., 1996; Benkő et al., 2001). This finding supports the hypothesis first proposed by Harrach (2000), for the squamatid origin of the genus *Atadenovirus*.

Of the detected types, agamid AdV-1 was found in 6 different cases (Aps.1-6), with 5 different sequence variants. The sequence variants possessed less than 2.5% nt variation in the examined region. One of the variants (Aps.1) was identical to that of the Austrian field isolate (Kübber-Heiss et al., 2006). This type of AdV is a known pathogen in bearded dragons (*Pogona vitticeps* [earlier: *Amphibolurus barbatus*]) and other related Australian agamid lizards bred in captivity worldwide. It was first recorded in New Zealand in association with inclusion body hepatitis (Julian & Durham, 1982). Hepatitis and enteritis were predominant in the later reports from the USA, although CNS signs also appeared in some cases, potentially due to encephalopathy caused by hepatonecrotic toxicosis (Frye et al., 1994; Jacobson et al., 1996; Kim et al., 2002; Moormann et al., 2009). Some of these cases were described with dependovirus and/or coccidial coinfection (Jacobson et al., 1996; Kim et al., 2002). In our six AdV positive bearded dragons, CNS signs dominated in three animals (Aps.1, 4, 6) and gastrointestinal disorders in one (Aps.5). Coccidiosis as a potential predisposing factor was found in one case (Aps.1). Interestingly, the sequence variation of our five German “strains” exceeded those of other multiple strain sequence studies from the USA (Parking et al., 2009) and from Hungary (Pénzes & Doszpoly, 2011) (Fig. 26), but they

all can be considered variants of one type. These data provide further support for the hypothesis that a single agamid AdV is circulating in the bearded dragon collections across Europe and the USA. This virus is believed to be the coevolved type for Australian agamids, as a similar variant was detected recently in wild captured netted dragons (*Ctenophorus nuchalis*) in Australia (Hyndman & Shilton, 2011). Unfortunately, we could not isolate the virus, and also could not obtain partial hexon gene sequences from a highly variable region (loop-2). This single round hexon PCR was found to be less sensitive than the nested consensus PCR for the DNA polymerase gene by Pénzes & Doszpoly (2011) as well, although it had been shown to enable a better genotype differentiation (Parking et al., 2009).

Figure 26. Bayesian tree of the available agamid AdV partial DNA polymerase gene sequences. Except for one, all sequences were obtained from bearded dragons (*Pogona vitticeps*). Eublepharid AdV was used as outgroup. For accession numbers see Appendix (Hungarian sequences were kindly provided by Judit Pénzes).

Another earlier reported lizard AdV type detected in our study was helodermatid AdV (Wellehan et al., 2004). The comparison of the partial polymerase sequences of our helodermatid sequences with those in GenBank has revealed that the two closely related host species, Mexican beaded lizards (Aps.7-8) and Gila monsters (Aps.9-11) possess two closely related, but different AdVs. The GenBank helodermatid AdV sequence from a Gila monster in the USA was identical to our Danish Gila sequences, whereas our beaded lizard isolate and sample sequences from the same enclosure differed from these. The difference (8.9/3.3% nt/aa) between the gene portions of the two helodermatid viruses was relatively small, in the range of being classified in the same species (Harrach et al., 2011), but two different types (Fig. 14, Fig. 15). This finding supported the host-specificity, coevolution-cospeciation theory of AtAdVs

(Harrach, 2000). However, recently an AdV was detected in wild caught western bearded dragons (*Pogona minor minor*) in Australia with complete identity to the Gila AdV sequence (Hyndman & Shilton, 2011). In Germany, a spiny-tailed monitor (*Varanus acanthurus*) was tested with an AdV, 99% identical to Mexical beaded lizard AdV (Inna Ball [Romanova], personal comm.). In both cases, the hosts have a more distant evolutionary relationship with the North American native helodermatid lizards, so most probably host switch(es) occurred. Further investigations are needed to unfold the evolutionary history of these two related viruses. Due to this apparently wider host range of these viruses, they have received the names lizard adenovirus type 1 (LAdV-1) and LandV-2, numbered in the chronological order of first description.

There were several interesting aspects of the AdV found in the emerald monitor (Aps.12). Although the histological examination of this animal showed hepatitis, no AdV was detected by PCR in the liver. The spleen and kidney were both PCR positive and the sequences obtained from these amplicons revealed that this varanid AdV is very different from every earlier described reptilian AdV. Using either nucleotide or amino acid sequence in the distance matrix analysis, the virus clustered not in but beside the genus *Atadenovirus* (Fig. 14). However, the sequences were short and the bootstrap values at this branching of the tree were under 50. Later acquired other varanid AdV types in Hungary from a white throated monitor (*Varanus albigularis*) (Pénzes et al., 2009) and in Germany from a water monitor (*Varanus salvator*) (Inna Ball [Romanova], personal comm.) have proven that the varanid AdVs are related to one another and all belong to genus *Atadenovirus* as the so far earliest known diverging branch. Thus, we can conclude that the varanid AdVs are apparently the closest descendants of the ancient common ancestor of all atadenoviruses discovered so far.

The asp viper (Aps.13) was the only animal that died in a collection of snakes in which an AdV was detected, although the companion animals were positive in a PMV RT-PCR only (Vip-2, Table 6, Table 15), and this animal had no pathognomic signs for AdV infection (it was detected in a blind survey). The phylogenetic analysis of the DNA polymerase gene portion of the AdV of this animal has clustered this virus into the genus *Atadenovirus*, representing probably a new species. An AdV with the same sequence was identified in a parallel study in another viper species in Hungary (Indonesian pit-viper [*Parias (Trimeresurus) hageni*]) (Farkas & Gál., 2008). However, the same short DNA polymerase sequence was amplified from AdVs detected in three colubrid snake species. Two of them were described in the USA (a California kingsnake [*Lampropeltis getulus californiae*] and a milksnake [*Lampropeltis triangulum*]) (Garner et al., 2008) and one later in Germany (a corn snake

[*Pantherophis (Elaphe) guttatus*] (Abbas et al., 2011). Consequently, the type has been named snake AdV-2.

In conclusion, our sequence data further support the theory on the squamatid reptilian origin of atadenoviruses. However, this theory needs to be extended to explain all of the recently described reptile AdVs. One open question is the occurrence of the same AdV type in different snake and lizard hosts, which clearly suggests some host switches. Additional work is necessary on the AdVs described here, as well as to characterise further reptilian AdVs, which will eventually result in the refining of their taxonomy.

7.1.2. Partial genome analysis of isolated lizard AdVs

Several papers have describied the occurrence of AdVs in different lizards (e.g. Kinsel et al., 1997; Wellehan et al., 2004; Hyndman & Shilton, 2011), but these viruses have never been isolated in cell culture . In the last decade there were some partially successful attempts to isolate lizard AdVs, especially agamid AdV, but a pure culture could not be established (reoviruses overgrew the replicating AdV; Silvia Blahak, personal comm.). Prior to the start of our studies, all reptilian AdV isolates originated from snakes (Jacobson et al., 1985; Ogawa et al., 1992; Juhász and Ahne, 1992) and there was evidence that some of these might represent the same virus type (Marschang et al., 2003). Our two viruses from swabs of two Gila monsters (LAdV-1) and from organs of a Mexican beaded lizard (LAdV-2) are the first pure lizard AdV isolates ever reported (Papp et al., 2009). Isolation was an important step in the further study of AdVs of reptiles and evolution of AtAdVs. Phylogenetic analysis of short sequence portions is possible with field samples, but virus isolation is essential for physico-chemical and ultrastructural characterisation as well as for pathogenicity studies.

At the University of Hohenheim, in Stuttgart, we have started the determination of genome sequences in cooperation with colleagues in the Veterinary Medical Research Institute of the Hungarian Academy of Sciences, in Budapest (Pénzes et al., 2012). Here, we present the preliminary partial sequence data determined in Stuttgart. We determined the nt sequence of genome fragments in over 13 kb length of the LAdV-1 and in over 17 kb of LAdV-2. Conserved, inner region genes and genome-end, variable genes were localised and analysed (Fig. 16). The genome fragments were collinear in the two viruses and shared many common features with SnAdV-1 and other AtAdVs. Short distances were found between the genes and in a number of cases they overlapped. For instance, the hexon and protease genes were overlapping with the

same length (4 nt) as in SnAdV-1 (Farkas et al., 2002) and DAdV-1, OAdV-7, BAdV-7 and BAdV-4 (Hess et al., 1997; Harrach et al., 1997; Dán et al., 1998). Further traits specific for the members of the genus *Atadenovirus* were: the presence of LH2 gene on the left hand side and the occurrence of E4.1 and RH1 genes on the right hand side. In LAdV-1, the sequenced fragments did not extend to cover all of these genes (Fig. 16). In LAdV-2, however, the URF-1 gene homologue of SnAdV-1 (Farkas et al., 2008) was found between E4.1 and RH1, a presumed synplesiomorph gene for squamatid AtAdVs, with yet unknown function.

The G+C content of LAdV-1 & LAdV-2 was lower in each of the examined genes (Table 9) than in the corresponding genes of SnAdV-1. For the concatenated fragments, LAdV-1 had a 43.31% and LAdV-2 had 43.82% G+C content, compared to 50.21% of SnAdV-1 genome (Farkas et al., 2008). These values are higher than those measured for the avian and mammalian AtAdVs, where biased G+C content was noticed, providing the name of the genus for A+T richness (Benkő & Harrach, 1998). It was hypothesized that the innate immune system enforces a strong negative selection against GpC dinucleotides when a new host is infected, as they are easier to recognise (Shackelton et al., 2006). Thus underbalanced G+C content can be regarded as a sign of a recent host switch event (Benkő & Harrach, 2003). In the case of the LAdVs, the slightly unbalanced G+C content may be connected with host switch events between reptile species, as described earlier (section 7.1.1). However, as mentioned for the partial DNA polymerase gene data above, stronger transition towards A+T richness has not been recognised in any squamatid AtAdV so far.

In regions determined for both isolates, few dissimilarities were found between the two LAdV genomes. Three genes (protease, fiber-2 and E4.3) and two IGRs had size differences between the two viruses. The nt identity values of the conserved and non-conserved genes ranged between 90-99% & 81-86%, respectively (Table 9).

These are the first cases when more than one fiber genes are present in an atadenovirus. This phenomenon has already been reported in three other genera: *Mastadenovirus*, *Aviadenovirus,* and *Ichtadenovirus*. In the sturgeon AdV genome (genus *Ichtadenovirus*) four fiber gene homologues were described in the left end of the genome (Doszpoly, 2011), but in all other examples the two fiber genes identified are positioned on the right describing strand downstream of the conserved late genes, before the E4 region (Fig. 2). The same orientation and positionining was determined in LAdVs (Fig. 16). In HAdV-F and HAdV-G mastadenoviruses, a shorter fiber-1 gene (347-387 aa) is followed by a longer fiber-2 gene (547-562 aa) and apparently both are expressed, so that the short and long fibers are incorporated alternately into the virion vertices, one per each penton base only (Kidd & Erasmus, 1989; Kidd et al., 1990,

1993; Davison et al., 1993; Yeh et al., 1994; Kovács et al., 2005). In contrast, in two fowl aviadenoviruses species (FAdV-A and FAdV-C) both fibers are expressed, and apparently both are incorporated at each vertex of the virion into the penton bases (Gelderblom & Maichle-Lauppe, 1982; Chiocca et al., 1996; Griffin & Nagy, 2011). The single serotype in FAdV-A contains a longer fiber-1 gene (corresponding to 793 aa) and shorter fiber-2 gene (410 aa), whereas FAdV-C members have two fiber genes coding similar size proteins (432-479 aa) (Marek et al., 2012). Other fowl AdV species (FAdV-D and FAdV-E) possess one gene only, but two fibers protrude from each vertex. Recently, two non-chicken aviadenovirus complete genomes were published (Kaján et al., 2010, 2012) containing two fiber genes, again. For these viruses there was no supporting EM data for the virion surface structure.

In the case of the LAdVs, analysis of the deduced protein sequences predicted that both fiber-1 and fiber-2 can be functional. The first fiber of LAdVs is shorter (331 aa) than the corresponding gene product of SnAdV-1 (415 aa). The fiber gene of SnAdV-1 has the highest homology to the fiber-1 genes of the LAdVs, with 52/51% nt and 58/57% identity values respectively (Table 9).

In the tail region of LAdV fiber-1 proteins, all three conserved motifs were localized, as follows: At the amino terminus, the “KRAK” motif, the nuclear localization signal was found (Hong & Engler, 1991). At a distance of 7 aa, the “PIYP” motif followed, which is responsible for interaction with the penton base protein (Caillet-Boudin, 1989). The third typical motif, “PPF”, with unknown function, is at another 10 aa distance. The second fiber genes are longer, coding for 431 and 433 aa in LAdV-1 and -2, respectively. The deduced protein sequence of fiber-2 was not found to be considerably similar to fibers of other known AdVs, but it also has the conserved motifs of the tail region. The nuclear localization signal “KRAR” at the amino terminus is in a distance of 8 aa from the penton base binding site “PVYP”, which is followed by a truncated “PPF” motif (only “PF”) at a distance of 13 aa. The presence of these motifs in the two fibers predict both of them to be functional. Additional proteomic analysis by mass spectrometry and detailed imaging of the virion surface by cryo-EM of the LAdV-1 isolate was performed at the Centro de Biología Molecular, in Madrid. These have shown that both fibers are expressed (Carmen San Martin, personal comm.).

It is unclear if there is a connection between the presence of multiple fibers on the surface of the virions and the occurrence of the LAdV types in distantly related lizards (helodermatids and agamids). The theory that these fibers are marks of adaptation to a new lizard hosts needs to be further investigated. Analysis of the complete genomes of LAdVs (Pénzes et al., 2012), as well as screening in wild and captive lizard populations by PCR and serological methods (Inna Ball [Romanova],

personal comm.), based on these investigations, are being carried out to refine our knowledge of the phylogeny and structural properties of reptilian AtAdVs.

7.2. Iridovirus studies

Our iridovirus studies were based on the findings of two German research groups. In 2001, isolation of IIV-like viruses was reported from the lung, liver, kidney, and intestine of two bearded dragons (*Pogona vitticeps*) and a four horned chameleon (*Chamaeleo quadricornis*) and from the skin of a frilled lizard (*Chlamydosaurus kingii*) (Just et al., 2001) was reported. Partial sequencing of the MCP gene of the isolates showed 100% identity to the nucleotide sequence of the cricket iridovirus GbIV (Kleespies et al., 1999; Just & Essbauer, 2001). In a parallel study by Marschang et al. (2002a), similar IIVs were isolated from two chameleons (*Chamaleo quadricornis, Ch. hoehnelii*) and an iguana (*Iguana iguana*). The chameleons were cachectic, whereas the iguana had skin lesions. Later, further IIVs were isolated in the Stuttgart laboratory from insectivorous hosts (lizards and a scorpion) and from the prey crickets (Table 3). A host-switch of this virus from prey insects to the predator lizards was postulated (Just et al., 2001). The Stuttgart isolates were compared to each other by partial genome sequencing and in bioassays. Transmission studies were performed and the isolates were used to elaborate novel diagnostic tools. Our results are as discussed in the following sections.

7.2.1. The qPCR as a diagnostic tool

Since GbIV-like IIVs are a significant problem for insect breeders (Kleespies et al., 1999) and are considered a potential threat to pet lizards, it was important to elaborate sensitive and specific tools for the detection of these viruses in clinical samples from a wide range of animals. The developed qPCR method is specific, and as sensitive as the previously described nested nPCR detecting the MCP (Weinmann et al., 2007), but it requires less handling steps and provides faster and more informative data. Both PCRs are 100 to 1000 times more sensitive than cell culture based detection methods.

The designed qPCR was used to detect IIVs in diagnostic samples from lizards and prey insects from four locations in Germany (Table 4, Table 11). Skin problems or cellular proliferations were the clinical signs observed in seven of the twelve IIV positive lizards. In the other four cases (Ips.4 to -7), in which entero-hepatic and CNS signs

were described, and oral and/or cloacal swabs were taken as samples, the results should be interpreted cautiously, since contamination from infected prey insects is also possible at these orifices (as suggested by the elevated viral quantity of the cloacal swabs from lizard Ips.4 to Ips.6). The qPCR results of diagnostic samples were consistent with those of the nPCR, the most sensitive diagnostic tool available previously. The lowest threshold cycle values (C_t: 14.3 to 24.6) and highest quantities (up to 10^8 copies) were measured in the positive crickets. In the case of the low copy number samples, it should be mentioned that the inter- and intra-assay coefficients of variation are elevated slightly compared to samples with high copy numbers (Table 10). A pre-assay extraction control with a host gene was not possible because of the variation in the targeted hosts, an exogenous internal control could, however, be considered in future to further improve the qPCR.

7.2.2. Comparison of genome fragments of different IIV isolates

The isolates found by us and included in this study (Table 3) were all identical to GenBank cricket iridoviruses: GbIV (Just & Essbauer, 2001) and CrIV (Jakob et al., 2002b) based on the available partial MCP gene data. The GbIV isolate kindly sent to our laboratory by Dr. Kleespies (Federal Biological Research Centre for Agriculture and Forestry, Institute for Biological Control, Darmstadt, Germany) has revealed identical sequences to our isolates in the four analysed genome regions (012L, 037L, MCP, VAB), and also in a PCR-RFLP (data not shown). The studied isolates were found to be identical to one another by RFLP and by comparing six complete and six partial gene sequences. A very low inter-isolate variance (up to 0.4%), however, was detected in two other genes (exonuclease II, DNA polymerase). The studied reptilian and invertebrate IIVs are therefore considered a single virus type and are hereafter, referred to as cricket iridovirus variants (GbIV-variants).

The MCP gene sequence of GbIV-variants was found to be 1428 nt (475 aa) long, the second longest reported in the family after IIV-9 (WIV) (Wong et al., 2011). This length seems to be rather conserved in members of the vertebrate IV genera (*Lymphocystivirus*: 459 aa, *Ranavirus*: 463 aa, *Megalocytivirus*: 453/452 aa) and all MCPs possess the consensus termination: “GFPV/IL”. IIV-3 of *Chloriridovirus* (466 aa) also has the same consensus termination. Of the six complete MCP gene sequences available from members of the genus *Iridovirus* so far, however, the size of this gene ranges from 462 to 484 aa, and two of the viruses (CIV and IIV-16) have this termination only. The MCP of GbIV-variants is not only 8 aa longer than that of CIV, but it has 13 additional mutation sites (Fig. 27). This rate of MCP divergence (5.5/4.5% for

nt/aa) is similar to that found between two separate types: IIV-1 (Tipula iridescent virus; TIV) and IIV-22 (Simulium iridescent virus; SIV) (Tidona et al., 1998).

Similarity plot analysis supported the hypothesis of recombination downstream of MCP and the closest relationship of this region to the homologous regions of group I iridoviruses, e.g. IIV-9 (WIV) ORF011, adjacent to the IIV-9 MCP gene (ORF010). In GbIV-variants, however, this region is a pseudogen, an unfunctional homologue of WIV ORF011, with two stop codons in the deduced protein sequence (Fig. 19).

We studied our IIV isolates for the presence of the sillucin homologue gene, since the Chilo iridescent virus was the first in which a functional viral antibiotic peptide (VAB) was detected in the genome (Jakob et al., 2001). The VAB gene was present in all GbIV-variants and found to be identical to one another. The leading signal peptide sequence had 3 point mutations and a 7 nt long deletion near the cleavage site compared to the VAB gene in CIV. This deletion results in a frame shift and a deduced active peptide sequence, which has no homology to any present GenBank entry. These changes indicate that the VAB gene in GbIV-variants is functionally different from that found in CIV. These changes in the gene, its transcriptional pattern, and its expression as a protein should further be investigated. The adjacent ORF downstream of VAB gene (ORF159), revealed another hypothetical recombination site (Fig. 20).

Despite the high similarity (97%) of the DNA polymerase gene of GbIV-variants (037L) to its homologue in CIV, the majority of the mutations and insertions in this gene clustered in close proximity. The structure and function of DNA polymerases have been characterised extensively (Rathwell & Waksman, 2005). Eukaryotic DNA polymerases contain three active centres, the DEDDY3´-5´exonuclease (aa 230-320), the POLBc domain (aa 470-560) and the POLB delta domain (aa 800-910). The DEDDY domain is responsible for substrate specificity and DNA repair. The mutations in the DEDDY domain of GbIV-variants result in the creation of a new alpha helix in the predicted structure of the protein (Fig. 21) and could lead to alterations in the catalytic properties of the enzyme. These structural alterations are significant and were detected by protein structure analysis.

The current taxonomical position of GbIV as a variant of CIV was based on biological and genetic data (Jakob et al., 2002b) with high similarity between the two viruses in susceptible insect host species and sequence data of 7 homologous genes (93-98% & 90-97% nt & aa). However, the genomic organisation of GbIV and CIV had been indirectly compared using RFLP (Kleespies et al., 1999) and found to be different.

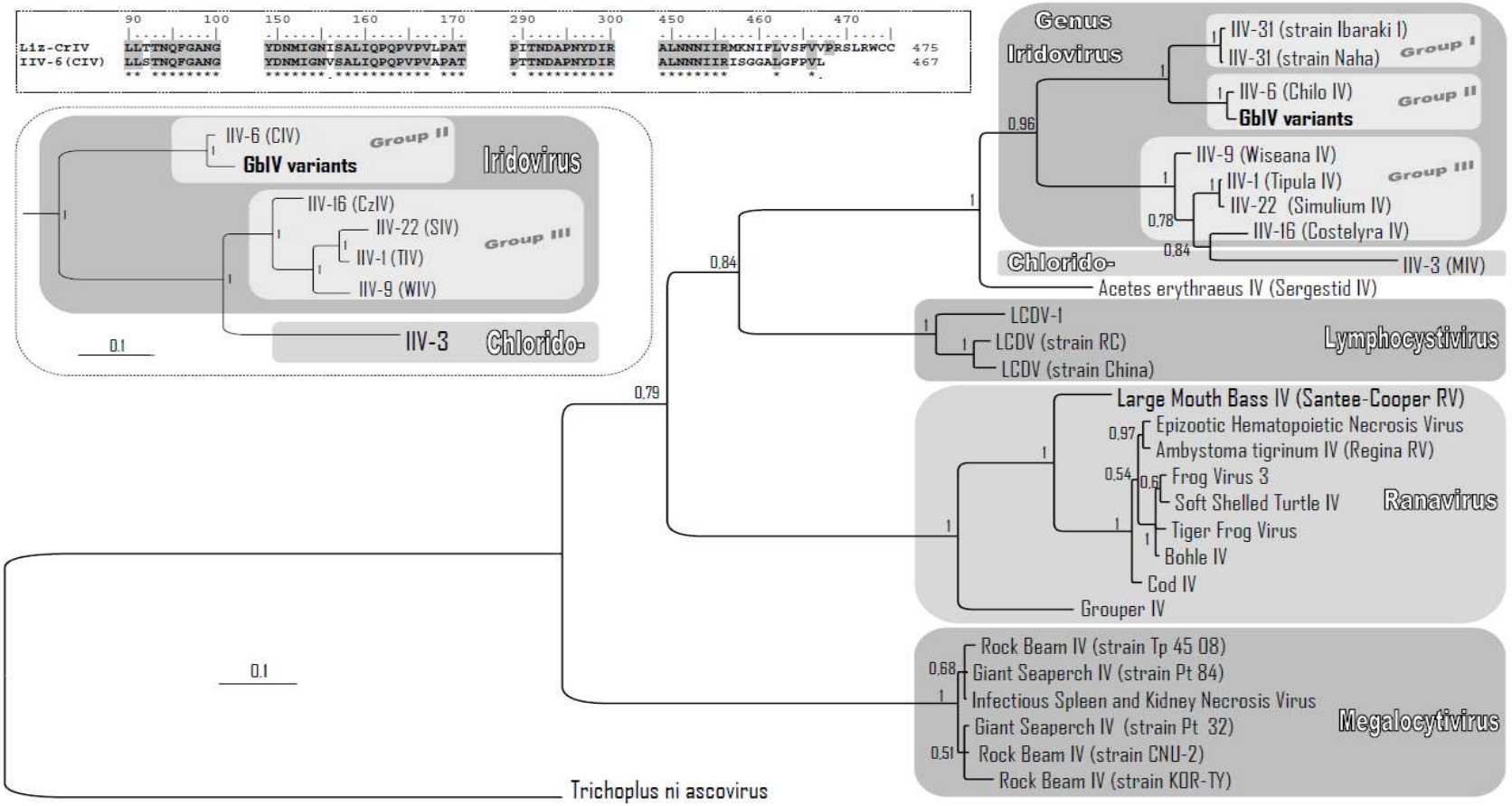

Figure 27. Phylogenetic tree reconstructions to resolve relationship of our IIV isolates (GbIV variants; in bold face). Bayesian tree calculated from partial (347 aa) alignment of the MCPs. In the middle left inserted box, a part of the tree calculated from complete MCP sequence (less available GenBank data) to demonstrate proportional differences of GbIV/CIV compared to IIV-1/IIV-22. A partial alignment of GbIV/CIV capsid proteins is shown top left inserted (asterisk means identical, dot indicates conserved positions). Posterior probability values are listed on the branches. An ascovirus was used as an outgroup. Note that the genus *Iridovirus* is apparently a paraphyletic group on the trees, and together with *Chloridovirus* member IIV-3, they form a monophyletic cluster.

We have found lower (44-98% & 46-100%) sequence identity across the examined genes. In addition to point mutations, patches of insertion and deletion were found in three of the fully sequenced genes, and evidence for recombination from other distantly related IIVs was found in two fragments. These latter ones either indicate actual recombination events or they are ancestral remnants of a non-coding region with low selection pressure. Phylogenetic tree reconstruction based on MCP gene data has shown that GbIV-variants and CIV form group II within the *Iridovirus* genus, with an observed distance similar to that between IIV-1 and IIV-22, two separate types. This distance exceeds that of variants of the same type, e.g. IIV-31 in group I (Fig 27). A phylogenetic tree based on DNA polymerase gene data showed similar results (not shown). Nucleotide sequence dissimilarity of GbIV-variants and CIV in this region (3.1%) resembles that of distinct species in the genus *Ranavirus*, e.g. frog virus 3 of amphibians (FV3) and the epizootic haematopoietic necrosis virus (EHNV) of fish (2.6%).

The species concept in virology is not straight-forward, but defined as a polythetic group (Regenmortel, 1992) which has its demarcation criteria determined for each genus. In the *Iridovirus* genus these criteria are listed as: amino acid sequence analysis of MCP (no limits established), RFLP, DNA-DNA dot-blot hybridisation, and serology (Jancovich et al., 2011). The latter two have not been applied to characterise cricket iridovirus(es), but RFLP showed that GbIV was distinct from all other iridoviruses (Kleespies et al., 1999). Our analysis of the complete MCP gene sequences indicated higher dissimilarity to CIV than those of earlier reports (Kleespies et al., 1999; Just & Essbauer, 2001; Jakob et al., 2002b) of partial MCP sequence, due to the mutations/insertions found at the 3' end. Thus, based on the considerable genomic differences described here, we suggest that GbIV be considered as a separate type of group II invertebrate iridoviruses (species *IIV-6)* and our IIV isolates as variants of this type. Despite the presence of several insertions, deletions, and recombination sites throughout the genome of GbIV-variants, all of the isolates studied are closely related or identical to one another in the studied genomic regions. Since the isolates studied were obtained from six different species, including lizards and arthropods over the course of six years, this indicates that GbIV has evolutionarily stable variants circulating in Germany.

Evolution of viruses is achieved mainly by two processes: On one hand, random point mutations accumulate in the genome and eventually lead to a pool of virus mutants from which the most successful pathogen is selected by its capability to multiply best in a particular host organism. On the other hand, major alterations occur in the viral genome by recombination, deletions, or insertions and can result in a virus

with completely different properties in a very short period of time (Shackelton & Holmes, 2004; Villarreal & Witzany, 2010). The limited number of mutation changes in the nucleotide sequence that we observe in the analysed genes are compatible with the hypothesis that the cricket iridovirus in question evolved relatively recently from CIV.

7.2.3. Comparison of three IIV isolates in a cricket bioassay

In the cricket bioassays, our aim was to compare the infectious characteristics of three different IIV isolates originating from three distinct hosts. The experiments were performed at two different regulated temperatures (20 & 30ºC). Due to technical reasons, not all the planned experiments could be perfectly accomplished in the planned two repetitions. Nevertheless, the correlations and differences revealed in this study are interesting (Table 13, for details see Suppl. Fig. 1 in Appendix).

We have found rates of mortality varying between 15 and 60%, which was like those seen in the cricket bioassays with the chameleon IIV isolate (Table 3, Ir.iso.1) performed earlier in our laboratory at unregulated temperatures (Weinmann et al., 2007). The mortality was much lower than the 93% reported by Kleespies et al. (1999) using the same infection method and a cricket isolate. However, the initial titre used by Kleespies et al. was five fold higher (2.2×10^{11} particles/ml), then the suspension used in our study, as they had propagated it in a more permissive insect cell line.

Mortality rates, however, are not the most representive measures for the rate of infection. Mortality also occurred in the negative control groups (especially at the higher temperature) as well as among individuals in the infected group which later tested negative. The developed new qPCR enabled a quick quantitation of viral particles in the infected crickets. Considering the appearance of qPCR results of up to 2 copies/µl in the negative control animals (data not shown) and by comparing the qPCR and nPCR data of the positives, the ratio of possible false positives could be kept at a minimum by disregarding the qPCR results below 5 copies/µl. This can be used as a guideline for the diagnostic samples in the future.

The so called “patently” infected crickets with very high virus loads and most often showing blue iridescence had accordingly high virus copy numbers: 10^7 to 10^{10} in the new qPCR, while the so-called covertly infected animals had considerably lower (10^2-10^4) copy numbers in their fat bodies. In the patently infected crickets it was possible to detect viral nucleic acid, using ISH, in the cytoplasym of the infected cells. If we compare the ratio of patently infected crickets and their mean survival time (MST) with the different virus isolates used, we find considerable differences (Table 13).

These data indicate that there might be differences in the pathogenecity of the different isolates (lower pathogenecity of the lizard isolate), but the repeated infection studies with the same isolate under the same conditions also varied significantly, so the observed differences between isolates could be due to interassay variability rather than true differences between the isolates. This was also consonant with the finding of an earlier study (Weinmann et al., 2007), when the infection rate greatly differed between 3 repeats of the same infection trial (Table 13). Based on the temperature data measured in that earlier study, it was hypothesized that greater variation in temperature adversely influences virus growth in crickets. However, according to our data, the higher temperature does not seem to affect the ratio of patently or covertly infected crickets, but slightly shortens the mean survival time (MST) of the infected animals. 30ºC, which is the temperature reported as the highest temperature at which CIV replication is still possible in *in vitro* systems (Williams et al., 2005), does not adversely affect these lizard-cricket IIVs in their replication *in vivo*.

The ratio of covert infection with middle range IIV load (isolation on reptilian cell-lines possible, qPCR measured copy numbers >100) was surprisingly low (0-10%), thus did not considerably vary between temperatures and isolates. This finding is again consonant with the results the crickets infection studies with the chameleon isolate at unregulated temperatures (Weinmann et al., 2007).

7.2.4. Bearded dragon transmission study

Koch's postulates could not be sufficiently fulfilled in this animal infection trial. None of the bearded dragons included in the study showed any signs of clinical disease during the course of the study. Virus was detectable by isolation and PCRs from the oral and cloacal swab of the infected animals from the 1st week on after the beginning of infection, while the negative control lizards remained negative in these tests.

The early death of lizard "A5", the smallest animal included in the study, could not be connected to the infection by iridovirus. The successful re-isolation of virus from every tissue and the positive nPCR results suggested viremia which was partly supported by the repeated qPCR results with the later established, re-evaluated controls. In the ISH and IHC tests, no viral DNA or proteins could be detected. In the EM examination, solitary viral particles could be found only, but no viral assembly sites could be detected, unlike in the the case of the infected crickets (Weinmann et al., 2007).

In the gastrointestinal tract, the large intestine (colon) showed the highest virus load both by virus isolation and qPCR, which is logical if we consider possible remnants of the infected food-crickets, despite the 2 weeks of vegetable diet at the end of the study. Relatively high virus loads were detected by qPCR (and virus isolation) from the proximal portions of the digestive tract of some survivors which is a rather surprising finding. Other organs (lung, heart, liver, skin) also had unexpectedly high virus loads (by qPCR) and in some cases the virus was also isolated.

These findings are new compared to the results of an earlier transmission study conducted at our laboratory (Weinmann, 2007) where the virus could be detected in these organs by nPCR only. But these results are in harmony with the findings in routine diagnostic lizard samples analysed in recent years in our laboratory (Table 4, 11) or other laboratories (Just et al., 2001) where these organs were sources for IIV isolation from diseased lizards, and skin seemed to be more affected. However, no macroscopic or histological changes could be detected in any of these organs, and the other detection methods (ISH, EM) were also unable to detect IIVs in the tissues. The yellowish friable liver, as sole pathological finding, resulted from glycogen depositing and lipidosis, might indicate hepato-toxic effect of the viral proteins, as it had been recorder in mice injected with CIV (Lorbacher de Ruiz et al., 1986).

Comparing the virus loads (according to qPCR data) of these lizard tissues to those found in patently infected crickets, these values are in a 3 to 6 fold lower range, which could explain the difficulties encountered in finding the virus in the tissues *in situ*, as the sensitivities of these tests are lower than the sensitivity of e.g. virus isolation (Weinmann et al., 2007). The elaborated IHC test seemed to give aspecific signals in several samples of the negative control animals as well, thus its results were omitted during the analysis.

7.3. Paramyxovirus studies

7.3.1. Studies on three genes of rPMV isolates

Although a number of different RT-PCRs have been described to detect squamatid PMVs (Ahne et al., 1999; Franke et al., 2001; Kurath et al., 2004), data so far has indicated that the RT-PCR targeting the L gene encoding the RNA dependent RNA polymerase, described by Ahne et al. (1999), is apparently the most sensitive and most broadly reactive. Our results were in concordance with these earlier comparisons between PCRs. The original HN gene RT-PCR amplified snake and lizard PMV

genome fragments only and gave weak bands in several cases (data not shown). Using the newly designed consensus HN gene primers (Suppl. Table 3 in Appendix), stronger bands were obtained in the RT-PCR, but the isolate from the tortoise was still not detected, indicating that the HN gene of this isolate differs considerably from that of squamate PMVs. This may be related to the function of the HN gene which encodes a surface glycoprotein with a more variable sequence than the internal RNA polymerase encoded by the L gene. The L gene has been shown to be the most conserved of the Newcastle disease virus genome as well (Wise et al., 2004).

We have successfully detected the U gene in all of the squamate PMVs used in this study, supporting the theory that this gene may be a distinguishing feature of these viruses (Kurath et al., 2004). The primers published by Kurath et al. (2004) were able to detect the squamatid PMVs of group “A” only. The use of the new consensus primers in a nested system (U-cons Fwd & Rev out, followed by U-cons Fwd & Rev in, Suppl. Table 3 in Appendix) led to a more broadly reactive RT-PCR.

The possible early translational start codon as described for the U-genes of FDLV, Gono-GER85 and Biti-CA98 isolates by Kurath *et al.* (2004), were missing in all of our isolates. Two consensus candidate genes could be outlined in the aligned U gene sequences (Fig. 24); one (ORF-1) coding a more variable (Table 14), but uniform length (82 aa) polypeptide, and a second one (ORF-2) which differs in length between the group “A”, “B” and “C” viruses (143/142/143-144 aa), but which has a lower sequence variation, more comparable to those of the partial L and HN proteins.

Unfortunately, no U gene sequence was detected in the tortoise PMV isolate (THer-GER99), although 4 additional consensus primers were designed based on the determined new squamatid sequences (Suppl. Table 3 in Appendix) and tested in various combinations with the previous primers. The unspecific amplicon that we obtained from the F gene is due to the fact that the second round primers attached partly to the transcription signals (stop-IGR-start) which are highly uniform among the different genes of FDLV. This motif was shown to be conserved with other genera of the subfamily (Lamb & Parks, 2007). The lack of specific U gene amplicon for the tortoise PMV may be rather due to a lack of the gene in this virus than to considerable sequence differences in the primer binding sites, since a number of consensus nested primers with low stringency conditions were tested. At the same time, the highest variability of sequences was observed in this gene from the three studied ones (Table 14). Thus we would extrapolate a 50-60% nt and 60-70% aa sequence identity between the hypothetical U gene of the tortoise PMV and that of the squamatid PMVs, and priming site mismatches cannot be completely ruled out.

7.3.2. Chelonid and squamatid ferlaviruses; novel grouping

Up to now, occurrence of PMVs has been described in the snake (sub)families: Boidae, Colubrinae, Elapidae and Viperidae. Our study included the first field isolate characterised from a homalopsid snake (Papp et al., 2012a). This study was also the first to partiallly characterise PMV isolates from varanid, xenosaurid and iguanid lizards, and from a chelonian host (Marschang et al., 2009). All sequence information obtained in the study indicated that ferlaviruses of snakes and lizards are closely related, while the tortoise isolate (THer-GER99) differed from the others, although part of a monophyletic cluster with the squamatid isolates (Fig. 23). This cluster could be identified as the genus *Ferlavirus* within the family *Paramyxovirinae*.

Since PMVs, among reptiles, are most commonly detected in snakes, these viruses have been called ophidian PMVs (oPMV). Phylogenetically, snakes clearly nest within the squamates, so that lizards are not monophyletic among the reptiles (Vidal & Hedges, 2005). Turtles, on the other hand, are monophyletic. Studies based on analysis of nuclear DNA-coded proteins have shown that turtles are the sister group to a monophyletic cluster of archosaurs (birds and crocodiles) (Iwabe et al., 2004). No PMVs have been described from crocodilians so far. PMVs from birds are found in the genera *Avulavirus* and *Metapneumovirus* (Lamb et al., 2005). The PMVs isolated from reptiles to date have all grown at temperatures below the body temperature of birds or mammals (Clark et al., 1979; Blahak, 1994). The same was true for the tortoise PMV used in this study. This is a possible common denominator among these reptilian PMVs, as they all cluster together on a single branch and appear to be monophyletic, although their hosts are not.

Based on our findings, the term: “ophidian PMVs” is inappropriate for the denomination of these viruses, as similar types appear in lizards and tortoises as well. First, we had suggested the use of the broader term: “reptilian PMVs” (rPMV) (Marschang et al., 2009), however, this is not appropriate if we consider the very recent studies from Australia (Hyndman et al., 2012a, 2012b) describing a new type PMV in snakes, which is phylogenetically very distant to these rPMVs. This virus was called Sunshine virus and clusters as a separate node between the *Paramyxovirinae* and *Pneumovirinae* subfamilies, very distant from the Fer de Lance virus (FDLV). The FDLV was nominated as the type species for the proposed genus *Ferlavirus* (Kurath, 2009) within the subfamily *Paramyxoviridae.* The new genus *Ferlavirus* has now been accepted by the ICTV (http://www.ictvonline.org/virusTaxonomy.asp?version= 2011). Thus, to avoid misunderstanding, the name “ferlavirus” is preferred to be used as a collective name for the candidate members of this genus as well.

The first molecular based (L and HN gene) classification of squamatid ferlaviruses by Ahne et al. (1999) described two distinct ‘‘subgroups’’ with very low inner variance, called ‘‘a’’ and ‘‘b’’ and a set of more variable intermediate isolates (Fig. 22). Later, Franke et al. (2001) described three clusters (A, B and C) on their L gene phylogenetic tree (not shown). Unfortunately, this latter publication did not include sequences from the earlier paper in the comparison, thus the denominations of their clusters do not refer to those of the subgroups (‘‘cluster A’’ corresponded to the earlier ‘‘subgroup b’’, ‘‘cluster B’’ to the ‘‘intermediate isolates’’, and ‘‘cluster C’’ to the earlier ‘‘subgroup a’’). This discrepancy in the names was resolved in our proposed grouping (Marschang et al., 2009; Abbas et al., 2011; Papp et al., 2012a). Considering the seniority rule in nomenclature, and the high bootstrap monophyletic clusters of the L, HN and U gene trees, a revised grouping for the squamatid ferlaviruses was suggested. A *sensu lato* group ‘‘A’’ contained the previous ‘‘subgroup a’’ and the old and new ‘‘intermediate isolates’’, while the *sensu lato* group ‘‘B’’ those contained those from ‘‘subgroup b’’ and similar squamatid PMVs. The lately described two field isolates from Germany and Hungary (PanGut-GER99 and HoBuc-HUN09) were equally distant from members of the previous groups, and a new group ‘‘C’’ was established from them (Fig. 23). The single tortoise isolate (THer-GER99), being apparently the most ancient branching offerlaviruses, is regarded as the first representative of a supposed separate chelonid group. Delineating dissimilarity values for these groups are listed in Table 14.

The close relationship between squamatid ferlaviruses from such divergent snake and lizard hosts indicates that these viruses may cross species barriers relatively easily. There were no epidemiological relationships among the isolates used in this study. They were all obtained from different collections at different times and, in several cases, from different countries. None of the studied squamate ferlaviruses clustered according to geographical origin or year of isolation. There was also no clear relationship between clinical signs observed in the host (Table 5) and clustering of the isolates. Many isolates were clearly associated with respiratory signs, while others were not, particularly Xeno-USA99 from a healthy xenosaurid lizard from Mexico. A number of studies have shown that captive and wild caught lizards may frequently have antibodies against PMVs, indicating that infections in lizards may be more common than observed clinical signs (Gravendyck et al., 1998; Marschang et al., 2002b; Lloyd et al., 2005).

7.3.3. PMV survey in snakes and a tortoise; multiple infections

Based on the above observations with the PMV isolates, we set up a more comprehensive survey with snake samples arriving to our Stuttgart laboratory. Virus isolation and nested RT-PCR targeting the L gene were used as a primary selective tool, and an additional RT-PCR to detect the HN gene was performed with the positive samples (Papp et al., 2010a).

PCR screening based on the L gene was positive in a total of 50 samples (24.6% of the 203 tested samples) from 28 (27.5%) different animals (Table 15). Cell culture isolation was attempted with all samples, but could isolate PMVs from three animals only (10.7% of PMV positive animals). Reoviruses were isolated from another three cases among the PMV positive animals and four times in the PMV negative ones (Suppl. Table 6 in Appendix). Nested RT-PCR for the HN-gene was also carried out with 47 of the L gene PCR positive diagnostic samples, and gave products of specific size in 16 (34%) cases only (Table 15). No connection was found between the characterised L gene sequence type and the reactivity of the HN gene PCR. It is assumed that the lower sensitivity of this PCR is not due to priming site mismatches only.

Considering the best tissue for the PMV diagnostics, our data support that the lung, intestine and kidney “triad” is the best choice from dead specimens. These tissues were simultaneously examined from 11 PMV positive and 19 PMV negative animals. There was not a single case in which these tissues were all negative and another organ was positive in the L-gene RT-PCR. Since no internal control was used at that time to monitor RNA preparation or the presence of inhibitory substances in the preparations, this result may not reflect the actual virus content of tissues, still these data give hints to practitioners for sampling. For the purpose of diagnosis of PMV infection in dead snakes, the lung appears to provide the most reliable positive results (Blahak, 1995), while including of intestine and kidney may help lower the number of obtained false negatives (Papp et al., 2010a). From live snakes, swabs (and trachea washes) were positives in 12 of the 46 examined cases (26.1%). This prevalence in swabs, similar to that detected in tissue samples, demonstrates sensitivity of this nested RT-PCR method.

In a parallel study at the Clinic for Birds and Reptiles, University of Leipzig (Pees et al., 2010), swabs from 100 living boid snakes were tested for ferlaviruses by the same RT-PCR method and by serology (HI) from collected blood. In this study, the prevalence of virus infection was measured much lower (9%) with a seroprevalance of 4.6% and there was no significant correlation between the two. Another similar RT-

PCR survey has been carried out at the Veterinary Medical Research Institute of the Hungarian Academy of Sciences, in Budapest (Szilvia L. Farkas, partly published data). Pooled organs of dead snakes (n=62) were tested, of which 3 animals (4.8%) were positives only (Papp et al., 2011). These striking differences in the measured prevalence of ferlavirus infection despite the same method might be due to several reasons. One explanation can be that the animals tested in the Stuttgart laboratory mainly originated from collections with known positive PMV history. This preselection must have shifted our numbers higher, and thus our study can be representative for this kind of collection only. The animals for the Leipzig study were collected according to opposed criteria, healthy specimen with negative viral disease history. However, they still measured a higher prevalence than the Budapest study from unselected dead specimens. This can either show differences between the two localities, or be the result of differences in preanalytical sample storage or RNA preparation methods (Tsui et al., 2002; Deng et al., 2005). Nevertheless, the higher number of positive animals in our tests and the separate treatment of different organs at the same time, enabled us to sequence characterise numerous ferlaviruses.

Thirteen different partial L gene sequences (“sequence variants”) were obtained from the 38 selected PCR amplicons, of which six cluster to “group A”, while the rest cluster to “group B”. Eight sequences were identical to earlier characterised isolates (Ahne et al., 1999; Franke et al., 2001; Marschang et al., 2009). Five new sequence variants were also identified, although these were also closely related to earlier described ones (Fig. 25). These new sequence variants could have also occurred due to failure by non-proofreading Taq polymerase or due to quasispecies diversity of virus strains. At the same time, the sequence variation represented here is comparable to those between earlier reported isolates. In order to correctly interpret these differences, a comprehensive study should be done using a proofreading DNA polymerase to reveal quasispecies diversity in the L-gene of ferlaviruses, similar to studies carried out with other PMVs (Kattenbelt et al., 2006).

Several cases, when amplicons from different tissues of the same animal or amplicons from different animals in the same collection were analysed, indicated that snakes can be infected with more than one ferlavirus during an outbreak (Table 15, Fig. 24). The multiple infection was also proven by cloning PCR-amplicons in two cases, where the direct sequencing results resembled a mixed sequence pattern. In the lung of a garter snake (Col-4) two distinct sequences could be identified, while in a ball python (Pyt-2) five different sequences were found in the kidney, belonging to two distinct cohorts of sequence variants. It was not proven, but very probable, that the cohorts represent single virus strains and the sequence variants represent part of the

quasispecies heterogeneity within the strains. Considering this phenomenon the cases of another dead garter snake (Col-3) and an Indian python (Pyt-6), where different sequences were obtained from different organs, but with 1 or 2 non-silent nucleotide changes only, can be regarded as heterogeneity of sequences within one virus strain. Ahne et al. (1999) have already proposed that members of “classical” subgroups “a” and “b” could be regarded as various strains in two distinct virus species. However, as the official demarcating criteria for strains, serotypes and virus species have not yet been determined in the new genus *Ferlavirus*, we have chosen to use the neutral “sequence variant” term. (At present, officially the genus *Ferlavirus* has a single accepted species: *Fer de Lance paramyxovirus*.)

In some cases, identical sequences were obtained from different animals of the same collection (Vip-2a & -2b & -2c) and from different organs of the same snake (Col-3 lung & kidney; Col-6 lung & kidney; Pyt-2 lung & liver; Pyt-4 lung, kidney & intestine; Vip-4 lung & intestine). This finding is more consistent with that of earlier publications (Ahne et al., 1999; Franke et al., 2001) where identical sequences were obtained from isolates from different animals in the same outbreak or population. However, virus isolates do not fully represent the original samples. Enrichment of one virus against the other in the case of multiple infection and adaptation to cell culture through continuous quasispecies-variant competition and selection during the isolation process can largely modify the final consensus sequence of an isolate compared to that of the original sample (Sauder et al., 2006).

This was the first study to characterise ferlaviruses directly from clinical samples of snakes. We found evidence for concurrent infection with several strains at the same time within populations and even within animals. Using the sequence data, suspected transmissions could be traced - best example in the collection is marked with one asterisk in Table 15: Col-2, Vip-2, Vip-4. Here three live asp vipers from a total of 8 live and one dead, kept in the same enclosure with 2 lineated ratsnakes (collectively Vip-2) tested PMV positive, all with the same sequence pattern (“variant B VI”). A dead mountain Mang pit viper from the same owner (Vip-4) two years later was also PMV positive with a slightly different pattern of the same strain (“B VII”, 0.4/0.6% nt/aa difference). The source for these infections could have been the leopard snake collection (Col-2) of the same owner, where half a year before the asp viper case a similar virus (“B VIII”, 0.2/0.6%) was detected from one of the survivors (Col-2a). However, another survivor (Col-2b) and the dead leopard snake (Col-2c) had differing PMV strains (variants “A I” and “B I”), suggesting a co-infection of the leopard snake population with three strains (two virus species?) at the same time, from which one could be passed over to the vipers.

In a separarate study, a surprising finding was the presence of multiple squamatid ferlaviruses in a leopard tortoise (Table 15, Fig. 25; “Geo”; Papp et al., 2010b) with severe pneumonia. This case was consonant with our finding of multiple PMVs in snakes, and also contributed to the hypothesis that a host switch from a squamatid to a chelonian can happen. The tortoise belonged to a group that had been imported from East Africa to Germany. Two years later several animals started to die with rhinitis, conjunctivitis and emaciation. The examined animal had lethargy and respiratory distress. Although bacteriological testing revealed the presence of several possible pathogens, antibiotic therapy was unsuccessful. Viruses commonly detected in tortoises include herpesviruses, picorna-like viruses (Marschang, 2000) and ranaviruses (Chen et al., 1999; Mao et al., 1997; Marschang et al., 1999; Johnson et al., 2007; Blahak & Uhlenbrok, 2010), or rarely AdVs (Farkas and Gál, 2009; Rivera et al., 2009). None of these viruses could be detected in this animal. The samples were tested for PMV only because all other tests were negative, not because this seemed to be the likely diagnosis, as descriptions of PMVs in chelonians are very limited (Oettle et al., 1990; Zangger et al., 1991; Marschang et al., 2009). RT-PCR targeting the L gene of ferlaviruses resulted in specific amplicons from the liver, heart, small intestine and cloacal samples. Since no histology was carried out in this case, it is not possible to clearly correlate the presence of ferlaviruses with specific changes in these tissues. It is interesting to note that no virus was detected in the lung of the tortoise, although this tissue was most severely affected and the lung is the most commonly positive organ in snakes (see above). It is possible that secondary bacterial infection led to rapid RNA degeneration, with a resulting negative RT-PCR despite the presence of PMV in the lungs. It is, however, also possible that the ferlaviruses were not directly involved in the observed disease, but was present due to immune suppression of the animal.

The sequencing results show that this tortoise was infected with several different PMVs, all of which were identical or very similar to known sequences of squamatid isolates (Fig. 25). Three sequences (small intestine, liver, heart) cluster to “group B” whereas the cloacal sequence clusters to “group A” of the squamatid ferlaviruses. Concerning sequence heterogeneity, at least two different ferlavirus species were present in different organs of the same animal. Both species were relatives of squamatid ferlaviruses, and not of the supposedly host specific chelonid PMV isolate (THer-GER99). Although the clinical impact of the PMV on the leopard tortoise is not clear, this finding underlines the apparent lack of strict species specificity of the squamatid ferlaviruses that appear to be able to infect a wide range of reptilian hosts.

8. New scientific results

1. Describing two novel squamatid adenoviruses, altogether five reptilian AdV types, that all proved to be members of genus *Atadenovirus*; supporting the theory of the coevolution and squamate origin of atadenoviruses.

2. Isolating the first pure lizard AdVs in culture, and determining their partial genome sequence; revealing the existence of two fiber genes in atadenoviruses for the very first time.

3. Establishing new methods (qPCR, ISH) for the detection of invertebrate iridoviruses (IIVs) in lizards and prey insects, and applying these methods in transmission trials.

4. Comparison of partial genom segments (14 kb) from 15 genes of IIV isolates from lizards, a scorpion and a cricket; finding mutations, insertions/deletions, and two spots with evidence for recombination compared to the type species CIV; proving a need for taxonomic revision and showing widespread presence of stable variants circulating in Germany.

5. With a transmission study, demonstrating that even the administration of high loads of IIV does not cause the clinical signs in bearded dragons, previously attributed to these viruses.

6. Molecular characterisation of novel paramyxovirus isolates from snakes, lizards, and (the first time) a tortoise; supporting their classification as members of the new genus *Ferlavirus*; the tortoise ferlavirus being the most ancient branch, while the other squamatid isolates separating into a new and two redefined groups. The genus characteristic U gene was undetectable in the tortoise isolate.

7. Describing fifteen ferlavirus sequence variants with surprisingly high (27.5%) prevalence, and (the first time) revealing concurrent infection with more than one PMV types in snake specimens and/or populations and in a leopard tortoise.

9. References

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10. Publications

10.1. Related articles

Weinmann, N., Papp, T., Alves de Matos, A.P., Teifke, J.P., Marschang, R.E.: **Experimental infection of crickets (*Gryllus bimaculatus*) with an invertebrate iridovirus isolated from a high-casqued chameleon (*Chamaeleo hoehnelii*),** J. Vet. Diag. Invest. 19, 674-679. 2007. IF: 0.984

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12. Appendix

12.1. Primers used in the study

Suppl. Table 1. Primers used for adenovirus studies.

Primer name	5' – Sequence – 3'	Reference
AdHex 4F	YTNTGGAAYCARGCNGTNGAY	Parkin et al., 2009
AdHex 5R	RTCNGGNARRTACATNGCNACRTT	
Adeno-pol Rout	GTDGCRAANSHNCCRTABARNGMR	Wellehan et al., 2004
Adeno-pol Fout	TNMGNGGNGGNMGNTGYTAYCC	
Adeno-pol Fin	GTNTWYGAYATHTGYGGHATGTAYGC	
Adeno-pol Rin	CCANCCBCDRTTRTGNARNGTRA	
Sn-HelodAdV 3723 F	YGTRCAKGCWGGRAACCKACC	this study
Sn-HelodAdV 5208 R	TAGGCRGGGAAARARRYCACG	
Sn-Helo AdV 5045 F	GAAMGCTCTGCTCCATGCTAG	
Helo AdV 5849 R	CAGTRGCAATGGCACGCTTCC	
Sn-Helo AdV 17063 F	CARMGRTGYTCYATCCAGTGG	
Helo AdV 20534 R	TGCCCCTTCKAGCTGGCGRTGCTG	
Sn-Helo AdV 21161 F	GTTCTAYYCTGARGAYTTYCACC	
Sn-Helo AdV 23888 R	CCTTYSTCATTKWSYAGCTSTTC	
Sn-Helo AdV 22450 F	CCKCCTTCMKCYTACAGMYTRGG	
Sn-Helo AdV 25303 R	TCTSCCAACTCTYTRCARTGC	
Sn-Gila AdV 24809 R	TSTRTKCACCTKTGTGACTTGCC	
SnHeloAdV 17579 F	AACATGGGYACTCTSACWGAC	
SnHeloAdV 20044 R	TTYAGCTCKGCMATCAGGYTSAC	
SnGilaAdV 24451 R	TKAAYYTGCCTGATAAGKTGGG	
SnHeloAdV 26051 F	TTTTCAARSCTGRYGYWGGGGC	
SnHeloHAdV 26819 R	CRAGAYRKGYCWAYGYTRAMTYCC	
SnGilaAdV 23180 R	AAWGGRTASAYGGGRTCTGGG	
Sn-HeloH 18139 F	TGYACCTGTGCTGGGTCTTGCGG	
Sn-HeloH 19681 R	TGCTGAGYAGGTTTTGAMGYTC	
Sn-HeloH 22482 F	AAGYTSACMAGAGAYGCCMTGCC	
Sn-HeloH 23392 R	GTSTYTCCKSTTTKMTGMARAGG	
Sn-HeloH 23691 F	TGGACWWTGGSCCAGGGTTGAC	
Sn-HeloH 24636 R	CCSACRCAGTAGTGRGCCACAT	
Sn-HeloH 24545 F	CAGTTRGTYCAAGGWAGRGGA	
Sn-HeloH 25306 R	TGYCACTGCARYTCTSCCAACTC	
Sn-Gila 23723 F	GGGTTGACMRTRGTSAATGGAA	
Sn-Gila 24535 R	AAGACTGTYTWYCAATTYRACTG	
Sn-Gila 21802 F	GGAAAYTWCGGRRYTCCTTCGTAC	
Sn-Gila 22415 R	TGCYARCTGCTCYCCYTCYGCTG	
Sn-Gila 18244 F	GASARAARAGYGGMATGGTRCC	
Sn-Gila 19623 R	TTCTRTCTYTTGCAGACATGTC	
Sn-HelodAdV 22132 F	GCWGGAGCCCGCCARAACTACGG	
Sn-HelodAdV 25469 R	CSYTKCAYGGYTYWTGTCCTCC	
Sn-HelodAdV 21543 F	AGCAGCYTGGACGGAGARCC	
Sn-HelodAdV 26166 R	GYRTYCTTYTMGCKTGYCAC	
Sn-HelodAdV 16270 F	AAYATYCCMGCWGTRGAGATG	
Sn-HelodAdV 21278 R	TCTCKGATGAGAGCCAATAGT	
Sn- AdV 23XXX F	CGGGAGTCTTGGAGTCCTAC	

Suppl. Table 2. (continue next page) Primers used for iridovirus studies.

Primer name	5' – Sequence – 3'	Reference
MCP-F1	GGTTTCATCGATATCGCCAC	Jakob et al., 2002b
MCP-F3	GGGCCGGAGATTATTTGTT	
MCP-R3	GACAATAGCATAGTTACCCCA	
MCP-R4	GAAAAGTAATCACTGCCCAT	
012L-R1	ATATGACCATTCCCAATCTGGAAC	
012L-F1	GGTTGATGATACAGCAGAATGGTC	
037L-R1	TGATCCTAAAAAATGTTTCGCCAC	
037L-F1	GAAGGAAGAGGAGCGTTAGCT	
075L-R1	TCATGGCATCCAAATTTCATATCTC	
075L-F1	GTCAGACACACAAGAATATAGCAT	
205R-R1	CTACAATTCAGCAATCTCCTTTCC	
205R-F1	TATTTCATCAAGGACAAATGTTTACC	
393L-R1	TTGAACCATTCATGTAATTCAAGCAA	
393L-F1	ATAAGTGGATTATTACAACTCATCGA	
IIV-012L 1010F	CAGATAACAATGGTTTGGGT	Spann, 2010; Papp et al., 2012c
IIV-012L 1036F	TCTTGTCAGGACGTAGTCTT	
IIV-012L 1642R	AATCAGAACTATCTGGAGGA	
IIV-012L 274F	GGAGTAGCTCCAAGAAGTAA	
IIV-012L 938R	GGAATATGTGGCAAGAAATC	
IIV-012L 262R	AACTGCAACTGCAACATC	
IIV-012L 436R	GGTTTGTTCTTCTACACG	
IIV-012L 975F	TGCAACTTCGTCATCATC	
IIV-012L 39F	TGTTCAATTCCGTGAAG	
IIV-012L 77F	ACAAGTTGGACGTCTTCTAG	
IIV-012L 744R	GCATAAATTCGGTTCCTG	
IIV-012L -237F	TCTAGCAGAAGCTTTGTGT	
IIV-012L 265R	GGAGCTACTCCGTCGATAC	
IIV-012L 501R	TTTGTGGAGCATCGGTNGT	
IIV-012L1582F	GCAACTTCGTCATCATCC	
IIV-012L 2196R	GGAACTGCAACTGCAACA	
IIV-012L -218F	TCAGAGAAACGTTCTTCA	
IIV-012L -27F	AGTTAAAGAAGATTAGTA	
IIV-012L 239R	TTGTATAACCATTCTGTC	
IIV-012L -1250F	GCTTTAACAGAACTTGCTGC	
IIV-012L 285R	TGGAGCTACTCCGTCGATACA	
IIV-012L 1532F	ACGACTGAGCCCCAGGAAAC	
IIV-012L 2863R	GTGGCGTTAAAACAACCCGTT	
IIV-037L 1577F	AATTATAGAGGTGAAGTGTT	
IIV-037L 2241R	TTCTTATATGTTCTTGTTCC	
IIV-037L 2455R	AACACACATTACACTCTTAC	
IIV-037L -12F	GTACATTATAAAATGGAG	
IIV-037L 715R	TTACCATCTTCAGAATATAC	
IIV-037L 1199F	CATATGGAACAACAGATT	
IIV-037L 1737R	GTTGACCATGAACATGTA	
IIV-037L 1639F	AGTGTTTTGGTAGCCGACAT	
IIV-037L 2742R	TGAATCGGTATCCCCATACA	
IIV-037L 3418R	CTTGACAAGGCAGTCGTTCT	
IIV-037L 2025F	ATGGGATGATCATGTTGCTTGT	
IIV-037L 2970F	GCGGAAAGGATGGAAATGTT	

Suppl. Table 2. (continued)

Primer name	5' – Sequence – 3'	Reference
IIV-037L 4084R	CTCATGGTCCTGCTTGGTCA	this study; Papp et al., 2012c
IIV-149L 61452F	AAGCATAGTAGAATCGTTTGCG	
IIV-155L 62425F	GCTTGTCTAAATTGAGGATCGG	
IIV-155L 62625R	CAGTGTAGATAATCATGTATTC	
IIV-157L Rev1	GATCTAATTTAAATGTATCC	
IIV-157L Rev2	GACTTTATCGATTTCCATTG	
IIV-158L 63444F	CGATTTCCATTGTAGATACAATG	
IIV-158L 63466R	CATTGTATCTACAATGGAAATCG	
IIV-159L Fwd1	CTGAAACGGTTATTACATGG	
IIV-159L Fwd2	CAAATGCTACATCTGCAGCTGG	
IIV-159L 64726R	CWATTACWGCTGGAGAATTAGC	
IIV-159L 64757F	CCGTAATTGTTCCTGCTGC	
IIV-159L 64795F	CCAGTAATTGTTCCTGCTGCG	
IIV-159L 65421F	CATTATTGTTAGCAGCGGATG	
IIV-160L 65107F	GCACAATTTCCAGAACAACAACC	
IIV-162L 66728R	CGTATACGGTTTACACTCCAC	
MCP 127908F	GGACAAGAAGAAAGCGCTTCTAC	
MCP 129276R	CCTAACTACGATATTCGCTTCTCTC	
MCP 128609F	GTAACAGAAAGTGGATATGATATAG	
MCP 128918R	GTATTGTACCAACTGCAAGCC	
MCP 129980F	GTTAGCTGATCCGTTTGCTCTAG	
MCP 130721R	GTTCTGTAAGATATGATTTTGCTAC	
MCP 127908F	GGACAAGAAGAAAGCGCTTCTAC	

Suppl. Table 3. Primers used for paramyxovirus studies.

Primer name	5' – Sequence – 3'	Reference
HN-1F	AAATCCTGCAGTACCGTGGCA	Ahne et al., 1999
HN-2R	AGATATCTGTGTAAACTCCTG	
HN-3F	GCACTCTACCCCAGTGTTGG	
HN-4R	TTCTATCCAGCTTATTCTTAT	
L-gene 5F	GCAGAGATTTTCTCTTTCTT	
L-gene 6R	AGCTCTCATTTTGTATGTCAT	
L-gene 7F	TAGAGGCTGTTACTGCTGC	
L-gene 8R	CATCTTGGCAAATAATCTGCC	
U-gene F	ATGTCACACCCCCATCAA	Kurath et al., 2004
U-gene R	CCACCCTGTTATCAACCA	
rPMV U-cons Fwd-out	ACCARYYATRRRCHTGYVRARATG	Marschang et al., 2009
rPMV U-cons Rev-out	GTTWGCCATTMTTACTGGATCTC	
rPMV U-cons Fwd-in	RARATGATTAAAGAAAACCTAGG	
rPMV U-cons Rev-in	ATCTCAGATACCTTTGATCCTAAG	
rPMV U-cons F1	ATGGARRNRGACMYGARRGAG	
rPMV U-cons R2	CKYCTRTATATAGNACTNGACC	
rPMV U-cons F3	TCRATGTGGAGACTGGTGAG	
rPMV U-cons R4	TTAGATAAATTCNCCAGTCTC	
rPMV HN-cons Fwd-out	AATGTYRTRGARGATGARAG	
rPMV HN-cons Rev-out	CRAGRCCWGGKAACRRBCCYTG	
rPMV HN-cons Fwd-in	CRAGRCCWGGKAACRRBCCYTG	
rPMV HN-cons Rev-in	ATAWCCYGTATTYWCTAYCC	

12.2. Accession of GenBank retrieved sequences

12.2.1. Adenoviruses

Agamid AdVs (AY576678, AAY83284, DQ077706, FJ196811 to FJ196819, HQ005515),

BAdV-1 (YP_094032),
BAdV-2 (AP_000006),
BAdV-3 (AP_000026),
BAdV-4 (AAK13183),
BAdV-4 Rus (not yet released),
Bat AdV (AB303301),
CAdV-1 (AAB05434),
CAdV-2 (AAB38716),
Chameleonid AdV (AY576679),
DAdV-1 (AP_000080),
Eublepharid AdV (AY576677),
FAdV-1 (AP_000410),
FAdV-5 (DQ159938),
FAdV-9 (AC_000013),
Frog (AAF86924),
Gekkonid AdV (AY576681),
HAdV-9 (CAI05960),
HAdV-12 (CAA51882),
HAdV-17 (AP_000143),
HAdV-40 (AAC13953),
HAdV-52 (ABK35035),

HAdV-B=HAdV-3 (ABB17778) + HAdV-7 (AP_000539) + HAdV-35 (AAN17476)

HAdV-E=HAdV-4 (AAS66917) + SAdV-25 (AP_000304)

Lizard AdV 1 (AAS89696),
MAdV-1 (AP_000342),
OAdV-7 (AAD45950),
PAdV-3 (AB026117),
PAdV-5 (AAK26504),
Parakeet AdV (EU056825),
SAdV-1 (AAX19399),
SAdV-3 (AAT84618),
Scincid AdV (AY576682),
Snake AdV 1 (AAL89790),
Sturgeon AdV (AY082701),
TAdV-3 (AAC64523),
Tortoise AdV (EU056826),
TSAdV (YP_068060)

12.2.2. Iridoviruses

ATV: AF080218,
Bohle IV: AY187046,
Cod IV: GU391284,
EHNV: AY187045,
FV3: DQ897669,
IIV-1: M39542,
IIV-3: NC_008187,
IIV-6: AF303741,
IIV-9: GQ918152,
IIV-16: AF025775,
IIV-22: M32799,
IIV-31(Ibaraki): AB686457,
IIV-31 (Naha): AB686462,
Sergestid IV: ABF82044,
LCDV-1: L63545,
LCDV (RC): AF103188,
LCDV (China): NC_005902,
Grouper IV: AY666015,
LMBIV: FR682503,
SSTIV: DQ335253,
TFV: AF389451,
RBIV (Tp4508): JF264352,
GSIV (Pt84): JF264354,
ISKNV: AB66996,
GSIV (Pt32): JF264346,
RBIV (CNU-2): AY849394,
RBIV (KOR-TY): AY532607,
Tricoplusia AV: AY197700

12.2.3. Paramyxoviruses

APMV-6=Avian PMV-6 (AY029299),
BVV (AF286043),
Canine distemper virus strain Onderstepoort (AF378705),
CrotX1-96 (AF349405),
CeraCe-98 (AF351137),
ElaGut-91 (AF349408),
FDLV (NC_005084),
GonoGER-85 (AF349404),
Hendra virus, AF017149,

HPIV-1 (AF457102),
Measles virus strain Edmonston (AF266289),
NDV = Newcastle disease virus (AF375823),
NTV = Neotropical rattlesnake virus (AF286045),
Mumps virus (NC_002200),
Nipah virus (NC_002728),
Pacific salmon PMV isolate SpeelWA00 (DQ172668.1),
Pacific salmon PMV isolate RinLyWA99 (DQ172679.1),
Pacific salmon PMV, isolate YakimWA03 (DQ172675.1),
Pacific salmon PMV isolate YaqOR82 (DQ172676.1),
Sendai virus (NC_001552),
SV-41=Simian parainfluenza virus 41 (NC_006428),
Tupaia paramyxovirus (AF079780)

The following sequences (Ahne et al., 1999) were kindly provided by Dr. Gael Kurath and Dr. William N. Batts (Western Fisheries Research Center, Seattle, WA, USA): Boa-CA98, Biti-CA98, Biti-GER 87, Call-GER88+, Crot1-VA95, Crot2-OH90, Crot3-CA98, Ela1-GER94, Ela2-GER93, Ela-FL93, Lamp-MD96, More-GER86, Pyth-GER88, Trim-MD97.

12.3. Cricket bioassay detailed results

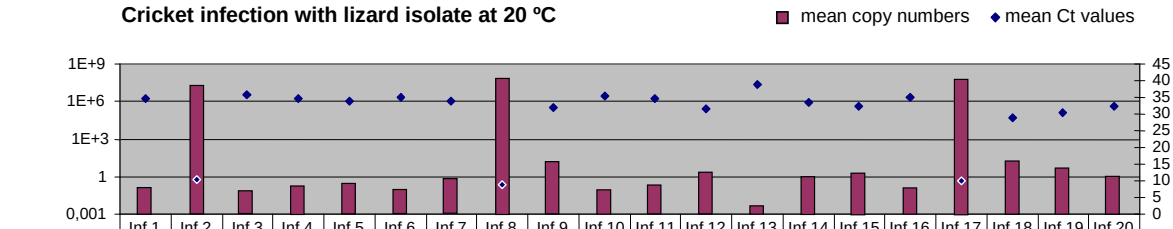
Supplementary Figure 1 (continue on next five pages): The figures section on the following pages have a top part showing a graph and a table showing the qPCR values recalculated for 20 mg tissue starting material for DNA extraction, and a lower table showing the evaluation of the qPCR, results of the nPCR and virus isolation.

The qPCR values for calculated copy numbers per µl template DNA were coded as follows: (+)= 1- 5 copies/µl, += 5-100 copies/µl. ++= 1xE2-1xE4 copies/µl, +++= 1xE4-1xE7 copies/µl, ++++= over 1xE7 copies/µl.

For nPCR results were interpreted on intensity of the second round MCP PCR products by electrophoresis, (+)= faint, += normal bands.

For virus isolation (+) means inconsistent results (contradicting in the repeats).

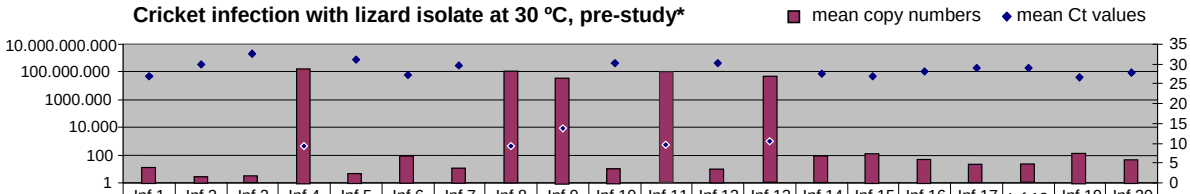
On the "survival" line, numbers indicate day of death post infection, or if marked with a star (*), then the day of euthanasia for "humane" reasons as the cricket was in a sublethal state. Smilies indicate survival until the end of study, (*) a sublethal state is marked with star. Abbreviations for ages: ad= adult, ny= nymph (last stage instar), la= larva (6th or 7th stage instar), ecd= in state of ecdysis, star (*) indicates visible malformation as shown on Figure 22.



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Mean copy No	0,159	2E+07	0,078	0,176	0,248	0,096	0,74	6E+07	14,53	0,099	0,194	1,852	0,005	0,841	1,355	0,142	5E+07	15,94	5,369	1,142
Mean Ct value	34,74	10,44	35,64	34,61	34	35,04	33,84	8,663	31,76	35,48	34,49	31,65	39,01	33,3	32,47	34,89	9,988	28,93	30,57	32,26

qPCR	—	++++	—	—	—	—	—	++++	+	—	—	(+)	—	—	(+)	—	++++	+	+	(+)
nPCR	—	+	—	—	—	—	—	+	+	—	—	(+)	—	(+)	(+)	—	+	+	+	(+)
Isolation	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—	+	(+)	—	—
Iridescence	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—	+	—	—	—
Survival (dpi)	☺	33	☺	☺	☺	☺	☺	35	55	☺	☺	59	☺	☺	59	☺	58	58	59	☺
Sex	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age at begin	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny
age at end	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

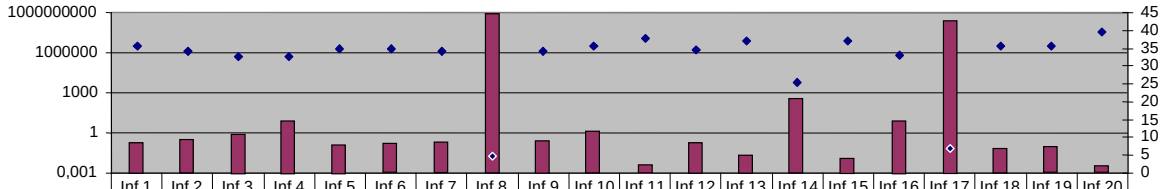
Cricket infection with lizard isolate at 30 ºC, pre-study*



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Mean copy No	11,063	2,6823	2,9659	2E+08	3,9926	95,999	13,08	1E+08	3E+07	9,0483	1E+08	9,5178	5E+07	69,839	122,72	43,992	22,705	22,116	149,55	59,228
Mean Ct value	26,97	29,823	32,685	9,1538	31,216	27,182	29,715	9,4215	13,902	30,199	9,6622	30,298	10,436	27,586	26,87	28,173	29,012	29,044	26,619	27,804

qPCR	+	(+)	(+)	++++	(+)	+	+	++++	++++	+	++++	+	++++	+	+	+	+	+	+	+
nPCR	+	—	—	+	—	+	—	+	+	—	+	—	+	+	+	(+)	(+)	(+)	+	+
Isolation	—	—	—	+	—	—	—	+	(+)	—	+	—	+	—	—	—	—	—	—	—
Iridescence	—	—	—	+	—	—	—	+	—	—	+	—	+	—	—	—	—	—	—	—
Survival (dpi)	☺	☺	35	26	☺	35	32	28	47	35	32	☺	☺	46	58	35	☺	☺	33	☺
Sex	♂	♂	♀	♀	♂	♀	♀	♀	♀	♀	♂	♂	♀	♀	♀	♀	♂	♂	♀	♂
age at begin	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad
age at end	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

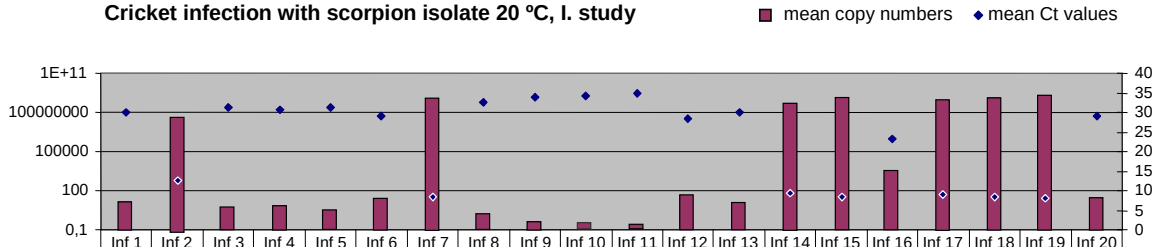
Cricket infection with lizard isolate at 30 ºC



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Mean copy No	0,153	0,265	0,779	8,27	0,131	0,162	0,227	8E+08	0,233	1,25	0,005	0,198	0,025	296,6	0,012	6,659	2E+08	0,075	0,085	0,004
Mean Ct value	35,63	34,1	32,74	32,61	34,98	34,72	34,29	4,815	34,26	35,47	37,86	34,47	37,06	25,25	36,96	32,94	6,72	35,7	35,53	39,41

qPCR	—	—	—	+	—	—	—	++++	—	(+)	—	—	—	++	—	+	++++	—	—	—
nPCR	—	—	—	+	—	—	—	+	—	+	—	—	—	+	—	+	+	—	—	—
Isolation	—	—	—	—	—	—	—	+	—	—	—	—	—	+	—	—	+	—	—	—
Iridescence	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	+	—	—	—
Survival (dpi)	☺	☺	☺	47	46	☺	☺	33	☺	56	☺	☺	☺	59	☺	54	40	☺	☺	☺
Sex	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age at begin	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny
age at end	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

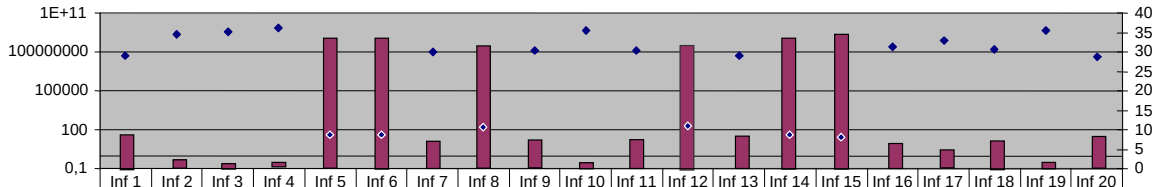
Cricket infection with scorpion isolate 20 ºC, I. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Mean copy No	15,37	4E+07	5,109	7,824	4,676	31,41	1E+09	1,403	0,473	0,401	0,234	60,7	13,67	6E+08	1E+09	4244	9E+08	1E+09	2E+09	35,77
Mean Ct value	29,9	12,49	31,2	30,69	31,3	29,06	8,301	32,71	33,99	34,19	34,82	28,29	30,04	9,321	8,352	23,3	8,929	8,424	8,138	28,91

		Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
qPCR		+	++++	+	+	(+)	+	++++	(+)	—	—	—	+	+	++++	++++	++	++++	++++	++++	+
nPCR		+	+	+	(+)	+	+	+	—	—	—	—	+	+	+	+	+	+	+	+	+
Isolation		—	+	—	—	—	—	+	—	—	—	—	—	—	+	+	+	+	+	+	—
Iridescence		—	+	—	—	—	—	+	—	—	—	—	—	—	—	—	—	+	+	+	—
Survival (dpi)		☺	52	☺	☺	☺	☺	31	46	☺	☺	33	☺	☺	31	37	☺	29	27	31	☺ *
Sex		♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age	**at begin**	ad	ad	ny	ny	ny	ny	ad	ad	ny	ny	ad	ad	ny	ad	ad	ny	ad	ad	ad	ny
	at end	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad*

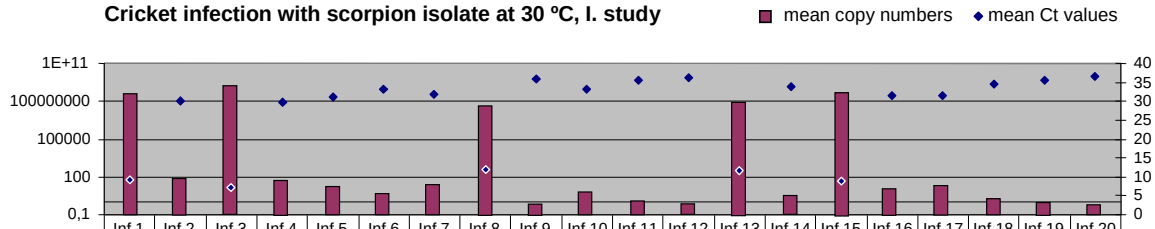
Cricket infection with scorpion isolate 20 ºC, II. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Mean copy No	36,07	0,292	0,174	0,145	1E+09	1E+09	14,52	2E+08	12,17	0,141	10,58	2E+08	28,12	1E+09	2E+09	5,269	1,364	8,281	0,131	38,57
Mean Ct value	28,9	34,56	35,17	36,15	8,821	8,814	29,97	10,55	30,18	35,41	30,34	10,88	29,19	8,795	7,976	31,16	32,75	30,63	35,5	28,82

		Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
qPCR		+	—	—	—	++++	++++	+	++++	+	—	+	++++	+	++++	++++	+	(+)	+	—	+
nPCR		+	—	—	—	+	+	+	+	+	—	+	+	+	+	+	+	—	(+)	—	+
Isolation		—	—	—	—	+	+	—	+	—	—	—	+	—	+	+	—	—	—	—	—
Iridescence		—	—	—	—	—	+	—	+	—	—	—	—	—	—	+	—	—	—	—	—
Survival (dpi)		☺	☺	☺	☺	22	41	☺	29	☺	☺	☺	18	18*	17	18	16	☺	☺	☺	☺
Sex		♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age	**at begin**	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny
	at end	ad	ad	ad	ad	ecd	ad*	ad	ad	ad	ad	ad	ecd*	ecd	ecd	ad*	ecd	ad	ad	ad	ad

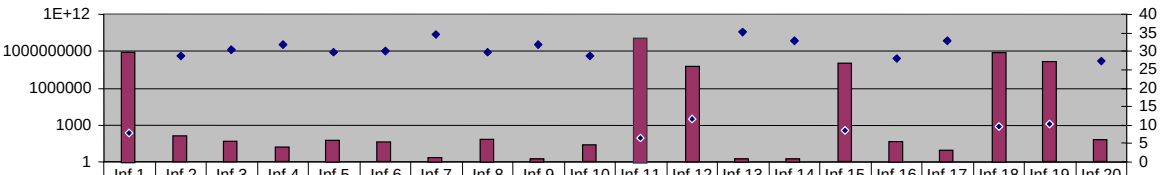
Cricket infection with scorpion isolate at 30 ºC, I. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Quantity Mean	3E+08	57,62	1E+09	67,64	25,5	6,209	18,31	4E+07	0,867	6,306	1,094	0,634	6E+07	3,512	4E+08	18,86	20,98	2,197	0,981	0,498
Ct Mean	9,268	30,12	7,157	29,9	31,22	33,12	31,66	11,95	35,77	33,1	35,45	36,19	11,48	33,89	8,975	31,62	31,48	34,52	35,6	36,51

qPCR	++++	+	++++	+	+	+	+	++++	—	+	(+)	—	++++	(+)	++++	+	+	(+)	—	—
nPCR	+	+	+	+	+	(+)	(+)	+	—	+	(+)	(+)	+	—	+	(+)	+	+	—	—
Isolation	+	—	+	(+)	—	—	—	+	—	—	—	—	+	—	+	—	—	—	—	—
Iridescence	—	—	+	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—
Survival (dpi)	11	53	19	52	☺	57	50	52	☺	☺	☺	☺	21	57	19	☺	☺	☺	46	53
Sex	♂	♀	♀	♀	♂	♀	♀	♀	♂	♂	♂	♂	♀	♀	♂	♂	♂	♂	♀	♀
age at begin	la	la	la	la	la	la	la	la	la	ny	ny	ny	la	la	la	ny	ny	ny	ny	ny
age at end	ny	ad	ny	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ny	ad	ad	ad	ad	ad

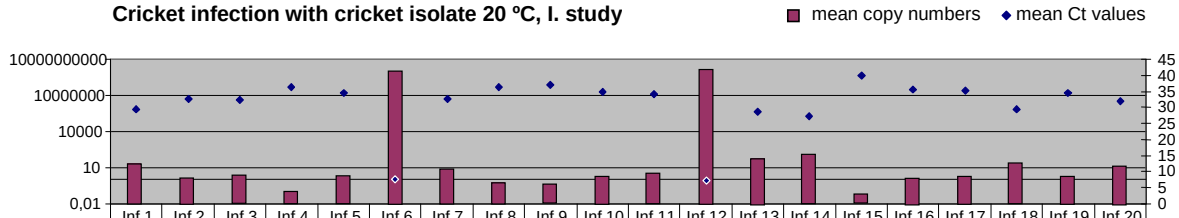
Cricket infection with scorpion isolate at 30 ºC, II. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Quantity Mean	9E+08	158,9	46,79	18,4	71,5	61,18	2,019	72,23	1,221	20,65	1E+10	5E+07	1,378	1,751	7E+07	31,71	8,464	6E+08	1E+08	75,63
Ct Mean	7,885	28,75	30,4	31,66	29,83	30,04	34,63	29,82	31,8	28,67	6,468	11,69	35,25	32,69	8,443	28,2	32,7	9,595	10,25	27,24

qPCR	++++	++	+	+	+	+	(+)	+	(+)	+	++++	++++	(+)	(+)	++++	+	+	++++	++++	+
nPCR	+	+	+	+	+	+	(+)	+	—	+	+	+	—	—	+	+	(+)	+	+	+
Isolation	+	+	—	—	—	—	—	—	—	—	+	+	—	—	+	—	—	+	+	—
Iridescence	—	—	—	—	—	—	—	—	—	—	+	+	—	—	—	—	—	+	+	—
Survival (dpi)	21	☺*	☺	☺	☺	30	30	☺	☺	☺	19	19	21	☺	59*	56	48	41	46	53
Sex	♀	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age at begin	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny*	ad	ad	ad	ny	ny
age at end	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ny*	ad	ad	ad	ad	ad

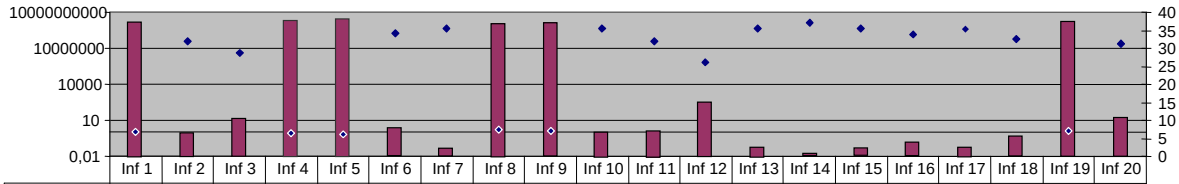
Cricket infection with cricket isolate 20 ºC, I. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Quantity Mean	26,792	1,8259	2,4679	0,098	2,2167	1E+09	8,8788	0,7068	0,4504	1,9416	3,3024	1E+09	46,568	147,11	0,0662	1,1906	1,6735	25,468	2,1428	12,457
Ct Mean	29,266	32,597	32,223	36,224	34,603	7,5436	32,505	36,332	37,013	34,803	34	7,2507	28,58	27,153	39,913	35,543	35,028	29,328	34,654	31,992

qPCR	+	(+)	(+)	—	(+)	++++	+	—	—	(+)	(+)	++++	+	++	—	(+)	(+)	+	(+)	+
nPCR	+	—	+	—	—	+	+	—	—	—	(+)	+	+	+	—	—	(+)	+	+	+
Isolation	—	—	—	—	—	+	—	—	—	—	—	+	—	(+)	—	—	—	—	—	—
Iridescence	—	—	—	—	—	+	—	—	—	—	—	+	—	—	—	—	—	—	—	—
Survival (dpi)	☺	☺	☺	☺	☺	**21**	☺	☺	☺	☺	☺	**49**	☺	☺	☺	☺	☺	**25**	☺	☺
Sex	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age at begin	ny	ny	ny	ad	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny
age at end	ad	ad	ad	ad	ad	ecd	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

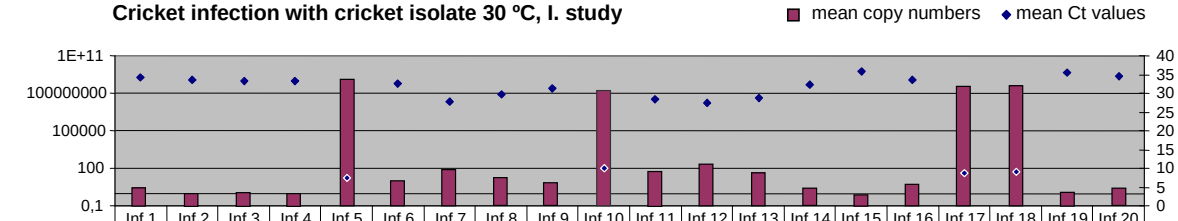
Cricket infection with cricket isolate 20 ºC, II. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Quantity Mean	2E+09	1,1869	19,224	2E+09	3E+09	2,6874	0,0426	1E+09	1E+09	1,1706	1,2303	369,36	0,046	0,0116	0,0446	0,1954	0,548	0,5244	2E+09	19,104
Ct Mean	6,9062	31,836	28,752	6,5862	6,2598	34,312	35,52	7,2676	7,2472	35,569	31,796	26,011	35,435	36,958	35,469	33,833	35,688	32,74	7,0066	31,346

qPCR	++++	(+)	+	++++	++++	(+)	—	++++	++++	(+)	(+)	++	—	—	—	—	—	—	++++	+
nPCR	+	+	+	+	+	(+)	—	+	+	—	+	+	(+)	—	—	—	—	(+)	+	+
Isolation	+	—	—	—	—	—	—	+	+	—	—	+	—	—	—	—	—	—	+	—
Iridescence	(+)	—	—	—	—	—	—	+	+	—	—	—	—	—	—	—	—	—	+	—
Survival (dpi)	**36**	☺	☺	**37**	**37**	**49**	**58**	**39**	**38**	☺	☺	**49**	☺	☺	☺	☺	☺	☺	**39**	**53**
Sex	♂	♂	♂	♂	♀	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age at begin	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny	ny
age at end	ad*	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

Cricket infection with cricket isolate 30 ºC, I. study



	Inf 1	Inf 2	Inf 3	Inf 4	Inf 5	Inf 6	Inf 7	Inf 8	Inf 9	Inf 10	Inf 11	Inf 12	Inf 13	Inf 14	Inf 15	Inf 16	Inf 17	Inf 18	Inf 19	Inf 20
Quantity Mean	2,6295	0,945	0,983	0,9756	1E+09	7,9966	92,882	19,923	5,3747	1E+08	58,494	247,24	41,792	2,66	0,9898	4,078	4E+08	4E+08	1,285	2,1428
Ct Mean	34,345	33,414	33,365	33,374	7,3717	32,663	27,724	29,633	31,258	10,069	28,297	27,474	28,714	32,13	35,822	33,681	8,8054	8,9157	35,428	34,654

qPCR		(+)	—	—	—	++++	+	+	+	+	++++	+	++	+	(+)	—	(+)	++++	++++	(+)	(+)
nPCR		+	—	—	—	+	+	+	+	—	+	+	+	+	—	—	(+)	+	+	—	+
Isolation		—	—	—	—	+	—	—	—	—	+	—	(+)	—	—	—	—	+	+	—	—
Iridescence		—	—	—	—	+	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—
Survival (dpi)		50	19	24	36	16	50	23	19	21	17	25	51	26	18	54	54	26	18	56	34
Sex		♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
age	**at begin**	ny	ad	ad	ad	ny	ad	ad	ad	ad	ny	ny	ny	ad	ad	ad	ad	ad	ny	ny	ny
	at end	ad	ad	ad	ad	ecd	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad	ad

12.4. Bearded dragon transmission study details

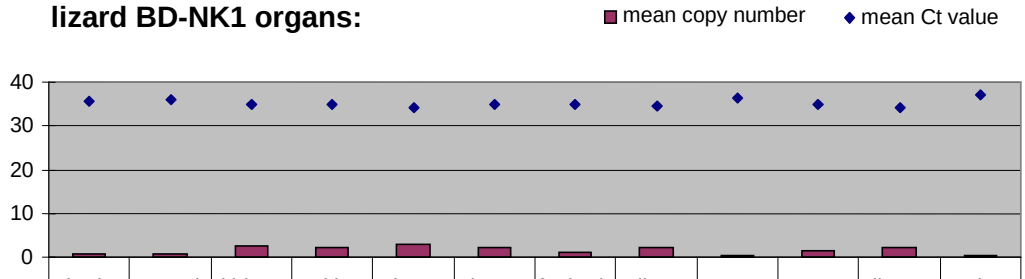
Suppl. Table 4: Bearded dragons included in the transmission studies.

Animal	Sex	Survival (60 dpi)	weight at (g)		length at (mm)		Coelomic inocoluate (ml)	Gastric inoculate (ml)
			begin	end.	begin	end.		
NK1	♂	living	30,6	43,3	95,0	106,5	1	1
NK2	♀	living	37,3	46,9	97,2	108,5	1	1
NK3	♂	living	11,4	16,1	67,5	75,5	1	1
NK4	♀	living	17,1	21,0	84,0	88,3	1	1
NK5	♂	living	11,5	13,6	66,8	70,6	1	1
A1	♀	living	23,4	26,6	85,3	91,0	—	1
A2	♀	living	25,0	31,4	92,2	92,7	—	1
A3	♀	living	9,8	12,6	64,5	69,8	—	1
A4	♀	living	12,0	16,2	71,0	76,8	—	1
A5	♂	† 16 days	5,7	5,1	53,0	53,0	—	0,8
B1	♀	living	28,1	36,4	96,5	107,2	1	1
B2	♀	living	10,8	12,2	62,7	65,4	1	0,9
B3	♀	living	8,7	9,3	57,6	59,8	1	1
B4	♂	living	18,9	25,3	85,2	92,0	1	0,9
B5	♂	living	23,8	27,2	88,7	91,9	1	1

Suppl. Figure 2 (continue next 5 pages): All of the figures segments on the following pages are divided into a top part with a graph and table showing the qPCR values recalculated for 20 mg tissue starting material for DNA extraction, and in the table below the results of the nPCR and virus isolation results are shown. (+) indicates a weak band for nPCR and inconsistent results (contradicting in the repeats) for virus isolation.

Negative control bearded dragons:

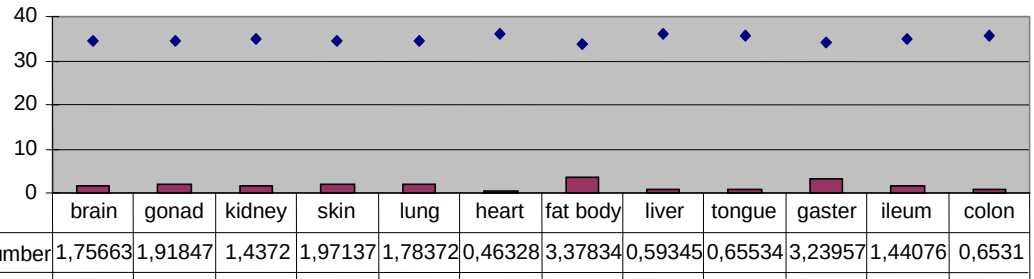
lizard BD-NK1 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	0,76625	0,63727	2,48949	2,15602	2,85199	2,19379	1,27941	2,19119	0,45466	1,55341	2,10567	0,18774
mean Ct value	35,6039	35,8151	34,8666	34,9817	34,0978	34,9681	35,0164	34,3998	36,2021	34,7941	34,0001	37,2157

nPCR	—	—	—	—	—	—	—	—	—	—	—	—
cell culture	—	—	—	—	—	—	—	—	—	—	—	(+)

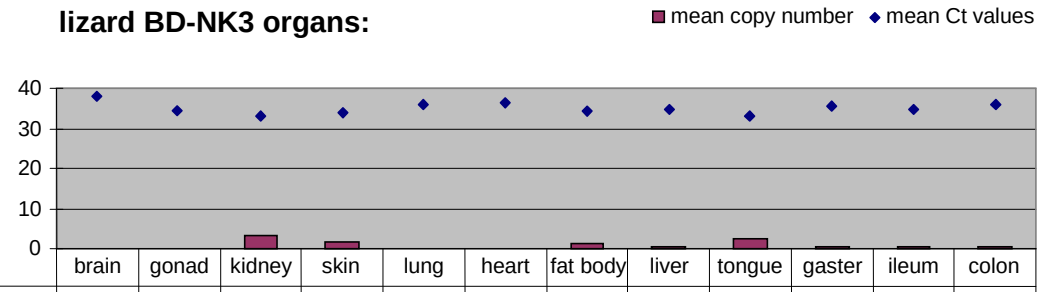
lizard BD-NK2 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	1,75663	1,91847	1,4372	1,97137	1,78372	0,46328	3,37834	0,59345	0,65534	3,23957	1,44076	0,6531
mean Ct value	34,6532	34,5522	34,8832	34,521	34,6356	36,1805	33,9037	35,8968	35,7831	33,9518	34,8803	35,787

nPCR	—	—	—	—	—	—	—	—	—	—	—	—
cell culture	—	—	—	—	—	—	—	—	—	—	—	(+)

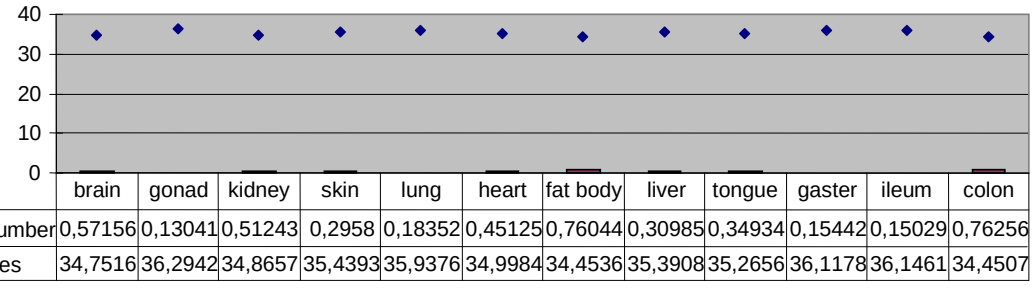
lizard BD-NK3 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	0,02996	undet.	3,09918	1,49828	0,15751	0,10863	1,02363	0,52267	2,52776	0,25996	0,5588	0,20852
mean Ct value	37,8296	>40	32,9869	33,7456	36,0971	36,485	34,1433	34,845	33,1996	35,5741	34,7752	35,8043

nPCR	—	—	—	—	—	—	—	—	—	—	—	—
cell culture	—	—	—	—	—	—	—	—	—	—	—	—

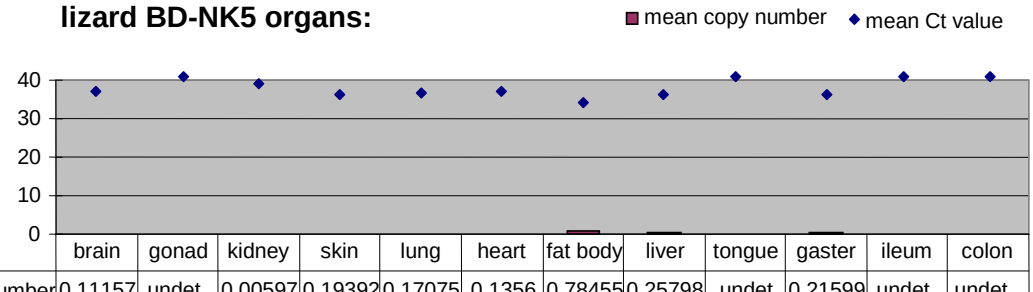
lizard BD-NK4 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	0,57156	0,13041	0,51243	0,2958	0,18352	0,45125	0,76044	0,30985	0,34934	0,15442	0,15029	0,76256
mean Ct values	34,7516	36,2942	34,8657	35,4393	35,9376	34,9984	34,4536	35,3908	35,2656	36,1178	36,1461	34,4507

nPCR	—	—	—	—	—	—	—	—	—	—	—	—
cell culture	—	—	—	—	—	—	—	—	—	—	—	—

lizard BD-NK5 organs:



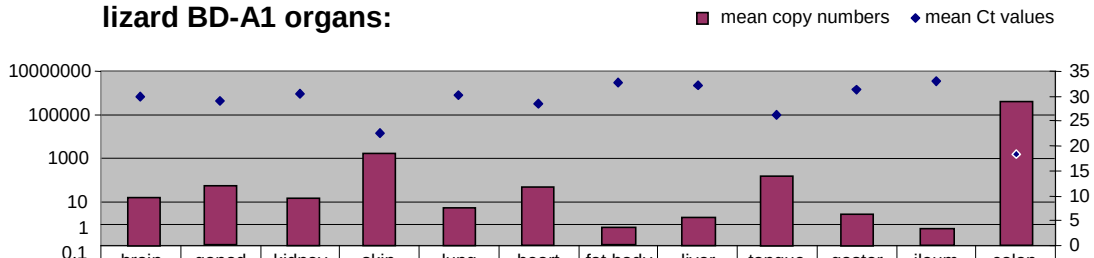
	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	0,11157	undet.	0,00597	0,19392	0,17075	0,1356	0,78455	0,25798	undet.	0,21599	undet.	undet.
mean Ct value	37,1425	>40	39,5816	36,402	36,4748	37,0259	34,3605	36,0581	>40	36,1586	>40	>40

nPCR	—	—	—	—	—	—	—	—	—	—	—	—
cell culture	—	—	—	—	—	—	—	—	—	—	—	—

“Single infected” bearded dragons:

Please note that the qPCR graphs have two different Y-axis scaling for the mean copy number /logarithmic/ and the mean Ct values /linear/.

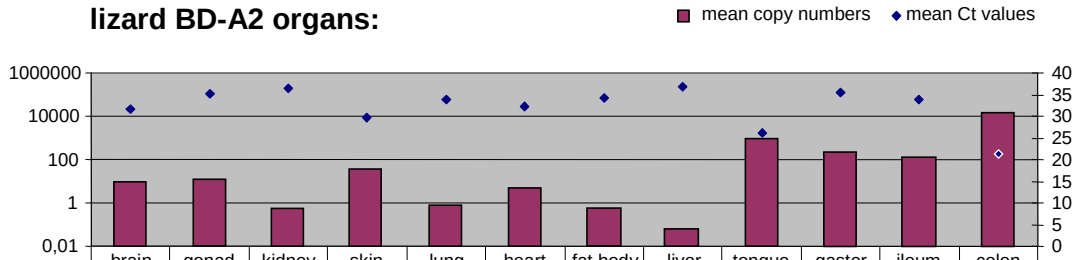
lizard BD-A1 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy numbers	17,33155	71,33109	11,38026	2431,141	7,027988	54,42955	0,895851	1,334234	138,9915	3,548637	0,77282	122223,5
mean Ct values	29,87605	28,94312	30,41985	22,58908	30,14686	28,39664	32,8098	32,29485	26,28859	31,24035	33,00078	18,42077

nPCR	+	+	+	+	+	+	—	—	+	+	—	+
cell culture	—	—	—	+	—	(+)	—	—	(+)	—	—	+

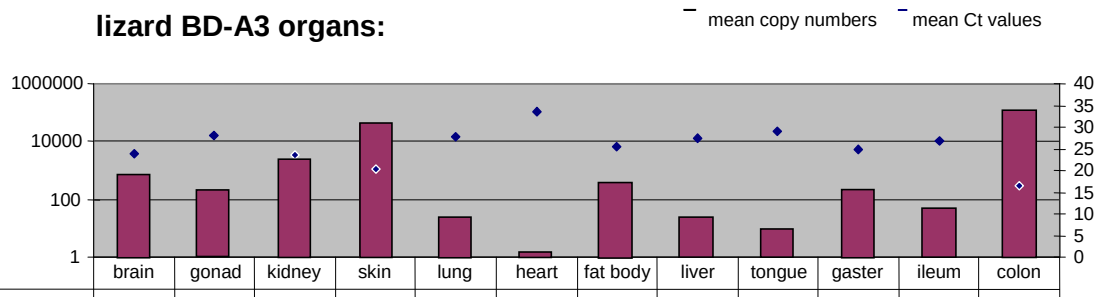
lizard BD-A2 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	10,53486	12,51816	0,535421	40,21178	0,934988	5,629609	0,736848	0,082606	766,2797	255,4266	127,7133	16194,93
mean Ct values	31,54414	35,19417	36,29218	29,8124	33,77917	32,3543	34,08707	36,78743	26,00187	35,53264	33,81247	21,16136

nPCR	+	—	—	+	—	—	—	—	+	+	+	+
cell culture	—	—	—	—	—	—	—	—	+	+	+	+

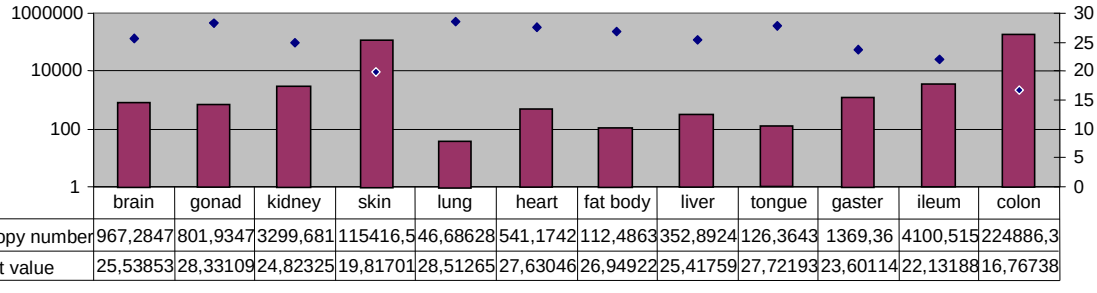
lizard BD-A3 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	836,6888	271,7185	3205,19	52269,95	24,05668	1,797944	484,032	28,12551	9,739005	267,391	56,41932	135959,2
mean Ct value	24,01612	27,99982	23,17293	20,4504	27,72127	33,41091	25,62544	27,51854	28,89444	24,73351	26,61539	16,5124

nPCR	+	+	+	+	+	—	+	+	+	+	+	+
cell culture	+	—	(+)	(+)	—	—	(+)	+	—	+	—	+

lizard BD-A4 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	967,2847	801,9347	3299,681	115416,5	46,68628	541,1742	112,4863	352,8924	126,3643	1369,36	4100,515	224886,3
mean Ct value	25,53853	28,33109	24,82325	19,81701	28,51265	27,63046	26,94922	25,41759	27,72193	23,60114	22,13188	16,76738

nPCR	+	+	+	+	+	+	+	+	+	+	+	+
cell culture	+	—	(+)	+	—	(+)	—	+	—	+	+	+

lizard BD-A5 organs:

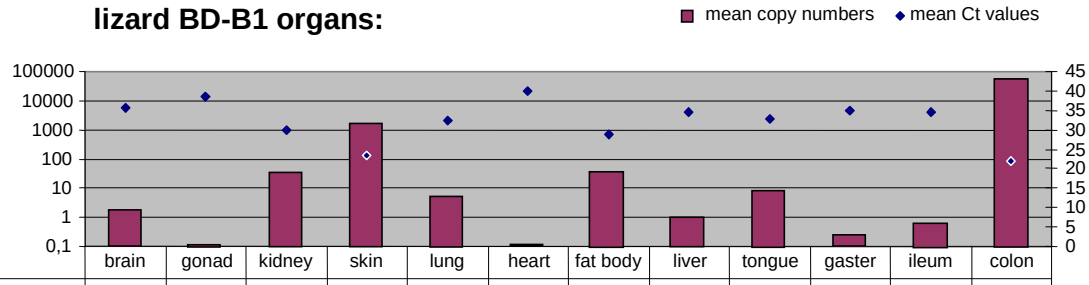
	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon	trachea
Quantity Mean	63,995	81,3966	100,488	465,979	1119,33	2790,60	135,44	6421,14	150638	111748	3108,3	3870119	13,002
mean Ct	29,4958	29,1865	28,9109	26,8958	26,2674	24,5476	28,513	23,6511	19,323	19,853	24,407	25,045	31,630

nPCR	+	+	+	+	+	+	+	+	+	+	+	+	+
cell culture	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	+	+	+	+	(+)

“Double infected” bearded dragons:

Please note that the qPCR graphs have two different Y-axis scaling for the mean copy number /logarithmic/ and the mean Ct values /linear/.

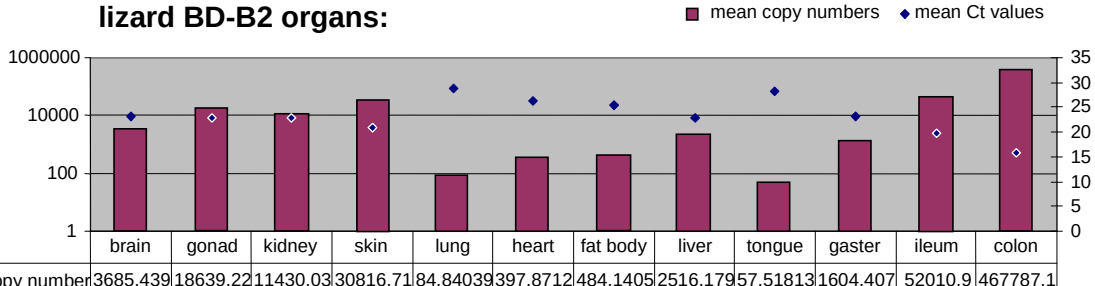
lizard BD-B1 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	2,068607	0,116842	35,93343	2108,226	5,721065	0,107455	43,07851	1,17953	9,383245	0,293217	0,628192	52041,57
mean Ct value	35,69173	38,49607	29,96328	23,51591	32,34272	39,84634	28,65036	34,45121	32,59034	34,75538	34,66544	22,09186

nPCR	(+)	—	+	+	+	—	+	+	(+)	—	—	+
cell culture	—	—	—	+	—	—	—	—	—	—	—	+

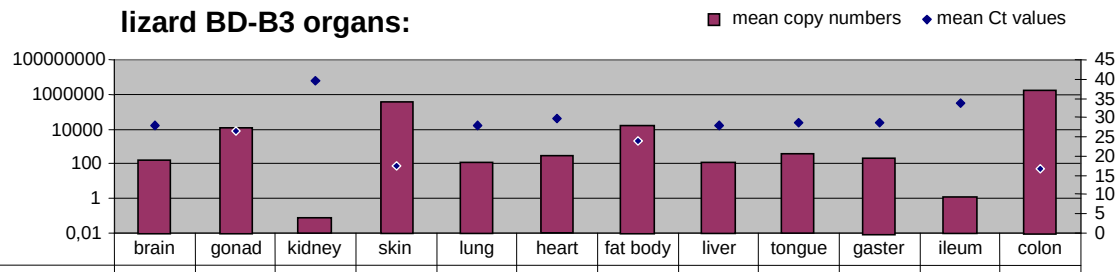
lizard BD-B2 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	3685,439	18639,22	11430,03	30816,71	84,84039	397,8712	484,1405	2516,179	57,51813	1604,407	52010,9	467787,1
mean Ct values	23,20341	22,88915	22,91463	20,90166	28,7988	26,18543	25,37937	22,78612	28,23314	23,26125	19,65734	15,78621

nPCR	+	+	+	+	+	+	+	+	+	+	+	+
cell culture	(+)	(+)	(+)	+	—	(+)	(+)	(+)	—	(+)	(+)	+

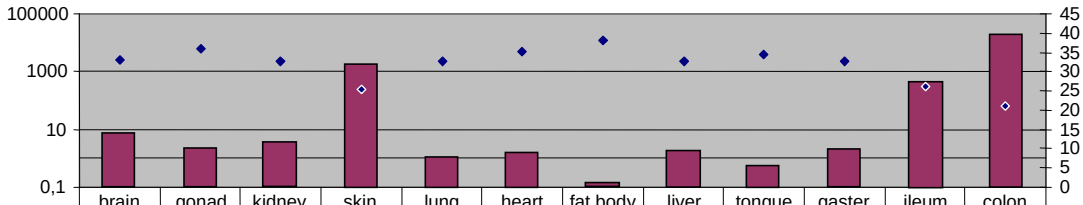
lizard BD-B3 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	157,0325	11753,08	0,07687	410447,3	115,2874	256,9836	17496,4	136,2962	430,2419	236,6456	1,387705	2195139
mean Ct value	28,07127	26,44616	39,56895	17,52804	27,79838	29,86099	23,97426	27,83133	28,53725	28,6946	33,89452	16,73113

nPCR	+	—	—	+	+	+	+	+	+	+	(+)	+
cell culture	+	(+)	—	+	(+)	—	—	(+)	+	+	—	+

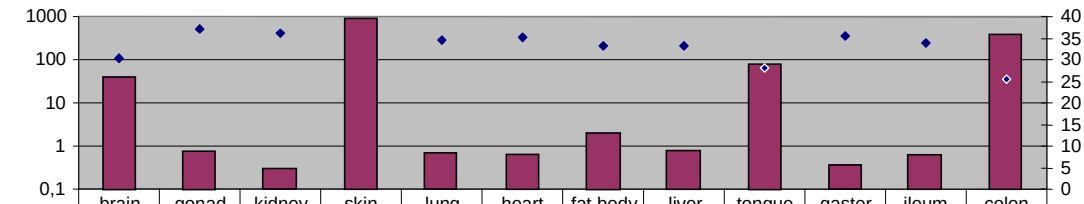
lizard BD-B4 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	9,187306	2,44995	3,948333	1738,796	1,120712	1,665637	0,148484	1,743048	0,581596	2,156099	502,7678	23303,19
mean Ct value	33,10958	36,03004	32,60687	25,43336	32,80478	35,37685	38,17025	32,7602	34,31198	32,69844	25,98483	20,94325

nPCR	—	(+)	(+)	+	+	—	—	+	(+)	+	+	+
cell culture	—	—	—	+	—	—	—	—	—	—	+	+

lizard BD-B5 organs:



	brain	gonad	kidney	skin	lung	heart	fat body	liver	tongue	gaster	ileum	colon
mean copy number	43,81467	0,818896	0,322029	935,0723	0,755868	0,707631	2,167637	0,85326	84,44505	0,389903	0,65162	448,0827
mean Ct value	30,43359	36,94032	36,10771	25,87417	34,64267	35,22318	33,34531	33,14054	27,98163	35,45784	33,97185	25,4272

nPCR	+	—	—	+	(+)	—	+	+	+	—	—	+
cell culture	—	—	—	(+)	—	—	—	—	(+)	—	—	+

12.5. List of negative samples

Suppl. Table 5. Diagnostic samples tested negative for AdV by nested PCR. (For positives see Table 2.)

Id.Nr.	Species	Sample type
2/06	star tortoise *(G.elegans)*	kidney, liver, lungs, genitals
3/06	bearded dragon *(P. vitticeps)*	liver
13/06	corn snake (*Pantherophis guttatus*	oral swab, cloacal swab
32/1-6/06	6 leopard snakes *(Zamenis situla)*	6 oral/cloacal swabs
32/06	leopard snake *(Zamenis situla)*	liver, pankreas, kidney, intestine, lungs
33/06	asp viper (*Vipera aspis*)	liver, pankreas, kidney, intestine, lungs
38/06	bearded dragon *(P. vitticeps)*	oral swab, cloacal swab
41/1/06	Mexican beaded lizard *(H. horridum)*	swab
41/2/06	leopard tortoise *(Stigmochelys pardalis)*	swab
41/3/06	leopard tortoise *(Stigmochelys pardalis)*	swab
50/06	rosy boa *(Lichanura trivirgata)*	liver, lungs, kidney, pankreas, small intestine
54/1/06	spiny-tailed lizard *(Uromastyx sp.)*	oral swab, cloacal swab
54/2/06	spiny-tailed lizard *(Uromastyx sp.)*	oral swab, cloacal swab
54/3/06	spiny-tailed lizard *(Uromastyx sp.)*	oral swab, cloacal swab
55/06	rainbow boa *(Epicrates cenchria)*	swab, liver
60/06	indian python *(Python molurus)*	liver, kidney, lungs, intestine
61//06/A2 to A9	8 asp vipers (*Vipera aspis*)	8 oral/cloacal swabs
66/06	indian python *(Python molurus)*	lungs, liver, kidney, intestine
67/06	bearded dragon *(P. vitticeps)*	oral swab, cloacal swab
68/06	bearded dragon *(P. vitticeps)*	cloacal swab
16/07	spiny-tailed lizard (*Uromastyx* sp.)	skin
18/07	spiny-tailed lizard (*Uromastyx* sp.)	oral swab, cloacal swab, crusts
24/07	rainbow boa (*Epicrates cenchria*)	lungs,kidney,intestine, trachea
62/1-2/07	2 african spurred tortoises (*Geochelone sulcata*)	2 livers

Suppl. Table 6 . Diagnostic samples tested negative for PMV by RT-PCR. (For positives see Table 6.)

Family	Identifying number Register number	Species name	Case history	Tissues tested	Comments, further findings (virus isolation, other PCRs)
Boidae	**Boa-4c to 4f** 24/2008	4 common boas *(Boa constrictor)*	PMV positive stock	four swabs	two animals were positive in this collection
	Boa-6a & 6b 18/2006	2 red tail boas (*Boa constrictor)*	respiratory disease, bad general condition	2 oral swabs	
	Boa-7 28/2006	rainbow boa *(Epicrates cenchria)*	respiratory disease	tracheal wash	
	Boa-8 50/2006	rosy boa *(Lichanura trivirgata)*	CNS signs	pancreas, liver, lung, kidney, brain, spleen, intestine	
	Boa-9 55/2006	rainbow boa *(Epicrates cenchria)*	CNS signs	liver, swab	
	Boa-10 24/2007	rainbow boa *(Epicrates cenchria)*	dehydration	liver, kidney, lung, trachea	adenovirus PCR positive
	Boa-11 38/2007	green anaconda *(Eunectes murinus)*	CNS signs	trachea, kidney, intestine	
	Boa-12 49/2007	common boa *(Boa constrictor)*	inclusion body disease (IBD)	liver	unspecified virus isolated
	Boa-13 50/2007	common boa *(Boa constrictor)*	stock PMV problem, inclusion body disease	kidney	
	Boa-14 51/2007	common boa *(Boa constrictor)*	stock IBD problem	trachea	
	Boa-15 52/2007	common boa *(Boa constrictor)*	stock IBD problem	kidney	
	Boa-16 83/2007	common boa *(Boa constrictor)*	anorexia, apathy, respiratory signs	blood, swab	
	Boa-17 29/2008	common boa *(Boa constrictor)*	anorexia, CNS signs	kidney, liver, lung, intestine, brain	
	Boa-18a & 18b 57/3 & 5/2008	common boa *(Boa constrictor)*	stock PMV problem	lung, kidney, intestine	adenovirus PCR positive
Colubridae	**Col-2d to g*** 32/2, 3, 5 & 6/ 2006	leopard snake (*Elaphe situla*)	PMV positive stock, samples from six living and one dead snake		The dead snakes and two living ones were PMV positive. *These animals are from the same owner as Vip-2 and Vip-4
	Col-7 76/2006	garter snake *(Thamnophis sirtalis)*	CNS signs	swab	

	Col-8 41/2007	corn snake *(Pantherophis guttatus)*	young animal	lung, intestine	reovirus isolated from lung and intestine
	Col-9 47/2007	garter snake *(Thamnophis sirtalis)*	stock PMV problem	lung, intestine, kidney	
	Col-10 3/2008	corn snake *(Pantheropphis guttatus)*	stock PMV problem	lung, intestine, kidney	reovirus isolated
	Col-11 23/2008	Amur ratsnake *(Elaphe schrenki)*	no report	lung, kidney, intestine	
	Col-12 30/2008	corn snake *(Pantherophis guttatus)*	stock PMV problem	lung, intestine, kidney	
	Col-13 57/4/2008	smooth snake *(Coronella austrica)*	stock PMV problem	lung, kidney, intestine	adenovirus PCR positive
Pythonidae	**Pyt-8b to 8m** 82/2007	12 ball pythons *(Python regius)*	PMV died animal earlier in stock	swabs[§]	Pyt-8a was PMV positive. Reovirus was isolated from Pyt-8c.
	Pyt-9 6/2006	ball python *(Python regius)*	pneumonia	swab[§]	
	Pyt-10 8/2006	ball python *(Python regius)*	CNS signs, torticollis	liver, pancreas	
	Pyt-11 60/2006	Indian python *(Python molurus)*	pneumonia	liver, kidney, lung, intestine	
	Pyt-12 71/2006	Green tree python *(Morelia viridis)*	stock PMV problem, pneumonia	kidney, liver, lung, oesophagus	
	Pyt-13 3/2007	white-lipped python *(Morelia viridis)*	died with CNS signs	oral swab, lung, intestine	
	Pyt-14 25/2007	ball python *(Python regius)*	endoparasitosis reported	lung, kidney, intestine	adenovirus PCR positive Reovirus isolated
	Pyt-15 28/2007	Indian python *(Python molurus)*	pneumonia	kidney, liver, intestine	
	Pyt-16 32/1/2007	ball python *(Python regius)*	accompanying animal (Pyt-5) died	tracheal wash	same enclosure as Pyt-5
	Pyt-17 53/2007	Indian python *(Python molurus)*	CNS signs, stomatitis	oral swab, cloacal swab	
	Pyt-18 55/2007	Indian python *(Python molurus)*	stock PMV problem	oral swab	
	Pyt-19 66/2007	Indian python *(Python molurus)*	dyspnoea	oral swab, tracheal wash	
	Pyt-20 78/2007	Indian python *(Python molurus)*	pneumonie, stomatitis	swab[§] tracheal wash	
	Pyt-21 4/2008	blood python *(Python curtus)*	pneumonia	kidney, lung, intestine	
	Pyt-22 5/2008	carpet python *(Morelia spilota)*	no report	kidney, lung, intestine	
	Pyt-23 27/2008	ball python *(Python regius)*	respiratory sypmtoms	tracheal swab	
	Pyt-24a 32/2008	carpet python *(Morelia spilota)*	stock PMV problem, 1 animal died	kidney, lung, intestine	
	Pyt-24b 32/2/2008	carpet python *(Morelia spilota)*		swab[§]	
	Pyt-25 37/2008	carpet python *(Morelia spilota)*	stock PMV problem	swab[§]	

	Pyt-26 41/2008	ball python *(Python regius)*	no report	kidney, lung, intestine	
	Pyt-27 43/2008	ball python *(Python regius)*	respiratory signs	oral swab	
	Pyt-28 47/2008	ball python *(Python regius)*	respiratory signs	oral swab	
Viperidae	**Vip-2d to i*** 61/2006-A1 to 5 61/2006-A7	6 asp vipers *(Vipera a. aspis)*	9 asp vipers & 2 lineated ratsnakes kept together in a PMV positive stock, one viper died.	intestine, liver, lung & kidney from the dead one (-2d) and swabs[§] from live ones were tested	*same enclosure as Col-2 and Vip-4, the dead viper was AdV positive but PMV negative.
	Vip-5 1/2007	meadow viper *(Vipera ursinii rakosiensis)*	CNS signs	cloacal swab	snake from Hungary[#]
	Vip-6 26/2007	Orlov´s viper *(Vipera orlovi)*	stock problem	intestine, lung, kidney	same owner as Vip-7
	Vip-7 27/2007	Asp viper *(Vipera aspis)*	stock problem	intestine, lung, kidney	same owner as Vip-6
Unknown	**Unsp-1** 46/2006	unspecified species	respiratory disease	tracheal wash	
	Unsp-2 79/2007	unspecified species	proliferative pneumonia	lung	