Tracking adenovirus infections in reptiles

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
Dr. med. vet.
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

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I hereby declare that I have completed the submitted doctoral thesis independently and without any unauthorised outside help and with only those forms of support mentioned in the thesis. All texts that have been quoted verbatim or by analogy from published and non‐published writings and all details based on verbal information have been identified as such. In the analyses that I have conducted and to which I refer in this thesis, I have followed the principles of good scientific practice, as stated in the Statute of Justus Liebig University Giessen for Ensuring Good Scientific Practice.

Inna Ball

To my parents and my life partner for their support and absolute faith in me.

Publications and presentations

Part of the work described here has been published in the following papers:

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Avian, Herpetological and Exotic Mammal Medicine (ICARE), April 20-26. Wiesbaden, Germany. p. 40.

Ball, I., Marschang, R. E. (2012) Establishment of an agamid cell line and first isolation of an adenovirus from a bearded dragon (*Pogona vitticeps*). 19th Annual Meeting of the Association of Reptilian and Amphibian Veterinarians. October 12-25. Oakland, USA. p. 11.

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Marschang, R. E., Funk, R. S., **Romanova, I.,** Ruckova, Z., Abbas, M. D. (2012) Detection of antibodies against adenoviruses and paramyxoviruses in native Arizona rattlesnakes. 61st WDA, 10th Biennial EWDA Conference „convergence in wildlife health“. July 23-27. Lyon, France. p. 214.

Abbas, M.D., **Romanova, I.,** Ruckova, Z., Öfner, S., Stöhr, A., Marschang, R. E. (2012) Concurrent infection of bearded dragons (*Pogona vitticeps*) in Germany with paramyxo- and adenoviruses. 22nd Annual Meeting of the Society for Virology. March 14-17, Essen, Germany, p. 187

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IV Abbreviations

°C	Degrees centigrade
α	Alpha
µl	Microliter
µm	Micrometer
A+T	Adenine-thymine content
A_{260}	Absorbance at 260 nm
aa	Aminoacids
Acc. No.	Accession number
AdV	Adenovirus
Agam-1 AdV	Agamid AdV-1
Anol-1 AdV	Anolis AdV-1
Anol-2 AdV	Anolis AdV-2
Anol-3 AdV	Anolis AdV-3
AtAdV	*Atadenovirus*
ATCC	American Type Culture Collection
BAdV	Bovine adenovirus
BDE	Bearded dragon embryos
Boa c. AdV	Boa constrictor AdV-1
Box-t AdV	Box-turtle AdV
BlueTM AdV	Blue tree monitor AdV
bp	Base pairs
C+G	Cytosine-guanine content
$CaCl_2$	Calcium chloride
Ch-1 AdV	Chameleon AdV-1
Ch-2 AdV	Chameleon AdV-2
cm^2	Square centimeter
CNS	Central nervous system
CO_2	Carbon dioxide
Com. a. AdV	Common agama AdV
CPE	Cytopathic effects
CsCl	Caesium chloride
CVUA	Chemisches und Veterinäruntersuchungsamt
DAdV	Duck AdV

DNA	Deoxyribonucleic acid
dNTP	Disoxyribonucleosidtriphosphat (dATP, dCTP, dGTP, dTTP)
DMEM	Dulbecco´s modified Eagle Medium
Dr.	Doctor
EastBT AdV	East box turtle AdV
EDTA	Ethylenedinitrilotetraacetic acid
EDS	Egg drop syndrome
e.g.	Exempli gratia
EM	Electron microscopy
et al	et alteri or et alii
Eubl AdV	Eublepharid AdV
FAdV	Fowl AdV
F-test	Fisher-test
FCS	Fetal calf serum
FrAdV-1	Frog AdV-1
g	Gram (Weight), Gravity
g/l	gram per liter
GLMM	General linear mixed models
GmbH	Gesellschaft mit beschränkter Haftung
Gr.std AdV	Green striped tree dragon AdV
h	Hours
H_2O	Water
HAdV	Human adenovirus
HBS	Hepes Buffered Saline
HCl	Hydrochloric acid
Hel-1 AdV	Helodermatid AdV-1
Hel-2 AdV	Helodermatid AdV-2
Herm. t AdV	Hermann’s tortoise AdV
http	Hypertext transfer protocol
hu	Hungary
HV	Herpes virus
IgH2	Iguana heart cells
IIV	Invertebrate iridovirus
ITR	Inverted terminal repeat

kbp	Kilo base pairs
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
kV	Kilovolt
Lab.	Laboratory
Map t. AdV	Alabama map turtle AdV
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimol
mRNA	Messenger RNA
n	Index of refraction relative to air
NA	Natural antibodies, not applicable (Phylogenetic tree)
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
$NaHPO_4$	Sodium phosphate
NaOH	Sodium hydroxide
ncbi	National Center for Biotechnology Information
n.d.	Not done
NEA	Non essential amino acids
ng	Nanogram
$(NH)_4SO_2$	Ammonium sulfate
No.	Number
nm	Nanometer
NT	Neutralization test
nt	Nucleotides
P	Probability value
Pan. t. AdV	Pancake tortoise AdV
PBS	Phosphate-Buffered Saline
pers. comm.	Personal communication
PCR	Polymerase chain reaction
pH	Potentia Hydrogenii (potential of hydrogen)

PMV	Paramyxovirus (ferlavirus)
Pond s. AdV	Pond slider AdV
Prof.	Professor
pTP	Protein tyrosine phosphatase
RAdV-1	Raptor AdV-1
RedES AdV	Red eared slider AdV
Reov	Reovirus
RNA	Ribonucleic acid
rpm	Rotation per minute
SD	Standard deviation
sec	Seconds
Sn-1 AdV	Snake AdV-1
sp., spp.	Species
St.	Saint
TAdV-3	Turkey AdV-3
TAE buffer	Tris-acetate-EDTA buffer
$TCID_{50}$	50 % Tissue culture infectious dose
TEMED	Tetramethylethylenediamine
TH-1	*Terrapene* heart cells
Ti	Titanium
TP	Terminal protein
Tris-Cl	Tris chloride
Tris-HCL	Tris hydrogen chloride
U	Units
U/µl	Units per microliter
U/l	Unit per liter
U/mg	Units per milligram
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
Varan. AdV	Varanid AdV
VH-2	Viper heart cells
WaterM AdV	Water monitor AdV
WSAdV-1	White sturgeon AdV-1

www	World wide web
Yell. BS AdV	Yellow-bellied slider AdV

1 Introduction

Adenoviruses (AdVs) are the most common pathogen found in lizards (Julian and Durham, 1985; Frye et al., 1994; Wellehan et al., 2004; Papp et al., 2009; Hyndman et al., 2011; Ball et al., 2012). Agamid AdV-1 infection is very common in central bearded dragons (*Pogona vitticeps*) (Jacobson et al., 1996; Kim et al., 2002; Wellehan et al., 2004; Moormann et al., 2009), which are the most popular pet lizards. The stability of AdVs in the environment and poor biosecurity in reptile breeding facilities has made this virus common in North American and European populations of bearded dragons (Latney et al., 2013). Aside from lizards, AdVs have also commonly been described in many different snake species (Heldstab et al., 1984; Farkas et al., 2002; Marschang et al., 2003; Garner et al., 2008; Papp et al., 2009; Abbas et al., 2011) as well as in chelonians (Wilkinson et al., 2004; Rivera et al., 2009; Farkas et al., 2009; Schumacher et al., 2012; Doszpoly et al., 2013). The most common method used for the detection of AdVs in reptiles is a polymerase chain reaction (PCR) targeting a portion of the DNA-dependent DNA polymerase gene followed by sequencing of PCR products (Wellehan et al., 2004; Papp et al., 2009). AdVs have also been diagnosed in reptiles by electron microscopy examination (EM) of histopathological sections (Jacobson, 2007).

All of the AdVs detected in squamates so far appear to belong in the genus *Atadenovirus*, and in many cases it has been hypothesized that individual viruses may represent host-specific viruses that have coevolved with their lizard hosts (Wellehan et al., 2004; Papp et al., 2009). Snake atadenoviruses, however have appeared to be less species specific (Marschang et al., 2003) than lizard AdVs. It is interesting to note that snakes are not basally divergent within the squamates and are closely related to the Iguanidae, Agamidae, and Chamaeleonidae families (Vidal et al., 2005; Pyron et al., 2013) so that the differences in species specificity between lizard and snake atadenoviruses can not be explained based on host evolution.

The host specificity of *Atadenoviruses* and their ability to switch hosts has not been studied yet due to difficulties in isolation of these viruses in cell culture. Isolation of lizard AdVs in cell culture is challenging. Therefore, there are no previous studies on serological cross-reactivity of lizard AdVs and no serological methods available for the detection of antibodies against AdVs in lizards. Up to 2009 AdVs had been

isolated only from snakes (Jacobson et al., 1985; Juhász and Ahne, 1993; Farkas et al., 2002; Marschang et al., 2003) and these viruses were all closely related to one another (Farkas et al., 2002; 2008; Marschang et al., 2003). In 2009, Helodermatid AdV-1 and 2 were isolated from different species of helodermatid lizards (*H. horridum* and *H. suspectum*) in cell culture (Papp et al., 2009). However, Agamid AdV-1 has never been isolated before. Since this virus seems to be species specific and was detected only in agamid lizards (Parkin et al., 2009), it was hypothesized that agamid AdVs could be isolated on a competent cell line from bearded dragons. The purpose of this project was to isolate squamate atadenoviruses in cell culture, to examine the serological relationships between lizard and snake AdVs, and to develop techniques for understanding how common adenovirus infections are among squamates, helping to ensure the establishment and maintenance of healthy populations. It was also intended to provide information on the importance of these viruses in various groups of lizards and snakes. An additional aim of the project was the characterization and comparison of adenoviruses from different reptiles.

2 Literature review

2.1 Adenoviridae

Adenoviruses (AdVs) are middle-sized (80-100 nm) nonenveloped, icosahedral, double-stranded DNA viruses. Their icosahedral capsid mostly consists of non-vertex capsomers (hexons) and vertex capsomers (pentons). The capsid also contains fibers, which protrude from the virion surface. The hexon, penton and fiber are three major antigenic proteins of AdVs (Benkő et al., 2005). The AdV genome is a linear molecule of double stranded DNA (30-38 kbp), containing an inverted terminal repeat (ITR) with the 5' ends of the genome linked covalently to a terminal protein (TP). The central part of the genome is conserved throughout the family, while the terminal parts show large variation in length and gene content (Harrach, 2008). AdV replication has been studied in detail in various human AdVs. Virus entry takes place via interactions of the fiber knob with specific receptors of the surface of susceptible cells. After uncoating, the virus core is delivered to the nucleus, which is the site of virus transcription, DNA replication and assembly. Virus infection mediates the shutdown of host DNA synthesis and later RNA and protein synthesis. Transcription of the AdV genome by host RNA polymerase II involves both DNA strands of the genome and initiates from five early, two intermediate, and the major late promoters. All primary transcripts are capped and polyadenylated, with complex splicing patterns producing families of mRNAs. Replication pathways between different AdV genera show considerable difference in gene layout between nonconserved regions of the genome (Harrach, 2008; Russell, 2009).

The family *Adenoviridae* is divided into five accepted (Wadell, 2011) and a sixth proposed genus (Doszpoly et al., 2013). The genus classification criteria are mostly based on genetic and genomic characteristics of these viruses and their host-specificity (Harrach, 2000).

2.1.1 Genus *Mastadenovirus*

All human AdVs (HAdV) and chimpanzee AdVs belong to the genus *Mastadenovirus*, and are divided into the seven species HAdV-A to HAdV-G. Bovine AdVs (BAdVs) types 1, 2, 3, 9 and 10 (classified as group I BAdVs) as well as ovine, canine, caprine, simian, equine, murine, and porcine AdVs also belong to this genus (Dán et al., 1998; Harrach et al., 2011). AdVs in humans can cause non-lethal respiratory

disease, conjunctivitis, diarrhea in infants and young children, and hemorrhagic cystitis (Wadell et al., 1984; de Jong et al., 1999). The genus *Mastadenovirus* contains the most well studied AdVs. Mastadenoviruses are believed to have coevolved with their mammalian hosts (Harrach, 2000).

2.1.2 Genus *Aviadenovirus*

The majority of AdVs isolated from birds belong to the genus *Aviadenovirus*. Among these are fowl adenoviruses (FAdV) with at least twelve different genotypes, classified into five species (A-E) (Marek et al., 2010) as well as two turkey AdVs (Kajan et al., 2010), goose AdV (Kajan et al., 2012), several falconid AdVs (Schrenzel et al., 2005; Tomaszewski et al., 2007), pigeon AdVs (Hess et al., 1997), Meyer's parrot AdV (Wellehan et al., 2005), psittacine AdV (Luschow et al., 2007) and gull AdV (Bodewes et al., 2013). AdVs in birds cause hepatitis, pancreatitis, diphtheritic stenosing tracheitis with occasional bronchitis, pneumonia, diarrhea, and poor hatchability (McFerran et al., 2000). Viruses in this genus are postulated to have coevolved with their avian hosts (Harrach, 2000).

2.1.3 Genus *Siadenovirus*

The host origin of this genus is not clear yet. Frog AdV-1 (FrAdV-1), isolated from a leopard frog (*Rana pipiens*) belongs to the *Siadenovirus* genus. This frog AdV was the first AdV strain from a cold-blooded vertebrate in which the full genome was completely analyzed (Davison et al., 2000). These analyses showed that FrAdV-1 is related to an unusual bird adenovirus, turkey AdV type 3 (TAdV-3) (Pitcovski et al., 1998), which was classified as a group II aviadenovirus until establishment of the genus *Siadenovirus* in 2002 (Davison et al., 2002). Budgerigar AdV-1 also belongs to the same group. Phylogenetic analysis of the genome of this virus demonstrated similarity to TAdV-3 and FrAdV-1 (Katoh et al., 2010). The genus *Siadenovirus* also includes several other avian AdVs, e. g. Raptor AdV-1 (RAdV-1), which was detected in multiple species of birds (Kovacs et al., 2009) as well as Great tit AdV-1, found in a great tit (*Parus major*) (Kovacs et al., 2010), Skua AdV-1, detected in south polar skuas (*Catharacta maccormicki*) (Park et al., 2012) and Psittacine AdV-2, detected in plum headed parakeets (*Psittacula cyanocephala*) and in umbrella cockatoos (*Cacatua alba*) (Wellehan et al., 2009). Sulawesi tortoise AdV-1, which has been detected in several turtle species (Rivera et al., 2009; Schumacher et al., 2012) also belongs to this genus. AdVs, which belong to group II aviadenoviruses cause

diseases such as hemorrhagic enteritis of turkeys, marble spleen disease of pheasants and avian adenosplenomegaly of chickens and budgerigars (Shivaprasad, 2008; Katoh et al., 2010). Also hepatomegaly, renomegaly as well as proventricular and ventricular dilation, ulceration, and erythema have been described in infected birds (Zsivanovits et al., 2006). Clinical signs in turtles will be described later. This genus has been hypothesized to have coevolved with amphibian hosts, with siadenoviruses found in birds and tortoises representing the results of interclass host switches (Harrach, 2008).

2.1.4 Genus *Ichtadenovirus*

This genus includes a single member, the only AdV isolated from a fish, a white sturgeon (*Acipenser transmontanus*) (Benkő et al., 2002). White sturgeon AdV-1 (WSAdV-1) was found associated with infections of the mucosa of the alimentary tract among juveniles (Hedrick et al., 1985). Phylogenetic analysis of WSAdV-1 based on the sequences of the pTP gene, part of the DNA polymerase gene, the hexon and protease genes showed a clear separation of WSAdV-1 from other AdV genera (Benkő et al., 2002; Kovacs et al., 2004). WSAdV-1 is often used as an outgroup in phylogenetic calculations.

2.1.5 Genus *Atadenovirus*

This genus contains AdVs from divergent hosts. Atadenoviruses were named after a bias toward high A+T content in the genomes of the first members of this genus studied. However, AtAdVs found in reptiles seem to have a balanced nucleotide content (Harrach, 2008). This genus was originally established to allocate several exceptional bovine (group II BAdVs) and ovine AdVs with unusual characteristics (Harrach, 2000). Thus the *Atadenovirus* genus includes several adenoviruses isolated from cattle (Dán et al., 1998; Benkő et al., 1998; Élő et al., 2003; Graham et al., 2005), goats (Lehmkuhl et al., 2001), sheep (Barbezange et al., 2000; Both, 2004), deer (Zakhartchouk et al., 2002), a marsupial (Thomson et al., 2002), and ducks (Duck AdV-1, DAdV-1) (Hess et al., 1997). Almost all squamate (snake and lizard) AdVs described so far belong to the genus *Atadenovirus* (Wellehan et al., 2004; Farkas et al., 2008; Garner et al., 2008; Papp et al., 2009; Ascher et al., 2013), except Varanid AdV-1, which was detected in an emerald monitor (*Varanus prasinus*) and clusters outside the genus *Atadenovirus* (Papp et al., 2009). BAdVs-6 and-7 were reported as causative agents of respiratory and enteric tract infections (Fent et

al., 2002; Graham et al., 2005). DAdV-1 causes egg drop syndrome (EDS) (Kraft et al., 1979). AdV detected in California black-tailed deer caused epizootic hemorrhagic disease (Lehmkuhl et al., 2001). Ovine AdV are generally considered nonpathogenic under field conditions, despite wide distribution (Both, 2004). Clinical signs in squamates are described below. The genus *Atadenovirus* has been hypothesized to have coevolved within squamate hosts and AdVs of other hosts represent later host switches (Harrach, 2000).

2.1.6 Proposed genus "Testadenovirus"

This novel genus was proposed for newly detected Testudinid AdVs. The first AdV to fall into this genetically distinct cluster was detected in an ornate box turtle (*Terrapene ornata ornata*) (Farkas et al., 2009). AdVs in other species of the superfamily Testudinoidea have recently been detected in a pancake tortoise (*Malacochersus tornieri*), eastern box turtles (*Terrapene carolina carolina*), red-eared sliders (*Trachemys scripta elegans*) and yellow-bellied sliders (*T. scripta scripta*). Phylogenetic analyses of DNA-dependent DNA polymerase and hexon gene partial sequences clustered these AdVs separately from all previously accepted genera of the family *Adenoviridae* (Doszpoly et al., 2013).

2.2 Adenoviruses in reptiles, host species

2.2.1 Adenoviruses in lizards

Table 1: Lizard species in which AdV infection has been reported

Common name	Scientific name	Reference
Eastern bearded dragon	*Pogona barbata*	Julian and Durham, 1985
Black-soil bearded dragon (also known as Rankin´s dragon lizard)	*Pogona henrylawsoni*	Frye et al., 1994
Central netted dragon	*Ctenophorus nuchalis*	Hyndman et al., 2011
Western bearded dragon	*Pogona minor minor*	Hyndman et al., 2011
Central bearded dragon	*Pogona vitticeps.*	Jacobson et al., 1996; Kim et al., 2002; Wellehan et al., 2004; Moormann et al., 2009;

Common name	Scientific name	Reference
Mexican beaded lizard	*Heloderma horridum*	Papp et al., 2009
Gila monster	*Heloderma suspectum*	Papp et al., 2009; Wellehan et al., 2004
Emerald monitor	*Varanus prasinus*	Papp et al., 2009
Savanna monitor	*Varanus exanthematicus*	Jacobson et al., 1986
Jackson´s chameleon	*Chamaeleo jacksonii* formerly *Trioceros jacksonii*	Jacobson et al., 1990
Mountain chameleon	*Chamaeleo montium* formerly *Trioceros montinum*	Kinsel et al., 1997, Wellehan et al., 2004
Fat-tail gecko	*Hemitheconyx caudicinctus*	Wellehan et al., 2004
Leopard gecko	*Eublepharis macularius*	Wellehan et al., 2004
Tokay gecko	*Gekko gecko*	Wellehan et al., 2004
Blue tongued skink	*Tiliqua scincoides intermedia*	Wellehan et al., 2004
Hispaniolan gracile anole	*Anolis distichus ignigularis*	Ascher et al., 2013
Hispaniolan gracile anole	*A. distichus ravitergum*	Ascher et al., 2013

2.2.2 Adenoviruses in snakes

Table 2: Snake species in which AdV infection has been reported

Common name	Specific name	Reference
Boa constrictor	*Boa constrictor*	Jacobson et al., 1985; Perkins et al., 2001; Ramis et al., 2000; Heldstab et al., 1984; Marschang et al., 2003;

Common name	Specific name	Reference
Corn snake	*Pantherophis guttatus* formerly *Elaphe guttata guttata*	Garner et al., 2008; Abbas et al., 2011; Farkas et al., 2002; Juhász and Ahne, 1993; Mahapatra et al., 2013
Royal python	*Python regius*	Abbas et al., 2011; Ogawa et al., 1992
Rosy boa	*Lichanura trivirgata*	Schumacher et al., 1994
Mountain kingsnake	*Lampropeltis zonata*	Wozniak et al., 2000a; Raymond et al., 2003
Mojave (Green) rattlesnake	*Crotalus scutulatus scutulatus*	Perkins et al., 2001
Palm pit viper	*Bothriechis marchi*	Raymond et al., 2002
Asp viper	*Vipera aspis*	Papp et al., 2009
Four-lined rat snake	*Elaphe quatuorlineata*	Heldstab et al., 1984
Aesculapian snake	*Zamenis longissimus* formerly *Elaphe longissima*	Heldstab et al., 1984
California kingsnake	*Lampropeltis getula californiae*	Garner et al., 2008
Milksnake	*Lampropeltis triangulum*	Garner et al., 2008
Gaboon viper	*Bitis gabonika*	Heldstab et al., 1984
Indonesian pit-viper	*Parias hageni*	Farkas et al., 2008
Death adder	*Acantrophis antarcticus*	GenBank accession number JN418933
Bull snake	*Pituophis catenifer sayi*	Garner et al., 2008

2.2.3 Adenoviruses in chelonians (turtles and tortoises)

Table 3: Chelonid species in which AdV infection has been reported

Common name	Specific name	Reference
Leopard tortoise	*Stigmochelys pardalis* formerly *Geohelone pardalis*	Wilkinson et al., 2004
Sulawesi tortoise	*Indotestudo forsteni*	Rivera et al., 2009
Ornate box turtle	*Terrapene ornata ornata*	Farkas et al., 2009
Impressed tortoises	*Manouria impressa*	Schumacher et al., 2012
Burmese star tortoise	*Geochelone platynota*	Schumacher et al., 2012
Pancake tortoise	*Malacochersus tornieri*	Doszpoly et al., 2013
Eastern box turtle	*Terrapene carolina carolina*	Doszpoly et al., 2013
Red-eared slider	*Trachemys scripta elegans*	Doszpoly et al., 2013
Yellow-bellied slider	*T. scripta scripta*	Doszpoly et al., 2013

2.3 Clinical signs in reptiles

Clinical signs described in infected squamates include inappetence, lethargy, dehydration, limb paresis, regurgitation, vomiting, diarrhea, anorexia, weakness, CNS signs including head tilt and circling, opisthotonus and spasms. Gastrointestinal problems have also been described (Julian and Durham, 1985; Frye et al., 1994; Kim et al., 2002; Garner et al., 2008; Papp et al., 2009; Mahapatra et al., 2013). There are several reported cases in which AdV infection was described without any clinical signs (Jacobson et al., 1986; Ogawa et al., 1992; Hyndman et al., 2011; Kubiak, 2013). Therefore, the primary pathogenic role of AdVs is not always clear. Nevertheless, the pathogenicity of these viruses has been demonstrated by an experimental transmission study in snakes, where an AdV isolated from a boa constrictor with hepatic necrosis was inoculated into a neonatal boa constrictor, which died 14 days after inoculation with hepatic necrosis (Jacobson et al., 1985).

Clinical signs described in infected turtles include lethargy, anorexia, mucosal ulceration, palatine erosions, nasal and ocular discharge, diarrhea and high mortality. Biliverdinuria, wasting and episodes of hemorrhaging have also been observed

(Wilkinson et al., 2004; Rivera et al., 2009; Schumacher et al., 2012). AdV infection has also been detected in clinically healthy tortoises (Schumacher et al., 2012).

2.4 Pathological and histological findings

Pathological lesions found in squamates frequently involve the liver, which may be diffusely pale and swollen (Frye et al., 1994). The liver can also be enlarged (Jacobson et al., 1986) and diffusely mottled. The intestine is also commonly affected, e.g. haemorrhage in the intestinal lumen (Jacobson, 2007; Hyndman et al., 2011; Marschang, 2011). Hepatic necrosis, enteritis, esophagitis, stomatitis, splenitis, and pneumonia have also been described (Jacobson, 2007). Histological changes described in infected lizards and snakes are basophilic and eosinophilic intranuclear inclusions in enterocytes, hepatocytes (Jacobson et al., 1996; Kim et al., 2002), epithelial cells of the bile ducts, lung, renal tubules, pancreatic acini and oral mucous membranes, as well as endothelial cells in the brain (Ramis et al., 2000; Raymond et al., 2003; Wellehan et al., 2004; Moormann et al., 2009).

Pathological changes in infected turtles include hepatosplenomegaly, epidermal hyperkeratosis, myositis, rhinitis, pharyngitis, tracheitis, pneumonia, vasculitis, vascular necrosis and thrombosis, myocardial fiber degeneration and fibrosis, stomatitis, gastro- entero- typhlocolitis, hepatitis, hepatic necrosis, hepatic lipidosis, pancreatitis, pancreas necrosis, nephritis, renal necrosis, bone marrow necrosis, lymphoid necrosis, splenic necrosis, sinus histiocytosis, neoplasia, adrenalitis, adrenal necrosis, gonadal degeneration and necrosis, choroid plexus meningitis, encephalitis, harderian gland necrosis, endothelial, epithelial and granulocytic karyomegaly and atrophy of fat (Rivera et al., 2009). Also diphtheritic plaques of the oral mucosa, edema of the wall of the colon and yellow colored liver were reported (Farkas et al., 2009; Schumacher et al., 2012). Histological examination demonstrated the presence of amphophilic to basophilic intranuclear inclusions in the bone marrow, within enterocytes, hepatocytes, biliary epithelial cells of the liver, reticuloendothelial cells in the spleen, renal tubular epithelial cells and epithelial cells of the testis. Intranuclear inclusions were also found in the pancreas, ovary, kidney, respiratory epithelium, renal epithelium, vascular and cardiac endothelium, and cerebral glia and choroid plexus (Rivera et al., 2009; Farkas et al., 2009; Schumacher et al., 2012).

2.5 Adenovirus species specificity

Their wide prevalence in various host species as well as significantly conserved genome organization make AdVs an ideal model for studying virus evolution (Harrach, 2008). The host specificity of the atadenoviruses and their ability to switch hosts is not fully understood. Thus, lizard AdVs seem to be relatively species specific and have mostly been found in single hosts. There are, however, a number of exceptions e.g. Eublepharid AdV-1 has been described in two different gecko genera, in a leopard gecko *(Eublepharis macularius)* and in a fat-tail gecko *(Hemitheconyx caudicinctus)* (Wellehan et al., 2004). An AdV with 99% identity to Helodermatid AdV-2, which was originally detected in a beaded lizard (*Heloderma horridum)* (Papp et al., 2009) was detected in a western bearded dragon *(Pogona minor minor*) in Australia (Hyndman et al., 2011). A closely related virus – Helodermatid AdV-1 – has been found only in Gila monsters (*Heloderma suspectum*) so far (Wellehan et al., 2004; Papp et al., 2009). An AdV identical to Helodermatid AdV-2 has also recently been found in a captive central bearded dragon in the USA (Wellehan et al., 2012). Agamid AdV-1 has been reported only in central bearded dragons (Parkin et al., 2009). A more marked absence of species specificity has previously been found in snake AdVs. Snake AdV-1 has been found in both colubrid and boid snakes (Farkas et al., 2002; Marschang et al., 2003), while Snake AdV-2 has been described in viperid and colubrid snakes (Garner et al., 2008; Papp et al., 2009), and Snake AdV-3 has been detected in different species of colubrid snakes (Garner et al., 2008).

Other AdVs described in reptiles have also demonstrated a mixed ability to switch hosts. Sulawesi tortoise AdV-1, which belongs to the genus *Siadenovirus*, was detected in Sulawesi tortoises, and then identical viruses were detected in impressed tortoises and Burmese star tortoises (Schumacher et al., 2012). Also the presence of identical AdVs in different turtle subspecies (yellow bellied and red-eared sliders) was described. This chelonian AdV belonged to the recently proposed genus *"*Testadenovirus*"* (Doszpoly et al., 2013).

2.6 Isolation of squamate adenoviruses in cell culture

There have been several reports of successful isolations of AdVs from snakes in cell culture. Snake AdV-1 was originally isolated in iguana heart cells (IgH2) from the internal organs of a red corn snake (*Pantherophis guttatus*) with clinical signs of

pneumonia (Juhász and Ahne, 1993). The red corn snake isolate was later randomly cloned and completely sequenced (Farkas et al., 2002; 2008), and thus serves as a prototype for reptilian AdVs. An identical virus was isolated from a boa constrictor (*Boa constrictor*) with inclusion body disease. In that case, the virus was isolated in viper heart (VH-2) cells from internal organs and blood cells (Marschang et al., 2003). Additional AdVs have been isolated from a boa constrictor with hepatic necrosis and basophilic intranuclear inclusions in the liver and small intestine (Jacobson et al., 1985), as well as from a royal python (*Python regius*) (Ogawa et al., 1992). A genetically distinct AdV was detected by PCR in colubrid snakes in the USA (Garner et al., 2008). This virus was named Snake AdV-2 and was later isolated in VH-2 cells from an intestine sample of a red corn snake, which died with clinical signs of dyspnea and vomitus (Abbas et al., 2011).

There is only one report available on the isolation of AdVs from lizards in cell culture. In that report, two closely related AdVs were isolated from helodermatid lizards (*Heloderma horridum* and *Heloderma suspectum*) in a permanent cell line from iguanas (IgH2) (Papp et al., 2009). These viruses have been completely sequenced and proposed to be renamed Lizard AdV-1 and -2 (Harrach, pers. comm.). However, none of the cell lines used previously for the isolation of adenoviruses in reptiles have been permissive for the growth of agamid AdVs.

2.7 Previous serological studies in reptiles

Previous studies have reported on the detection of neutralizing antibodies against AdVs in snakes by serum neutralization test. In the first case, plasma from 12 different groups of snakes collected in four different countries were tested against a Snake AdV-1 isolate from a boa constrictor. Neutralizing antibodies were found in 15 of 113 (13.3%) snakes tested from Costa Rica and Germany. The positive tested snakes included a wild boa constrictor from Costa Rica (Marschang et al., 2003). In another study, antibodies against Snake AdV-1 were found in boid snakes in Germany, these were detected in only four out of 86 sera samples (4.7%) (Pees et al., 2010). All of these findings demonstrate that atadenovirus infections are common in snakes and are present in snakes both in captivity and in the wild. Serological assays are a powerful tool for diagnostics and for screening collections of reptiles for exposure and infection with AdV.

Since atadenoviruses seem to be wide spread among reptiles we hypothesized that screening of reptiles from various species would lead to the detection of AdVs and that characterization of these AdVs would demonstrate a close relationship to previously described AdVs of reptiles. Due to difficulties previously reported in the isolation of lizard AdVs, we hypothesized that Agamid AdV-1 could be grown in cell culture using bearded dragon specific cells. This project was also intended to develop serological tests using previously isolated squamate atadenoviruses as well as new isolates. It was hypothesized that neutralizing antibodies against these viruses could be detected in various lizard and snake species and that genetically distinct reptilian atadenoviruses would also be serologically distinct.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and reagents

100 bp-DNA-Ladder	Fermentas, St. Leon-Rot
6×Ladder	Fermentas, St. Leon-Rot
10 DG column	Bio-Rad, Munich
Agarose ultraPURE™	Bioenzym, Oldendorf
Amphotericin B	Biochrom, Berlin
Bromphenolblue	Carl Roth, Karlsruhe
$CaCl_2$ x 2 H_2O	Merck, Darmstadt
Caesium chloride	Bio-Rad, Munich
DMEM-Powder	Biochrom, Berlin
D-glucose	Biochrom, Berlin
dNTPs	Thermoscientific, Darmstadt
EDTA	Carl Roth, Karlsruhe
Ethanol 99.8%	Carl Roth, Karlsruhe
Ethidiumbromide	Carl Roth, Karlsruhe
FCS (Fetal calf serum)	Biochrom, Berlin
Gentamycinsulfate (640 U/mg)	Biochrom, Berlin
Glacial acetic acid	Carl Roth, Karlsruhe
Glycerol	Carl Roth, Karlsruhe
HEPES dry substance	Biochrom, Berlin
KCl	Merck, Darmstadt
KH_2PO_4	Merck, Darmstadt
L-glutamin	Biochrom, Berlin
$NaHCO_3$	Merck, Darmstadt
Na_2HPO_4 x 12 H_2O	Merck, Darmstadt
NaCl	Merck, Darmstadt
NEA	Biochrom, Berlin
Penicillin-G (1664 U/mg)	Biochrom, Berlin
Saponin	Sigma-Aldrich Chemie, Munich
Streptomycin sulfate (758 U/mg)	Biochrom, Berlin
TEMED	Merck, Darmstadt

Tris Pufferan®≥99.9%	Merck, Darmstadt
Trypsin-dry substance	Biochrom, Berlin
Trypsin solution 10×	Sigma-Aldrich Chemie, Munich
Versen (Titriplex III)	Merck, Darmstadt

3.1.2 Consumables

10 DG disposable chromatography column	Bio-Rad, Munich
96-well microtest cell culture plates, flat bottom	BD Biosciences Discovery Labware, Heidelberg
CO_2	Westfalengas, Münster
Cell culture bottles with gas exchange cap 25cm^2, 75cm^2, and 175cm^2	BD Biosciences Discovery Labware, Heidelberg
Copper grids 3.05	Plano, Wetzlar
Erlenmeyer flasks, 250 ml	Hirschmann, Eberstadt
Glass pipettes, 1 ml, 2 ml, 5ml, 10 ml, 20 ml and 50 ml	Hirschmann, Eberstadt
Sterile scalpel blades	C. Bruno Bayha, Tuttlingen
Liquid nitrogen	Messergriesheim, Bad Soden
Pipette tips	Biozym, Hessisch Oldendorf
Ultracentrifuge plastic tubes 13×51mm, 25×89 mm	Beckman, Weinheim
Polypropylene tubes 1.5 and 2 ml	Eppendorf, Hamburg
Polypropylene conical tubes 15 ml and 50 ml	Corning, New-York
Sterile syringes	Th. Geyer, Renningen
Syringe filters 0.2 µm and 0.45 µm	Th. Geyer, Renningen
Tissue culture dishes, 30 mm diameter	Cellstar, Greiner Bio-One GmbH, Frickenhausen

3.1.3 Equipment

Autoclave	Systec, Wettenberg
Bench centrifuge 5415 D	Eppendorf AG, Hamburg
Dry sterilizer	Willi Memmert, Schwabach
Egg incubator (Kunstglucke FB 50 E-Reptilien, 12 Volt)	Jäger, Wächtersbach
Electronic weighing balance	Mettler-Toledo GmbH, Giessen
Fluorescence microscope, BZ-9000	Keyence, Neu-Isenburg
Gel electrophoresis system	Biometra, Göttingen
Hemocytometer, Neubauer	Carl Roth, Karlsruhe
Incubator for cell cultures (Standard CO_2 incubator, CB210)	Binder, Tuttlingen
Inverted light microscope	Will, Wetzlar
Magnet stirrer	Heidolph Instruments, Schwabach
Mastercycler gradient	Eppendorf, Hamburg
Milli-Q	Millipore, Schwalbach
Photometer (Ultraspec 2100 pro)	Amerham Bioscience, Glattbrugg
Refractometer	Krüss, Hamburg
Dry bath incubator	Kisker, Steinfurt
Transmission electron microscope JEM-1011	JEOL, Tokyo
UV Box	Vilber, Eberhardzell
Ultracentrifuge L7-65, rotors SW 41 Ti and SW 28	Beckman, Weinheim
Ultralow temperature freezer (-80), New Brunswick™	Eppendorf, Hamburg
Ultrasonic, Branson Sonifier 250, Danbory	Gerhard Heinemann, Schwäbisch Gmünd
Varifuge 3.2 RS, rotor 5315	Thermoscientific, Darmstadt
Vortex shaker, EU Plug	VWR International, Darmstadt

3.1.4 Solutions and Buffers

1.5% agarose gel	100 ml 1× TAE-Buffer add 1.5 g Agarose Ultrapure™, heat in microwave until agarose is completely dissolved, then add 5 µl of ethidium bromide, mix and pour in the gel chamber. Let stand at room temperature until polymerized
Amphotericin B-solution	250 µg Amphotericin B resuspend in 1 ml of double distilled water and store at 4°C
Caesium Cloride (CsCl) light (n=1.354)	12 g CsCl dissolve in 45 ml of 10 mM Tris-HCl (pH 8.1). Autoclave at 121°C for 30 min and 2 bar. Store at 4°C
CsCl heavy (n=1.376)	28.5 g CsCl dissolve in 43 ml of 10 mM Tris-HCl (pH 8.1). Autoclave at 121°C for 30 min and 2 bar. Store at 4°C
DMEM (Dulbecco modified Eagle medium)	13.4 g/l of DMEM powdered medium, 4.5 g/l of D-glucose, 4.5 g/l L-glutamin and 2.2 g/l of $NaHCO_3$, dissolve in double-distilled water, sterile filter and store at 4°C
Ethidiumbromide solution	10 g/l Ethidiumbromide dissolved in double distilled water. Store at -20°C in darkness.
Gentamycin sulfate-solution	5 mg of Gentamycinsulfate dissolve in 1 ml double-distilled water. Store at 4°C
Growth medium	DMEM supplement with 5% FCS and 1% NEA. Store at 4°C
HBS (Hepes Buffered Saline)	20 mM HEPES dissolve in double distilled water, adjusted pH to 7.8 with 0.15 M NaCl, sterilize by filtration though a 0.2 µm filter. Store at 4°C
Medium with two times antibiotics	To 500 ml of DMEM add 2 ml each Gentamycin sulfate-solution, Penicillin-G-solution, Streptomycin sulfate-solution and 4 ml of Amphotericin B-solution. Store at 4°C
Nutrient medium	500 ml of DMEM supplement with 2% FCS, 1 ml of Gentamycin sulfate-solution, Penicillin-G-solution, Streptomycin sulfate-solution and 2 ml of Amphotericin B-solution. Store at 4°C

PBS (Phosphate-Buffered Saline)	8.00 g/l (137 mM) NaCl, 0.20 g/l (26 mM) KCl, 0.12 g/l (1.23 mM) KH_2PO_4, 0.91 g/l (5.1 mM) Na_2HPO_4 x 2 H_2O. Dissolved in in Millipore-water, adjust to pH 7.5 with 1 M NaOH. Store at 4°C
Penicillin-G-solution	60 mg of Penicillin-G resuspend in 1 ml of double-distilled water. Store at 4°C
Streptomycinsulfate-solution	256 mg Streptomycin sulfate resuspend in 1 ml of double-distilled water. Store at 4°C
50×TAE-Buffer for agarose gel electrophoresis	242 g/l (0.2 M) Tris (hydroxylmethyl) aminomethan, 57.1 ml/l glacial acetic acid, 18.612 g/l (0,5 M) EDTA. Dissolve in double distillated water, Store at room temperature. Dilute 1:50 before use
Trypsin-versene solution 0.05%	8.00 g/l (136 mM) NaCl, 0.20 g/l (3 mM) KCl, 0.20 g/l (1 mM) KH_2PO_4, 2.31 g/l (6 mM) Na_2HPO_4 x 12 H_2O, 0.132 g/l (0.9 mM) $CaCl_2$ x 2 H_2O, 0.5 g/l Trypsin-dry substance, 1.25 g/l (3 mM) Versen (Titriplex III), 0.05 g/l (37,900 U/l) Streptomycin sulfate, 0.06 g/l (100,000 U/l) Penicillin-G dissolve in Millipore-water, adjust to pH 7.00 with 1 M NaOH, sterile filter. Store at -20°C and melt at 4°C before use

3.1.5 External services

Eurofins MWG	Ebersberg

3.1.6 Kits

DNAeasy® kit	Qiagen GmbH, Hilden
PCR kit (Taq Polymerase S)	Genaxxon, Ulm
peqGOLD Extraction Kit	PEQLAB Biotechnology, Erlangen,

3.1.7 Cell cultures

Table 4: Cell lines and their origins and sources

Cell line	Source	Origin
Permanent cell lines		
Iguana heart cells (IgH2)	American Type Culture Collection ATCC, CCL 108 (Teddington, Middlesex, UK)	Epithelial heart cells of a lizard (*Iguana iguana*)
Viper heart cells (VH-2)	American Type Culture Collection ATCC, CCL 140 (Teddington, Middlesex, UK)	Fibroblast heart cells of a snake (*Vipera russelli*)
Terrapene heart cells (TH-1)	American Type Culture Collection (ATCC), CCL 50 (Teddington, Middlesex, UK)	Epithelial heart cells of a tortoise (*Terrapene carolina*)
Primary cell line		
Bearded dragon embryo fibroblasts (BDE)	Established during our study	Fibroblasts from Bearded dragon embryos (*Pogona vitticeps*)

3.1.8 Viruses

Table 5: Virus isolates used for neutralization tests

AdV	Host species	Cell line	Reference
Helodermatid AdV-1	Gila monster (*Heloderma suspectum*)	IgH2	Papp et al., 2009
Helodermatid AdV-2	Mexican beaded lizard (*Heloderma horridum*)	IgH2	Papp et al., 2009
Snake AdV-1	Boa constrictor (*Boa constrictor*)	VH-2	Marschang et al., 2003

AdV	Host species	Cell line	Reference
Snake AdV-2	Eastern corn snake (*Pantheropis guttatus*)	VH-2	Romanova et al., 2011
Agamid AdV-1	Central bearded dragon (*Pogona vitticeps*)	BDE cells	Ball et al., 2014

3.1.9 Sample origins

3.1.9.1 Tissue and swab samples

Tissue and swab samples were sent to our laboratory between 2008 and 2013 from veterinary practices and diagnostic laboratories. The samples were collected form reptiles considered suspicious for AdV infection. Also reptilian samples, which were sent for other virus testing, were “blind” tested for the presence of AdV infection. Lizard tissue and swab samples were collected from 668 animals from ten families and 40 different species. Snake samples were collected from 420 animals from six families and 29 different species. Testudines samples were collected from 70 animals from two families and twelve different species. The samples were sent from different European countries as well as from the USA (see Table 17 in appendix). The samples were tested by PCR using the protocol described in chapter 3.2.2.

3.1.9.2 Plasma samples

Plasma was collected between 2006 and 2013. Lizard plasma was collected from 263 lizards, including twelve different species from five different families. Ten of 263 (3.8%) lizards were wild-caught helodermatid lizards, approximately 101/263 (38.4%) were captive-bred. The origins of the rest were unknown. Snake plasma was collected from 141 snakes from 28 different species from four families. 104 of 141 (75.2%) snakes were wild-caught, 2/141 (1.4%) were captive-bred and the origins of the rest of the snakes were unknown (see Table 18 in appendix). The plasma samples were tested for the presence of antibodies against different AdVs using neutralization tests (NT) described in chapter 3.2.14.

3.2 Methods

3.2.1 DNA preparation

Organ tissue or cotton heads of swab samples were sonicated in three ml of medium with a double concentration of antibiotics. Sonicated samples were centrifuged at 2,000 × g to remove cells debris and bacteria. DNA was extracted from 200 µl of the supernatant of previously sonicated samples, using the Qiagen DNAeasy® kit following the instructions of the manufacturer. Briefly, 200 µl of sonicated, centrifuged supernatant were mixed with 20 µl of proteinase-K and 200 µl of AL-Buffer, vortexed and incubated at 56°C for 10 min. Afterwards, 200 µl of Ethanol was added to the samples, then they were vortexed and transferred onto a two ml membrane column. After several washes with different buffers and centrifugation steps, DNA was collected on the membrane and diluted in 100 µl of AE-Buffer.

3.2.2 PCR screening

A consensus nested PCR described previously (Wellehan et al., 2004) was used for the screening of diagnostic samples. Two degenerate primer pairs (Table 6) based on a consensus sequence of a portion of the DNA-dependent DNA polymerase genes of different AdVs from three genera, were designed by Wellehan et al. (2004). This PCR amplifies an approximately 320 bp long portion of the polymerase gene. PCR mastermix was prepared as described in Table 7. The temperature protocol from Table 8 was used for the first and the second rounds of the PCR.

Table 6: PCR primers designed by Wellehan et al. (2004)
Used for detection of a portion of the DNA-dependent-DNA-polymerase gene of various AdVs

PCR round	Primer	Sequence
I	Pol-Fouter	5’- TNMGNGGNGGNMGNTGYTAYCC-3’
	Pol-Router	5’- GTDGCRAANSHNCCRTABARNGMRTT-3’
II	Pol-Finner	5’- GTNTWYGAYATHTGYGGHATGTAYGC-3’
	Pol-Rinner	5’- CCANCCBCDRTTRTGNARNGTRA-3’

Table 7: PCR protocol (Wellehan et al., 2004)

Reagent	Volume (µl)	Final concentration
I round of PCR		
Millipore (double-distilled water)	12.4	-
10x Buffer $(NH)_4SO_2$	2.5	1 ×
$MgCl_2$ (25 mM)	1.5	1.5 mM
dNTP (2 mM)	2.5	0.2 mM
Primer (Pol-Fouter) (10 mM)	2.5	1 mM
Primer (Pol-Router) (10 mM)	2.5	1 mM
Taq-Polymerase (5 U/µl)	0.125	0.6 U
Sample (DNA)	1	-
Final volume	25	
II round of PCR		
Millipore (double-distilled water)	10.875	-
10x Buffer $(NH)_4SO_2$	2.5	1 ×
$MgCl_2$ (25 mM)	1.5	1.5 mM
dNTP (2 mM)	2.5	0.2 mM
Primer (Pol-Finner) (10 mM)	2.5	1 mM
Primer (Pol- Rinner) (10 mM)	2.5	1 mM
Taq-Polymerase (5 U/µl)	0.125	0.6 U
Sample (DNA)	2.5	-
Final volume	25	

Table 8: Temperature protocol for the first and the second rounds of the PCR (Wellehan et al., 2004)

Time	Temperature	Function
Step 1 (1 Cycle)		
5 min	94°C	Initial denaturation
Step 2 (45 Cycles)		
30 sec	94°C	Denaturation
1 min	46°C	Annealing
1 min	72°C	Extension

Time	Temperature	Function
Step 3 (1 Cycle)		
7 min	72°C	Final extension
∞	4°C	Cooling

This nested PCR has been shown to be an effective tool for the detection of AdVs in reptiles (Benkő et al., 2006; Papp et al., 2009). As a positive control, DNA from a Snake AdV-1 isolated from a boa constrictor (Marschang et al., 2003) was used.

3.2.3 Gel electrophoresis

Electrophoresis was carried out on a 1.5% agarose gel, containing 0.5 µg ethidium-bromide in TAE-Buffer. PCR samples after the second round were mixed with 6×Ladder, and 10 µl of the mixture were placed into slots of the gel. Slots were positioned toward the minus pole. 5 µl of 100 bp DNA-ladder was added to one slot per row for size determination of the bands after electrophoresis. The samples were run for about 30 min at a voltage of 80 kV. Afterwards, the gel was visualized under 320 nm UV light.

3.2.4 Gel purification

All PCR amplicons of approximately 320 bp were cut out with a sterile scalpel blade and placed into 1.5 ml polypropylene tubes. The PCR amplicons were then purified with a peqGOLD Extraction Kit. Briefly, pieces of the gel with DNA were mixed with binding buffer and heated at 60°C until the gel was completely melted. Afterwards, the gel and DNA mixture was transferred into two ml membrane columns and after several centrifugation and washing steps, purified DNA was collected on the membrane, followed by elution in 30 µl of elution-buffer.

3.2.5 Sequencing

15 µl of purified sample, containing five ng per ml of DNA was placed into 1.5 ml polypropylene tubes and sent, together with 20 µl of primer, to the commercial company Eurofins MWG for sequencing.

3.2.6 Analysis of sequences

Raw sequences were processed by the ABI Sequence Analysis Programme 5.1.1 (Applied Biosystems, Foster City, USA). Then sequences were compared using the STADEN Package version 2003.0 Pregap4 and Gap4 programmes (Bonfield et al.,

1995). All sequences, after editing out primers, were compared to the data in GenBank (National Center for Biotechnology Information, Bethesda USA) online (http://www.ncbi.nih.gov) using BLASTX and BLASTN. For analysis of the sequences, multiple alignments were performed with the ClustalW algorithm of the BioEdit Sequence Aligment Editor programme (Hall, 1999) using default settings. For this, homologous nucleotide (nt) or amino acid (aa) sequences of DNA-dependent DNA polymerase genes of AdVs were retrieved from GenBank through the non-redundant AdV-database of the Molecular Virology Group at the Veterinary Medical Research Institute, Budapest (http://www.vmri.hu/~harrach). Amino acid sequences were used for phylogenetic calculation. Calculation of phylogenies using multiple methods is helpful for understanding the possible true topology of the resulting trees. Phylogenetic calculation was performed using Mr. Bayes analysis with the TOPAli v2 platform (Milne et al., 2008) as well as maximum likelihood (ML) method based on the Dayhoff matrix model (Schwarz et al., 1979) using the Mega 5.05 programme (Tamura et al., 2011) and Fitch–Margoliash method with global rearrangements, using PHYLIP program Package version 3.6. (Felsenstein, 1989).

3.2.7 Cell line from central bearded dragon embryos (BDE)

3.2.7.1 Establishment of BDE cells

Fertilized bearded dragon eggs were obtained from a private breeder. The eggs were incubated for six to eight weeks at 28°C and 75–85% humidity. The cells were prepared from a pool of 12 eviscerated six to eight-week-old central bearded dragon embryos (BDEs) as described previously for chicken embryo fibroblasts (Schat and Purchase, 1998). After the incubation period, the eggs were disinfected with 70% Ethanol and opened in a sterile workbench. BDEs were eviscerated and decapitated. Afterwards, BDEs were homogenized with scissors in a small amount of PBS buffer and transferred into 250 ml Erlenmeyer flask. Homogenates were stirred with PBS buffer on a magnet stirrer for five min at 100 rpm. Then, homogenates were let stand for ten min, and the supernatant was discarded. The same procedure was repeated three to four times with PBS buffer until the supernatant became clear. Afterwards, 1× trypsin solution was added to the cell sediment (to fill approximately 1/3 of the Erlenmeyer flask), stirred for 30 sec, let stand for ten min and then the supernatant was discarded. Trypsin was added again, stirred for ten min and let stand for another ten minutes. This supernatant was transferred into 50 ml polypropylene conical tubes

with one ml of FCS and centrifuged at 800 rpm for ten min. This process was repeated three times. The supernatant from the 50 ml tubes was discarded and cells in the sediment were resuspended in five ml growth medium. Afterwards, two ml of the cell suspension were transferred into a 30 mm-diameter tissue culture dish and the density of the cells was controlled with an inverted light microscope. Since cell density seemed to be high, the cell suspension was diluted 1:2 with growth medium. Then two ml of diluted cell suspension were added to each 30 mm-diameter tissue culture dish. The fibroblasts were grown at 28°C, with 5% CO_2 and 85% humidity. After reaching confluency the cells were transferred into 25cm^2 cell culture flask.

3.2.7.2 Passaging of cells

3.2.7.2.1 Passaging of IgH2, VH-2 and TH-1 cell lines

These cell lines were grown in 75cm^2 cell culture flasks at 28°C in an atmosphere of 5% CO_2. Every five to seven days the cells were sub-cultured at a split ratio of 1:5. For sub-culturing, growth medium was removed, the cells were washed twice with five ml of 0.05% trypsin–versene. After the second wash, four ml of trypsin versene were removed. Cells were incubated at room temperature with the remaining one ml of trypsin versene and detached from the tissue culture flask bottom. Then four ml of growth medium were added and mixed thoroughly with a glass pipette. Afterwards, four ml of the cell and medium suspension were removed and 20 ml of the growth medium was added. The cell lines were also used for the preparation of 30 mm-diameter tissue culture dishes, wherefore one ml of the cells and medium suspension were mixed with ten ml of growth medium and two ml of diluted suspension were added to each dish. For the preparation of 96-well microtest cell culture plates, 100 µl of diluted suspension were added to each well.

3.2.7.2.2 Passaging of BDE cell line

The BDE cells were passaged after reaching confluency, approximately 2 days after seeding as described above. The cells were first passaged into 25 cm^2 tissue culture flasks and later into 75 cm^2 tissue culture flasks. Afterwards, the cells were passaged every week as described above, at a split ratio of 1:2.

3.2.7.3 Calculation of density

Calculation of the cell density was performed using a hemocytometer. Cell medium suspension, which was collected during sub-culturing of the cells (see above), was

used for the calculation. The hemocytometer and a cover slip were cleaned with 70% ethanol. The cover slip was placed on the hemocytometer and cell suspension was added with a pipette. The hemocytometer was then placed under an inverted light microscope, cells in four large squares of the hemocytometer were counted and the average number of cells per square was multiplied by 10^4 to calculate the cell concentration per ml (Lindl, 2002).

3.2.8 Isolation of viruses

For snake samples the VH-2 cell line, for lizard samples the IgH2 cell line, for chelonian samples TH-1 cell line, and for bearded dragon samples the BDE cell line were used for virus isolation. 200 µl of the supernatant of the previously sonicated and centrifuged samples (see 3.2.1) were inoculated onto approximately 70% confluent, 1 day old cell monolayers in 30 mm-diameter tissue culture dishes from which the medium had been removed. The dishes were then incubated for two hours at 28°C after which two ml of nutrient medium was added to each dish. Cells were examined for cytopathic effects (CPE) every three days with an inverted light microscope. In positive cases, extensive CPE was seen seven to ten days after inoculation, and the dishes were frozen at –80°C. Dishes showing no CPE were also frozen at –80°C after two weeks of incubation for blind passaging. Additional passages were performed after a single freeze (–80°C)-thaw (room temperature) cycle and low speed centrifugation. The dishes demonstrating CPE were also tested by PCR (see 3.2.2) for the presence of AdV. For this, DNA was extracted from 200 µl of the cell culture supernatant.

3.2.9 Electron microscopy

Electron microscopic examination was performed with bearded dragon virus isolates. For this, cell culture supernatants were negatively stained with 2% potassium phosphotungstate at pH 7.3 on 3.05 mm copper grids. The isolates were examined with a JEM-1011 transmission electron microscope for the presence of viral particles. Electron microscopic examinations were carried out by Dr. Marc Hoferer at the Chemisches und Veterinäruntersuchungsamt (CVUA) Stuttgart, Germany.

3.2.10 Propagation of viruses

All viruses were propagated in cell lines in which each virus was originally isolated, until achievement of an appropriate titer for NT. Each cell line was sub-cultured in 175cm^2 cell culture flasks. After 24 hours of incubation, when the cell monolayer was

70% confluent, the growth medium was removed and 5 ml of virus suspension was added onto the cell monolayer and incubated for two hours at 28°C. Afterwards, 50 ml of nutrient medium was added to the flask and incubated at 28°C and 5% CO_2. In the case of Helodermatid AdVs, nutrient medium without FCS was added. The flasks were checked after 24 hours for cytopathic effects (CPE). Complete destruction of the cell monolayer occurred within two to ten days, after which the flasks were placed in the freezer at –80°C. The virus suspension was then thawed, removed from the flasks, centrifuged at 3,000 × g for 15 min, aliquoted in 10 and 50 ml sterile plastic tubes and stored at –80°C.

3.2.11 Virus titration

The virus titrations were carried out by end point serial dilution method in 96-well microtitration plates. 0.9 ml of nutrient medium was added to each of eight sterile plastic tubes. 0.1 ml of the virus suspension was added to the first tube, mixed by vortexing and 0.1 ml of the suspension was transferred to the next tube. Serial tenfold dilutions were made from $1:10^0$ to $1:10^8$. 70% confluent 96-well cell culture plates were used for the titrations. Growth medium was removed from the plates and 100 µl of the virus-containing nutrient medium was added to the respective wells of the cultured cells (four wells per dilution step). Plates with snake and helodermatid AdVs were incubated for ten days and plates with central bearded dragon AdVs were incubated for 10-14 days at 28°C and 5% CO_2 and checked afterwards for CPE by light microscopy. A complete destruction of the cell monolayer was considered as positive for virus growth. Mean tissue culture infective dose ($TCID_{50}$) values were calculated by the Spearman-Kärber method (Villegas, 1998).

3.2.12 Purification of lizard AdVs

Helodermatid AdV-1 and -2 were purified by equilibrium centrifugation in CsCl gradients. 40 cell culture flasks (175cm^2) of each virus were propagated. Viruses were propagated until first signs of CPE were seen (one to two days post inoculation). The cells, which were not detached at the time of harvesting, were removed from the cell culture flasks by hitting the side of the flasks. Infected cells were placed into 50 ml sterile plastic tubes and centrifuged for 30 min at 1,000 rpm. Afterwards, the medium was carefully removed with a glass pipette and the cells were resuspended in 17 ml of 10 mM Tris-Cl (pH 8.1). Cells were destroyed using four freeze-thaw cycles in liquid nitrogen and then centrifuged at 3,000 rpm for one

hour. Supernatant was collected and the first gradient was prepared by adding ten ml of heavy CsCl (4°C) to Beckman plastic tubes (25×89 mm). Ten ml of light CsCl was then added on top. Virus containing supernatant was added above the CsCl. Centrifuge buckets of the rotor (SW 28) were balanced using Tris-Buffer and precooled together with the rotor to 4°C. Viruses were ultracentrifuged for two hours at 53,000 x g at 4°C. After ultracentrifugation, two white bands were seen. Full virus particles appeared as the lower of the two bands. To collect the virus particles the side of the Beckman plastic tube was punctured with a 18½ gauge needle and the lower band was collected. The upper band, which represented empty virus particles, was then collected with another needle into a separate tube. Virus was then diluted with 10 mM Tris to a final volume of four ml. A second column purification was prepared as described above, but in this case, four ml each of heavy and light CsCl were added to each Beckman plastic tube (13×51 mm) as well as the four ml of diluted virus from the first round. The tubes were placed into the buckets of the SW-41 Ti rotor and the viruses were ultracentrifuged over night at 49,000 x g at 4°C. Afterwards, the side of the tube was punctured with a needle as described to collect the lower viral band. A 10 DG disposable chromatography column was then placed into a stander, buffer in the column was poured out and 20 ml of HBS solution was added to flow through the column. When the last of HBS had run through, the virus collected from the gradient was added. Afterwards, one ml fractions were collected as the virus entered the column. When the virus had completely entered the column, ten ml of HBS was added and collection of 0.5 ml fractions continued. The A_{260} of all fractions were measured by spectrophotometer to determine the presence of DNA. Virus fractions which demonstrated the presence of DNA were collected in one tube and diluted 1:10 in HBS with 10% glycerol and stored at -80°C. After purification, viruses were titrated as described above.

3.2.13 Polyclonal antibodies from rabbits

The purified viruses (described in 3.2.12) Helodermatid AdV-1 with a titer of $ID_{50}=10^{5.5}$ $TCID_{50}$ per ml and Helodermatid AdV-2 with a titer of $10^{6.5}$ $TCID_{50}$ per ml were used to inoculate rabbits. Two rabbits were used for each virus. The initial immunization was performed via intradermal injection with 500 µl of purified virus suspension combined with 500 µl of adjuvant (saponin). The immunization was repeated after two and four weeks. Blood was collected from the saphenous vein of each animal before each immunization. The final hyperimmune plasma was collected

every week after final immunization, over a period of three weeks. The inoculation of the rabbits was carried out following notification of the appropriate committee (Landesdirektion Leipzig, Referat 24) as an “Anzeige von Eingriffen und Behandlungen zur Herstellung, Gewinnung, Aufbewahrung oder Vermehrung von Stoffen, Produkten oder Organismen” according to §10a of the German Animal Protection Law. Date of the notification: November 30, 2009. It was carried out at the Veterinary Medical Faculty of the University of Leipzig.

3.2.14 Neutralization tests

All plasma samples were clarified by low speed centrifugation (800 x g) for ten min and incubated at 56°C for 30 min. Plasma from the rabbits were first tested for the presence of antibodies against the Helodermatid AdVs-1 and -2 by NT. These plasma were used as positive controls for all NTs.

Lizard and snake plasma were pre-diluted 1:10 with PBS buffer to avoid toxic effects for the cells and to allow testing for antibodies against a wider range of viruses. The plasma were tested against five virus isolates by NT (Table 5). 96-well tissue culture plates were used for NT. 25 µl of nutrient medium were pipetted to each well. Afterwards, 25 µl plasma were added in the first well, mixed with the medium and 25 µl of mixture were transferred to the next well. Thus, plasma were serially diluted from 1:20 until 1:2560. 25 µl of virus suspension containing 100 $TCID_{50}$ of virus was added to the plasma in each well. The tissue culture plates were incubated for two hours at 28°C in an atmosphere of 5% CO_2. Afterwards, for NTs with Snake AdVs-1 and -2 freshly sub-cultured VH-2 cells were added to each well. For the NTs with Helodermatid AdVs-1 and -2 LgH2 cells and for NTs with Agamid AdV-1 BDE cells were added to each well. The plates were read after 10-14 days. A titer ≥ 1:20 was considered significant.

3.2.15 Statistical analysis

General linear models and general linear mixed models (GLMM) were performed using the procedures PROC GENMOD and PROC GLIMMIX of SAS software Version 9.4 (SAS Instituts, 2013). The GLMM allows the calculation of odds ratios and relative risks for significant effects. Separate analyses were performed for lizards and snakes. For both we assumed that sampled animals were independent from one another. First we compared the probability of finding antibodies depending on

families, species, sex and habitat for each virus. We assumed that data follow a binomial distribution, therefore we used the logit link. We fitted fixed main effects for families, species within families, sex, and habitat and tested them using an F-test. In a second analysis, we wanted to test whether the probabilities for finding antibodies against viruses are independent or not. We therefore fitted a loglinear model and included fixed main effects for four viruses and all possible interactions between them. We performed a likelihood ratio test for testing main effects and interactions.

4 Results

4.1 PCR screening

4.1.1 Reptilian samples tested during this study

During this study 1075 samples (tissues or swabs) from 668 lizards were tested by PCR for the presence of adenoviruses. 78 (7.3%) lizard samples from 71 (10.6%) animals demonstrated positive results. In addition, 786 snake samples from 420 snakes were tested by PCR. 35 (4.5%) snake samples from 26 (6.2%) animals showed positive results. 88 chelonian samples from 70 tortoises and turtles were screened and 3 (3.4%) samples from 3 (4.2%) animals demonstrated the presence of AdVs (see Table 17 in appendix). Thus, AdVs were detected in captive-bred and wild-caught reptiles as well as in reptiles with different clinical signs and apparently healthy animals (Tables 9, 10, 11).

4.1.2 Newly detected AdVs

Screening of lizard, snake and chelonian samples for AdVs using PCR and sequencing of the PCR products led to the detection of six new lizard AdVs (Table 9), one new snake AdV (Table 10) and three new chelonian AdVs (Table 11) in this study. Thus, AdVs were detected in the intestine samples of the common agama (168/3/10) – Common agama AdV-1, in green anole (59/12) – Anolis AdV-3, in the liver sample of the Jackson's chameleon (117/12) – Chameleon AdV-2 and in intestine samples of three out of five green striped tree dragons (96/11) - Green striped tree dragon AdV-1. Also new AdVs were detected in two different species of *Varanid* lizards: in liver and brain samples of a blue tree monitor (55/12) - Blue tree monitor AdV, and in two water monitors in intestine, skin, and liver samples of one of two animals (64/1/12) and in gastrointestinal tract and brain sample of the other animal (64/2/12) – Water monitor AdV (Table 9). The newly described snake AdV - Boa constrictor AdV was detected in kidney, lung, and intestine samples of a boa constrictor (57/3/08) (Table 10). Three new chelonian AdVs were found in mixed oral and cloacal swabs of a pond slider (106/36/11) – Pond slider AdV, a Hermann's tortoise (106/41/11) – Hermann's tortoise AdV and in a cloacal swab of an Alabama map turtle (86/17/11) – Alabama map turtle AdV (Table 11).

Table 9: Lizard AdVs detected during this study by PCR
Case histories, results of virological screening, parasitological, bacteriological, cytological and coprological examinations (* new AdVs)

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
85/08	Central bearded dragon (*Pogona vitticeps*)	No information available	oral swab	+	n. d.	+	n. d.	Agamid AdV-1	IIV isolated (lgH2)	
			cloacal swab	+	n. d.	+	n. d.			
57/09	Central bearded dragon	No information available	oral swab	+	n. d.	+	n. d.	Agamid AdV-1	IIV isolated (lgH2)	
			cloacal swab	+	n. d.	+	n. d.			
75/8/10	Central bearded dragon	No information available	cloacal swab	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	
75/11/10	Central bearded dragon	No information available	cloacal swab	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	
75/14/10	Central bearded dragon	No information available	cloacal swab	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	
75/16/10	Central bearded dragon	No information available	cloacal swab	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	
92/10	Central bearded dragon	Captive-bred animal. Demonstrated central nervous system signs consisted of tremor and circling	oral/ cloacal swab	+	-	-	n. d.	Agamid AdV-1	AdV isolated (BDE)	
120/10	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
126/10	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
148/10	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
158/3/10	Central bearded dragon	No information available	cloacal swab	+	+	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
158/3/10	Central bearded dragon	No information available	cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
168/3/10	Common agama (*Agama agama*)	2-year-old male, purchased at a reptile trade fair. The animal was believed to have been wild-caught. Animal was severely dehydrated and emaciated with abscess in the oral cavity causing osteolysis.	liver	-	-	-	n. d.	Common agama AdV-1*	No viruses isolated	Bacteriological testing demonstrated a moderate number of *Proteus vulgaris* and *Morganella morganii* (*Proteus morganii*) in the oral cavity. Fecal examinations (native and flotation methods) showed *Choleoeimeria sp.* oocysts and pinworm eggs.
			kidney	-	-	-	n. d.			
			heart	-	-	-	n. d.			
			intestine	+	-	-	n. d.			
7/11	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
28/11	Central bearded dragon	No information available	cloacal swab	+	-	-	-	Agamid AdV-1	No viruses isolated	
34/11	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	-	Agamid AdV-1	No viruses isolated	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
47/1/11	Central bearded dragon	Captive-bred animal with clinical signs of egg binding	oral swab	+	-	n. d.	-	Agamid AdV-1	AdV isolated (BDE)	
			cloacal swab	-	-	n. d.	-		No viruses isolated	
72/3/11	Central bearded dragon	Animal from Reptile Rescue Station (Munich). Did not show any clinical signs during captivity	cloacal swab	+	-	n. d.	+	Agamid AdV-1	No viruses isolated	
87/11	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
96/1/11	Green striped tree dragon *(Japalura splendida)* animal 1	Group of animals imported from China via Florida to Germany. Demonstrated dehydration, lethargy, CNS signs and dermatitis. Gross pathology: haemorrhagic, oedemateous gastrointestinal tract, ecchymotic haemorrhages in the fatty tissue and the liver.	skin	-	-	+	-	Green striped tree dragon AdV-1*	No viruses isolated	Coprological examination demonstrated the presence of flagellates, *Heterakis spp., Spirurida spp., Choleoeimeria spp.*and larval ascarids (probably *Hexametra spp*.)
			lung	-	-	+	-			
			liver and kidney	-	-	-	-			
			intestine	+	-	-	-			
96/2/11	animal 2		skin	-	-	+	-			
			lung	-	-	+	-			
			liver and kidney	-	-	+	-			
			intestine	+	-	+	-			
96/5/11	animal 3		skin	-	-	+	-			
			lung	-	-	+	-			
			liver and kidney	-	-	-	-			
			intestine	+	-	+	-			

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
106/28/11	Central bearded dragon	Animal from Reptile Rescue Station (Munich). Clinically healthy.	oral/ cloacal swab	+	-	n. d.	-	Agamid AdV-1	No viruses isolated	
106/29/11	Central bearded dragon	Animal from Reptile Rescue Station (Munich). Clinically healthy.	oral/ cloacal swab	+	-	n. d.	-	Agamid AdV-1	No viruses isolated	
106/30/11	Central bearded dragon	Animal from Reptile Rescue Station (Munich). Clinically healthy.	oral/ cloacal swab	+	-	n. d.	-	Agamid AdV-1	No viruses isolated	
106/31/11	Central bearded dragon	Animal from Reptile Rescue Station (Munich). Clinically healthy.	oral/ cloacal swab	+	-	n. d.	-	Agamid AdV-1	No viruses isolated	
119/11	Central bearded dragon	Sample sent from University Leipzig. Obtained from animal with high obstipation and inapetance	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
127/11	Central bearded dragon	Male with bloody diarrhea.	cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	Bacteriological examination: Salmonella were detected
135/62/11	Central bearded dragon	No information available	stomach	+	-	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
135/63/11	Central bearded dragon	No information available	intestine	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
			brain	-	-	n. d.	n. d.			
20/12	Central bearded dragon	No information available	crusts	-	-	-	n. d.	Agamid AdV-1	No viruses isolated	
			oral/ cloacal swab	+	-	-	n. d.			

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
21/12	Central bearded dragon	No information available	heart	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	
			lung	-	-	-	n. d.			
			kidney	+	-	-	n. d.			
			liver	-	-	-	n. d.			
			intestine	-	-	-	n. d.			
33/12	Central bearded dragon	No information available	oral swab	+	-	-	-	Agamid AdV-1	No viruses isolated	
			cloacal swab	-	n. d.	-	-			
38/1/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/2/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/5/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/6/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/17/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/20/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
38/21/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
38/23/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/24/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
38/30/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/35/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/36/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/37/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/38/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
38/39/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/40/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
38/41/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/43/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
38/44/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/45/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/46/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/48/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	AdV isolated (BDE)	
38/49/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/54/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/56/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/58/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
38/60/12	Central bearded dragon	Clinically healthy. Captive bred. Sent from USA	oral swab	+	n. d.	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
39/12	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	AdV name	Virus isolation (LgH2, BDE)	Other findings
55/12	Blue tree monitor (*Varanus* (*Euprepios aurus*) *macraei*)	Virus detected in one of two wild-caught animals. Clinical signs: acute spasms and death.	liver	+	+	n. d.	-	Blue tree monitor AdV (Varanid AdV-1-like)*	No AdVs isolated	
			brain	+	+	n. d.	-			
59/12	Green anole *(Anolis carolinesis)*	Imported from Florida to Germany, obtained from a group of animals, which demonstrated weakness, dermatitis, and increased mortality rate.	skin	-	+	+	n. d.	Anolis AdV-3*	No AdVs isolated	Parasitological examination demonstrated the presence of flagellates, *Coccidia,* oxyurids*, Oochoristica spp.*
			liver	-	+	-	n. d.			
			intestine	+	+	-	n. d.			
64/1/12	Water monitor (*Varanus salvator*) animal 1	Obtained from a group of animals with 70% morbidity and 100% mortality. Gross lesions: poor body condition, dehydration, prominent spinous processes with no detectable subcutaneous and intra-coelomic body fat, markedly sunken eyes, wrinkling and tenting of the skin with tacky membranes, reddening around the mouth and gingiva with sloughing of skin and yellow plaques. Mucoid material with numerous of white worms in stomach and small intestine. Flattened worm admixed with mucoid material Within the small and large intestine. Distention of the cloaca, with dry, brown, granular feces in the coprodaeum and dry, white, granular urates in the urodaeum.	formalin fixed tissues	-	-	-	n. d.	Water monitor AdV*	No AdVs isolated	Histological examination: marked subacute to chronic, erosive and ulcerative, necrotizing stomatitis, rhinitis, sinusitis and esophagitis.
			intestine	+	-	-	n. d.			
			skin	+	-	+	n. d.			
			liver	+	-	-	n. d.			
			lung	-	-	-	n. d.			
64/2/12	animal 2		gastrointestinal tract	+	-	+	n. d.		No AdVs isolated	
			head	-	-	+	n. d.			
			lung+heart	-	-	+	n. d.			
			liver + kidney	-	-	-	n. d.			
			brain	-	-	-	n. d.			

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	IIV PCR	PMV PCR	PMV PCR	Virus isolation (LgH2, BDE)	Other findings
78/12	Central bearded dragon	No information available	cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
91/12	Central bearded dragon	No information available	oral/ cloacal swab	+	-	n. d.	n. d.	Agamid AdV-1	No viruses isolated	
117/12	Jackson's Chameleon *(Chamaeleo jacksonii)*	Cachexia. Gross pathology: diffusely black coloured liver. Pathohistologically: melano phagocyte hyperplasia of liver, lungs and spleen, mild diffuse lymphocytic infiltrations in the small intestine, few to moderate numbers of multifocal eosinophilic and basophilic large intranuclear inclusion bodies within enterocytes.	liver	+	-	+	n. d.	Chameleon AdV-2*	No AdVs isolated	Bacteriological testing: *Proteus vulgaris* in liver, lungs, heart and intestine. *Salmonella* Blijdorp in the intestine. Cytological examination (DiffQuik): liver, lung, kidney: Melano macrophages heterophilic granulocytes, lymphocytes, macrophages, uniform small bacillary bacteria.
37/13	Central bearded dragon	No information available	oral swab	-	-	n. d.	-		No viruses isolated	
			cloacal swab	+	-	n. d.	-	Agamid AdV-1		
61/13	Blue tree monitor (*Varanus* (*Euprepiosaurus) macraei*)	Obtained from animal with crusts on the lips. The tissue seems to grow invasively in a cone shape.	crusts in NaCl	+	-	-	n. d.	Varanid AdV-1	No AdVs isolated	
85/13	Central bearded dragon	No information available	pooled organs	+	-	-	n. d.	Agamid AdV-1	No viruses isolated	

Table 10: snake AdVs detected during this study by PCR
Case history, results of virological screening, mycological, and cytological examinations (* new AdVs)

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	Reo PCR	PMV PCR	AdV name	Virus isolation (VH-2)	Other findings
57/3/08	Boa constrictor (*Boa constrictor*)	Obtained from a group of animals, which was imported from the USA. Demonstrated vomiting and rapid mortality. Gross lesions: stomach moderately filled with turbid water with flocculent content. Small intestine and rectum highly diffuse white plaque, which cannot be removed without loss of substance, mucosa highly thickened. Genitals: juvenile. Pathohistology: in liver, activation of Kupffer cells with hemosiderinphagocytosis, in intestine subacute diphtheroid ulcerative enteritis	kidney	+	-	n. d.	-	Boa constrictor AdV (Snake AdV-1-like AdV)*	No AdVs isolated	Cytological examination (DiffQuik): heterophilic granulocytes, lymphocytes, macrophages in liver. Intestine: *Hypotrihomonas* sp., *Proteromonas* sp., *Proteus vulgaris*. Stomach: *Proteromonas* sp. Liver, lungs, kidney, heart: *Pseudomomonas aeruginosa*. Native preparations: Stomach, small intestine, rectum: *Trichomonas* sp., *Proteromonas* sp. Mycological examination: Intestine: *Candida albicans*.
			lung	+	-	n. d.	-			
			intestine	+	-		-			
105/12/08	Indian python (*Python molurus*)	Without any clinical signs	oral/ cloacal swab	+	n. d.	-	-	Snake AdV-GER09 (Snake AdV-1-like AdV)	AdV isolated (VH-2)	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	Reo PCR	PMV PCR	AdV name	Virus isolation (VH-2)	Other findings
105/16/08	Indian python	Died after a period of inappetence	oral/ cloacal swab	+	n. d.	-	-	Snake AdV-GER09 (Snake AdV-1-like AdV)	AdV isolated (VH-2)	Pathological and bacteriological investigation were inconspicuous
6/3/09	Eastern corn snake (*Pantherophis guttatus*)	Died in a new collection	lungs	-	n. d.	-	-	Snake AdV-2	PMV isolated	
			kidney	+	n. d.	-	+			
			intestine	+	n. d.	-	+			
14/2/09	Eastern corn snake	Same collection that 6/3/09. Swabs taken one month later from survivors.	oral/ cloacal swab	+	n. d.	-	+	Snake AdV-1	AdV isolated	
14/3/09	Eastern corn snake		oral/ cloacal swab	+	n. d.	-	+	Snake AdV-1	Reov isolated	
54/1/09	Eastern corn snake	Clinically healthy	oral/ cloacal swab	+	n. d.	-	-	Snake AdV-3	No viruses isolated	
54/4/09	Eastern corn snake	Clinically healthy	oral/ cloacal swab	+	n. d.	-	-	Snake AdV-1	AdV isolated	
54/6/09	Eastern corn snake	Clinically healthy	oral/ cloacal swab	+	n. d.	-	-	Viperid AdV	AdV isolated	
59/09	Eastern corn snake	No information available	lungs	+	n. d.	n. d.	-	Snake AdV-1	AdV isolated	
			kidney	+	n. d.	n. d.	-			
			intestine	+	n. d.	n. d.	-			

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	Reo PCR	PMV PCR	AdV name	Virus isolation (VH-2)	Other findings
60/09	Eastern corn snake	No information available	lungs	+	n. d.	n. d.	+	Snake AdV-1	AdV isolated	
			kidney	-	n. d.	n. d.	-			
			intestine	-	n. d.	n. d.	-			
136/1/10	Eyelash Viper (*Bothriechis schlegelii*)	No information available	oral/ cloacal swab	+	n. d.	n. d.	-	Snake AdV-1	No viruses isolated	
136/4/10	Eyelash Viper	No information available	oral/ cloacal swab	+	n. d.	n. d.	-	Snake AdV-1	No viruses isolated	
152/10	Eastern corn snake	Demonstrated clinical signs of respiratory disease	oral/ cloacal swab	+	n. d.	n. d.	-	Snake AdV-2	AdV isolated	
30/11	Eastern corn snake	A group of 10 adult animals was bought from a zoological shop. 6 snakes died within the first 4-5 months. Pathological examination: 4 years old male corn snake was in a bad body condition, intestinal wall of rectum was edematous thickened and contained yellow crumbly soluble content, which couldn't be removed without loss of substance as well as edema in submucous. *Monocercomonas* sp. were found in intestinal contents. Histopathologically: in rectal mucosa villous atrophy, inflammatory reaction with fibrin and heterophile and lymphocytic infiltration. In the inflamed mucosa flagellates and yeast (Candida) were found.	lungs, kidney and intestine mixed	+	-	n. d.	-	Viperid AdV	AdV isolated	Microbiological examination: *Candida albicans* was detected and in a high amount.

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	Reo PCR	PMV PCR	AdV name	Virus isolation (VH-2)	Other findings
		No intranuclear inclusion bodies in the epithelium of the intestine. A diphteroid colorectitis and mild myoglobinurische pigmentnephrosis were diagnosed.								
35/11	Boa constrictor (*Boa constrictor ortonii*)	Dermatitis was observed. Mites were detected	oral/ cloacal swab	+	-	n. d.	-	Viperid AdV	No viruses isolated	Bacteriological testing: Salmonella were detected
104/1/11	Eastern corn snake	No information available	oral/ cloacal swab	+	-	n. d.	-	Snake AdV-2	AdV isolated	
104/2/11	Eastern corn snake	No information available	oral/ cloacal swab	+	-	n. d.	-	Snake AdV-2	AdV isolated	
104/3/11	Eastern corn snake	No information available	oral/ cloacal swab	+	-	n. d.	-	Snake AdV-2	AdV isolated	
104/4/11	Eastern corn snake	No information available	oral/ cloacal swab	+	-	n. d.	-	Snake AdV-2	AdV isolated	
122/11	Indian python	No information available	oral/ cloacal swab	-	n. d.	n. d.	-		No viruses isolated	
			brain	-	n. d.	n. d.	-			
			lung	-	n. d.	n. d.	-			
			pancreas	-	n. d.	n. d.	-			
			kidney	+	n. d.	n. d.	-	Snake AdV-1		
			intestine	-	n. d.	n. d.	-			
19/2/12	Eastern corn snake	No information available	oral/ cloacal swab	+	n. d.	n. d.	-	Snake AdV-1	No viruses isolated	

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	Reo PCR	PMV PCR	AdV name	Virus isolation (VH-2)	Other findings
25/4/12	Indian python	No information available	pancreas	-	-	n. d.	-		No viruses isolated	
			kidney	-	-	n. d.	-			
			lung	-	-	n. d.	-			
			intestine	+	-	n. d.	-	Viperid AdV		
25/5/12	Eastern corn snake	No information available	lung	+	-	n. d.	-	Viperid AdV	AdV isolated	
			intestine	+	-	n. d.	-			
			kidney	+	-	n. d.	-			
74/3/12	Common death adder (*Acanthophis schistos*)	No information available	liver	-	-	n. d.	-			
			kidney	-	-	n. d.	-			
			intestine	-	-	n. d.	-			
			lung	+	-	n. d.	-	Viperid AdV	No viruses isolated	
48/1/13	Eastern corn snake	No information available	heart	+	-	n. d.	-	Snake AdV-1	AdV isolated	
			intestine	+	-	n. d.	-			
			liver	+	-	n. d.	-			

Table 11: Chelonian AdVs detected during this study by PCR
Case history, results of virological screening (* new AdVs)

Lab. No.	Species	Case history	Sample	AdV PCR	Rana PCR	HV PCR	AdV name	Virus isolation (TH-1)	Other findings
106/36/11	Pond slider (*Trachemys scripta*)	Animal from Reptile Rescue Station (Munich). Clinically healthy	oral/cloacal swab	+	-	-	Pond slider AdV*	No AdVs isolated	-
106/41/11	Hermann's tortoise (*Testudo hermanni*)	Animal from Reptile Rescue Station (Munich). Demonstrated clinical signs of pneumonia	oral/cloacal swab	+	-	-	Hermann's tortoise AdV*	No AdVs isolated	-
86/17/12	Alabama map turtle (*Graptemys pulchra*)	No information available	cloacal swab	+	-	-	Alabama map turtle AdV*	No AdVs isolated	-

4.1.3 Sequence analysis

All of the obtained AdV sequences were 272 nt long after editing out the primers. The nt and aa sequences of newly detected AdVs were used to construct an identity matrix (see Table 19 in appendix). The AdV detected in a group of green striped tree dragons had the highest aa identity value of the partial DNA-polymerase sequence with Helodermatid AdV-1 (GenBank acc. No. AAS89696) and Eublepharid AdV (AY576677) (71.1%), while nt identity was highest with Helodermatid AdV-1 (68.7%). The AdV from the green anole showed the highest aa identity with Agamid AdV-1 (DQ077706), and Anolis AdV-2 (KC544016) (70.3%), although nt identity was highest with the newly detected green striped tree dragon AdV (65.3%). The AdV detected in the Jackson's chameleon had the highest aa and nt identity (71.1% and 63.1%) with Agamid AdV-1 (DQ077706). The AdV detected in a common agama showed highest aa and nt identity (74.4% and 65.5%) with Eublepharid AdV. The AdV detected in a boa constrictor showed the highest aa and nt identity with the Snake AdV-1 (AAL89790) (95.5% and 91.8%). The AdV detected in a water monitor demonstrated the highest aa identity with the previously detected Varanid AdV (ACH86253) (80%) and the highest nt identity (73.4%) with the newly detected blue tree monitor AdV. The AdV detected in a blue tree monitor showed the highest aa and nt identities (98.8% and 89.6%) with Varanid AdV. The Alabama map turtle AdV demonstrated the highest aa and nt identities (91.2% and 87.2%) with the newly detected pond slider AdV, while the pond slider AdV showed the highest aa and nt identities (94.5% and 92.3%) with the previously described Red eared slider AdV (JN632580). The Hermann's tortoise AdV demonstrated the highest aa and nt identities (64.1% and 53.4%) with the previously detected Pancake tortoise AdV (JN632575). 61 AdVs detected in central bearded dragon samples demonstrated 99-100% aa and nt identity of the partial DNA polymerase gene sequence to Agamid AdV-1. In one case an AdV detected in a blue tree monitor (61/13) demonstrated 100 % aa and 99% nt identity to Varanid AdV-1 (ACH86253). Several different AdVs were detected in snake samples. AdVs detected in two Indian pythons (105/12/08 and 105/16/08) had 100% aa and nt identity to Snake AdV-Ger09 AdV (HQ148088). Also an AdV 100% identical to Snake AdV-1 in aa and nt sequences was found in seven eastern corn snakes, two eyelash vipers and in one Indian python. Also in six eastern corn snakes AdVs, which demonstrated 100% aa and nt identity to Snake AdV-2 (ACH91014) were detected. Three AdVs found in three eastern corn snakes, an AdV found in the

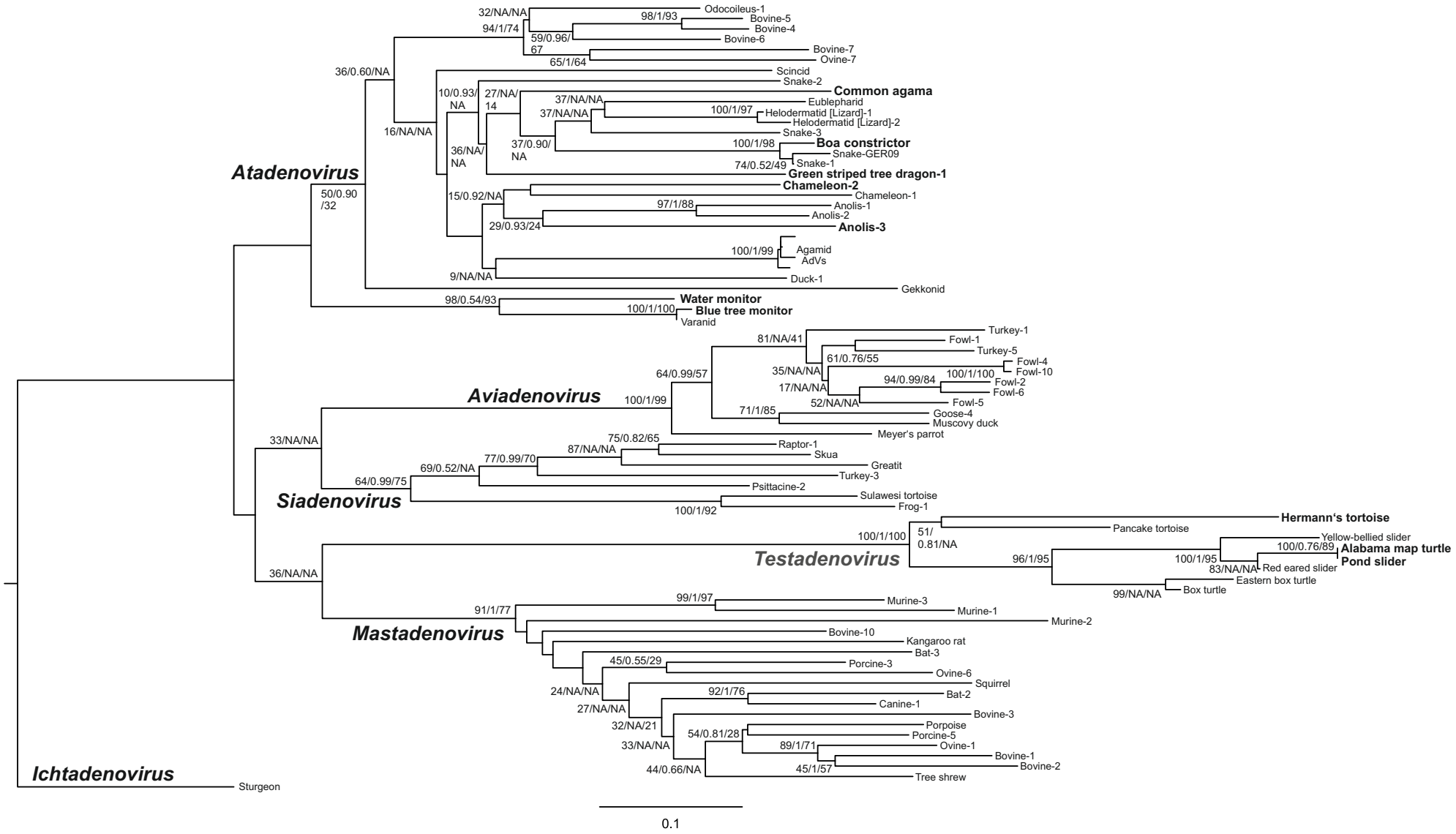
common death adder, Indian python and common boa constrictor demonstrated 100% aa and nt identity to Viperid AdV (EU914209). An AdV detected in an eastern corn snake (54/1/09) demonstrated 100% nt and aa identity to the Snake AdV-3 (FJ012164). All of the newly detected AdVs were named according to the reptile species in which they were found.

75 amino acid sequences (92 amino acids long) were used for the calculation of a phylogenetic tree (Figure 1). Phylogenetic analysis clustered the newly detected Green anole AdVs, Jackson's chameleon AdV, Green striped tree dragon AdV, Common agama AdV, and Boa constrictor AdV within the genus *Atadenovirus*. The Varanid AdVs clustered outside of all accepted genera together with the previously detected Varanid AdV-1. The newly detected chelonian AdVs all clustered within the proposed genus "Testadenovirus" (Doszpoly et al., 2013), together with previously described chelonian AdVs. Phylogenetic analysis of the newly detected Anolis AdV-3 demonstrated that this virus belongs to a clade that includes the sequences from previously reported Anolis AdV-1 and -2 and Chameleonid AdV-1. The sequence of the new Chameleonid AdV-2 belongs to the same clade, although the sequence demonstrated the highest identity to Agamid AdV-1. This clade is, however, not well supported. The newly detected Boa constrictor AdV clusters together with Snake AdV-1, which was well supported by all phylogenetic analyses. The newly detected Green striped tree dragon, Common agama and Boa constrictor AdVs form a well-supported clade together with Snake AdV-1 as well as other snake, gecko, helodermatid, and agamid AdVs. The relationships between the newly detected Anolis AdV-3 and two previously detected Anolis AdVs, along with both chameleonid AdVs within one clade, and kinship between newly described Green striped tree dragon AdV, Common agama AdV and Boa constrictor AdV and other agamid, helodermatid, snake, and gecko AdVs in another clade are all well supported by the Bayesian posterior probability values. The newly detected Blue tree monitor AdV clusters together with the previously detected Varanid AdV-1 and forms a well-supported cluster together with the newly detected Water monitor AdV. All monitor AdVs together seem to form a paraphyletic cluster with the *Atadenoviruses*. However, this cluster is not well supported.

All three of the chelonian AdVs detected cluster in the proposed genus “Testadenovirus”. The newly detected Alabama map turtle AdV and Pond slider AdV cluster together and this kinship is well supported. These two newly detected AdVs form a well supported clade together with the previously described Red eared and Yellow-bellied slider AdVs. The Hermann’s tortoise AdV clusters together with the previously described Pancake tortoise AdV. This clade is well supported only by Bayesian posterior probability and Fitch–Margoliash bootstrap values (Figure 1).

Figure 1: Phylogenetic distance tree

The phylogenetic distance tree of partial adenovirus DNA polymerase amino acid sequences (92 aa) is shown. Fitch–Margoliash bootstrap values /posterior probabilities generated by the Bayesian method/ maximum likelihood bootstrap values are shown on branches, separated by slashes. New viruses are shown in bold lettering. Abbreviations and GenBank accession numbers (in brackets): Agamid AdVs (DQ077706, EU914205, FJ196812, ACH86251), Anolis AdV-1 (KC544015), Anolis AdV-2 (KC544016), Anolis AdV-3 (KF886534), Bat AdV-2 (JN252129), Bat AdV-3 (AB303301), Bovine AdV-1 (YP_094032), Bovine AdV-2 (AP_000006), Bovine AdV-3 (AP_000026), Bovine AdV-4 (AAK13183), Bovine AdV-5 (not released yet), Bovine AdV-6 (YP_007346998), Bovine AdV-7 (U57335), Bovine AdV-10 (AF238882), Box turtle AdV-1 (EU828750), Canine AdV-1 (AAB05434), Chameleon AdV-1 (AY576679), Chameleon AdV-2 (KF886533), Common agama AdV (KC155825), Duck AdV-1 (AP_000539), Eastern box turtle AdV (JN632579), Eublepharid AdV-1 (AY576677), Fowl AdV-1 (AP_000410), Fowl AdV-2 (HM853995), Fowl AdV-4 (GU188428), Fowl AdV-5 (DQ 159938), Fowl AdV-6 (HM853999), Fowl AdV-10 (HM854003), Frog AdV (AAF86924), Gekkonid AdV (AY576681), Goose AdV-4 (JF510462), Great tit AdV (ACW84422), Green striped tree dragon AdV-1 (KF886532), Helodermatid AdV-1 (AAS89696), Helodermatid AdV-2 (EU914207), Kangaroo rat AdV (not released yet), Mascovy duck AdV (not released yet), Parrot AdV (AY644731), Murine AdV-1 (AC_000012), Murine AdV-2 (HM049560), Murine AdV-3 (EU835513), Odocoileus AdV (AF361168), Ovine AdV-1 (not released yet), Ovine AdV-6 (not released yet), Ovine AdV-7 (AAD45950), Pancake tortoise AdV (JN632575), Psittacine AdV-2 (EU056825), Porpoise AdV (JN377908), Porcine AdV-3 (AB026117), Porcine AdV-5 (AAK26504), Raptor AdV (NC_015455), Red eared slider AdV (JN632580), Scincid AdV (AY576682), Squirrel AdV (GU735083), Skua AdV (YP_004935931), Snake AdV-1 (AAL89790), Snake AdV-2 (ACH91014), Snake AdV-3 (FJ012164), Snake-Ger09 AdV (HQ148088), Sturgeon AdV (AY082701), Sulawesi tortoise AdV (EU056826), Tree shrew AdV (AF258784), Turkey AdV-1 (GU936707), Turkey AdV-3 (AC_000016), Turkey AdV-5 (YP_008719852), Varanid AdV (ACH86253), Yellow-bellied slider AdV (JN632578).


Odocoileus-1
Bovine-5
Bovine-4
Bovine-6
Bovine-7
Ovine-7
Scincid
Snake-2
Common agama
Eublepharid
Helodermatid [Lizard]-1
Helodermatid [Lizard]-2
Snake-3
Boa constrictor
Snake-GER09
Snake-1
Green striped tree dragon-1
Chameleon-2
Chameleon-1
Anolis-1
Anolis-2
Anolis-3
Agamid
AdVs
Duck-1
Gekkonid
Water monitor
Blue tree monitor
Varanid
Turkey-1
Fowl-1
Turkey-5
Fowl-4
Fowl-10
Fowl-2
Fowl-6
Fowl-5
Goose-4
Muscovy duck
Meyer's parrot
Raptor-1
Skua
Greatit
Turkey-3
Psittacine-2
Sulawesi tortoise
Frog-1
Hermann's tortoise
Pancake tortoise
Yellow-bellied slider
Alabama map turtle
Pond slider
Red eared slider
Eastern box turtle
Box turtle
Murine-3
Murine-1
Murine-2
Bovine-10
Kangaroo rat
Bat-3
Porcine-3
Ovine-6
Squirrel
Bat-2
Canine-1
Bovine-3
Porpoise
Porcine-5
Ovine-1
Bovine-1
Bovine-2
Tree shrew
Sturgeon
Atadenovirus
Aviadenovirus
Siadenovirus
Testadenovirus
Mastadenovirus
Ichtadenovirus
0.1

4.2 BDE cells

4.2.1 Establishment of the BDE cells

A cell line was established from BDEs in order to be able to isolate agamid AdVs in cell culture. The established cells grow well at 28°C, are mid-sized, and have a polymorphic and elongated form (Figure 2). The cells form a monolayer two days after seeding and can be sub-cultured at a split ratio of 1:2 every 1–2 weeks. Seeding density of the cells after the third passage was 2.66×10^6 cells/ml. The cells have been carried in continuous culture for 27 passages. The morphological characteristics of the cells remained consistent in all 27 passages as well as during the 1-2 weeks incubation between subcultures.

Figure 2: Noninfected bearded dragon embryo cells
Second passage. Eight days old cells. Bar = 100 µm

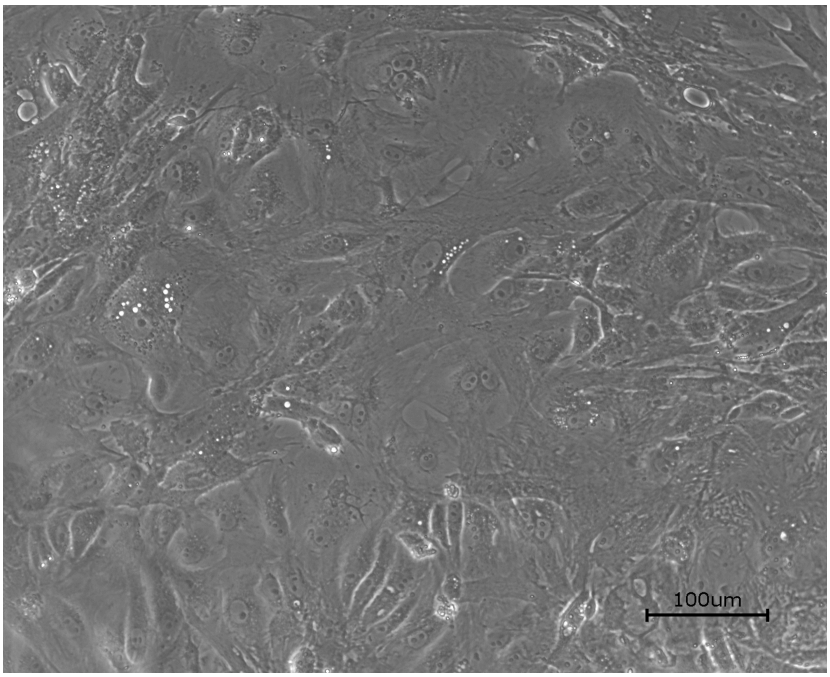
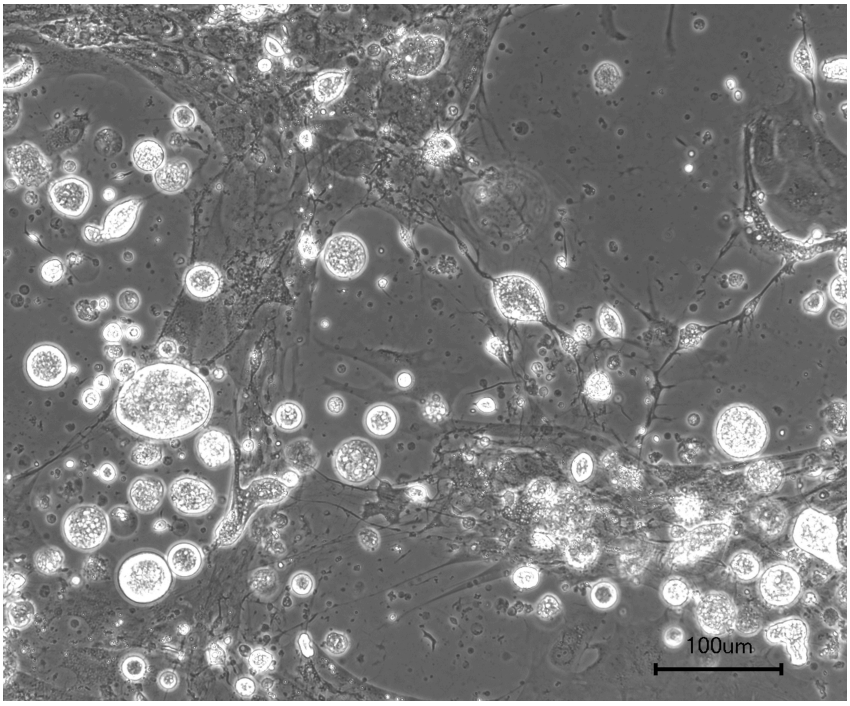


**Figure 3: Adenovirus isolate from a central bearded dragon (*Pogona vitticeps*)
Cytopathic effect in bearded dragon embryo cells, seven days after inoculation, sixth passage of an isolate. Bar =100 µm**

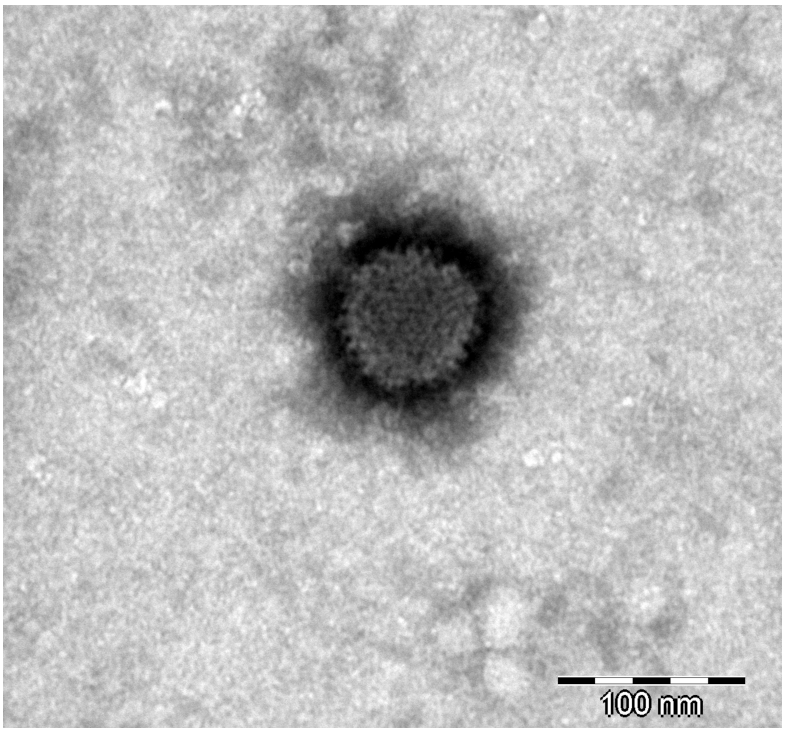


4.2.2 Isolation of bearded dragon AdVs

60 samples from central bearded dragons, collected from 2010, where presence of AdV was detected by PCR and sequencing (Table 9), were inoculated onto BDE cells. Thus, 11 AdV isolates were obtained during this study. All isolated viruses demonstrated a CPE with rounding and detachment of cells (Figure 3). All isolates have been serially passaged on the established BDE cells. 5-10 additional passages of each virus isolate have been performed to date, following freeze–thaw cycles and low speed centrifugation (Table 12). No difference was observed in adenovirus replication between different cell passages.

The highest virus titers were 10^4 and $10^{3.5}$ mean tissue culture infectious dosage ($TCID_{50}$). Only two isolates (92/10 and 47/1/11) were identified as AdVs by electron microscopic examination of the cell culture supernatants. Nonenveloped particles, with a diameter of approximately 80 nm, were detected by negative staining electron microscopy (Figure 4).

Figure 4: Electron micrograph (courtesy of Mark Hoferer, CVUA, Fellbach)
Electron micrograph of icosahedral nonenveloped virus particles in cell culture supernatant from sample 90/10 of the central bearded dragon (*Pogona vitticeps*) isolate (fifth passage on bearded dragon embryo cells). Bar = 100 nm.



4.2.3 PCR and sequence identification of isolated bearded dragon AdVs

The AdVs isolated in BDE cells were identified as AdVs by PCR and sequencing. DNA was prepared from cell culture supernatant showing clear CPE and subjected to an AdV specific PCR as described. The sequencing results were identical before and after isolation. The partial DNA-dependent DNA polymerase gene sequences obtained from all isolates were 272 nucleotides long and were identical to one another and to the corresponding part of the Agamid adenovirus 1 genome (DQ077706). DNA from the last passage of each isolate was also prepared, and PCR and sequencing carried out as described. Sequencing results remained identical to previous results. AdVs were isolated mostly from oral and cloacal swabs (Table 12). These sequences were also used to prepare a sequence identity matrix (Table 13).

Table 12: Central bearded dragon samples from which AdVs were isolated.
Their highest passage numbers, BLASTN values, $TCID_{50}$ per ml and results of electron microscopy examination

Lab. No.	Sample type	Passage No.	BLASTN result	GenBank Acc. No	$TCID_{50}$	EM
92/10	oral/cloacal swab	10	Agamid AdV 100%	DQ077706	10^4	AdV
120/10	oral/cloacal swab	8	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
119/11	oral/cloacal swab	6	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
135/62/11	stomach	5	Agamid AdV 99%	DQ077706	$10^{2.5}$	-
38/20/12	oral swab	5	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
38/24/12	oral swab	5	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
38/38/12	oral swab	5	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
38/40/12	oral swab	5	Agamid AdV 99%	DQ077706	$10^{2.5}$	-
38/43/12	oral swab	5	Agamid AdV 100%	DQ077706	$10^{2.5}$	-
38/48/12	oral swab	5	Agamid AdV 100%	DQ077706	10^3	-
47/1/11	oral swab	8	Agamid AdV 99%	DQ077706	$10^{3.5}$	AdV

Table 13: Identity matrix
Identity matrix of the 272 nt long sequence of the amplified DNA-polymerase gene region of isolated bearded dragon AdVs

Lab. No.	38-48-12	38-38-12	38-20-12	38-24-12	38-43-12	92-10	119-11	120-10	135-62-11	38-40-12	47-1-11
38-48-12	ID										
38-38-12	1,000	ID									
38-20-12	1,000	1,000	ID								
38-24-12	1,000	1,000	1,000	ID							
38-43-12	1,000	1,000	1,000	1,000	ID						
92-10	1,000	1,000	1,000	1,000	1,000	ID					
119-11	1,000	1,000	1,000	1,000	1,000	1,000	ID				
120-10	1,000	1,000	1,000	1,000	1,000	1,000	1,000	ID			
135-62-11	0,994	0,994	0,994	0,994	0,994	0,994	0,994	0,994	ID		
38-40-12	0,989	0,989	0,989	0,989	0,989	0,989	0,989	0,989	0,983	ID	
47-1-11	0,989	0,989	0,989	0,989	0,989	0,989	0,989	0,989	0,983	0,989	ID

4.3 Neutralization tests (NT)

4.3.1 NT with polyclonal antibodies from rabbits

Post immunization polyclonal antibodies from rabbits were tested for the presence of antibodies against the original viruses using NTs. By NT, antibody titers of 2560 (end point of the dilution series) were detected against both Helodermatid AdVs in plasma from rabbits inoculated with each virus. No titer differences were detected in titers against the homologous and the heterologous virus. No antibodies were detected against either of the Snake AdVs or the Agamid AdV.

4.3.2 NT with lizard plasma

263 lizard plasma samples were tested by NT, 89 (33.8%) samples had detectable antibodies against Agamid AdV-1. Of all lizard plasma samples tested, 53 (20.2%) had antibodies against Helodermatid AdV-2, 27 (10.3%) against Helodermatid AdV-1, only three (1.1%) against Snake AdV-1 and none (0%) against Snake AdV-2 (Table 16). Antibodies against two or more AdVs were detected in 32 of 263 (12.2%) of the lizards tested.

Looking at specific lizard families, 12/27 (44.4%) of the helodermatid lizards tested had antibodies against Agamid AdV-1 with titers ranging from 1:20 to 1:80. 18 of 27 (66.7%) of the helodermatid lizards had antibodies against Helodermatid AdV-2 with titers ranging from 1:20 to 1:1280 and 12/27 (44.4%) had antibodies against Helodermatid AdV-1 with titers ranging from 1:40 to 1:320. In one case, antibodies against Snake AdV-1 were detected in a helodermatid lizard with a titer of 1:20. 74 of 223 (33.2%) agamid lizards had antibodies against Agamid AdV-1 with titers ranging from 1:20 to 1:640, 34/223 (15.2%) had antibodies against Helodermatid AdV-2 with titers ranging from 1:20 to 1:640, 13/223 (5.8%) had antibodies against Helodermatid AdV-1 with titers ranging from 1:20 to 1:40 and in two cases agamid lizards had antibodies against Snake AdV-1, both with titers of 1:20. In one case, an iguanid lizard had antibodies against Agamid AdV-1, in one case against Helodermatid AdV-2 and in two cases against Helodermatid AdV-1, with titers of 1:20 in all cases. Antibodies were only detected in scincid lizards in two cases, in both against Agamid AdV-1 with titers ranging from 1:20 to 1:40. The varanid lizards tested did not demonstrate any neutralizing antibodies against any of the five isolates (Table 14).

Table 14: Results of serological testing (NT) of lizard plasma
Percent positive (number positive/total)

AdV / Lizard family (n)	Agamid 1	Helodermatid (Lizard) 1	Helodermatid (Lizard) 2	Snake 1	Snake 2
Agamidae (223)	33.2% (74/223)	5.8% (13/223)	15.2% (34/223)	0.9% (2/223)	0% (0/223)
Helodermatidae (27)	44.4% (12/27)	44.4% (12/27)	66.7% (18/27)	3.7% (1/27)	0% (0/27)
Iguanidae (6)	16.7% (1/6)	33.3% (2/6)	16.7% (1/6)	0% (0/6)	0%(0/6)
Scincidae (2)	100%(2/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)
Varanidae (5)	0% (0/5)	0% (0/5)	0% (0/5)	0% (0/5)	0% (0/5)

4.3.3 Neutralization test with snake plasma

All together 141 snake plasma samples were tested by NT. 62/138 (44.9%) demonstrated the presence of antibodies against Agamid AdV-1, while 14/140 (10%) of the tested snakes had antibodies against Helodermatid AdV-2. No antibodies against Helodermatid AdV-1 were found in any of the snakes tested. 17 of 141 snakes (12.1%) had antibodies against Snake AdV-1, and 5/141 (3.5%) had antibodies against Snake AdV-2. 14 of 141 (9.9%) of the snake plasma tested had antibodies against two or more viruses (Table 15).

Looking at specific snake families, the majority of viperid snakes tested were wild-caught animals and sent to our laboratory from the USA. The viperid snake samples demonstrated a seroprevalence of 16/109 (14.7%) against Snake AdV-1 with titers ranging from 1:40 to 1:640. Three of 109 (2.8%) viperid snakes had antibodies against Snake AdV-2, with titers ranging from 1:20 to 1:80. 59 of 106 (55.7%) viperid snakes had antibodies against Agamid AdV-1 with titers ranging from 1:20 to 1:320. 14/108 (13%) of the viperid plasma samples contained antibodies against Helodermatid AdV-2 with titers ranging from 1:20 to 1:1280. Pythonid snake samples had a seroprevalence of 1/20 (5%) against Snake AdV-1 with a titer of 1:80 and 3/20 (15%) had antibodies against Agamid AdV-1 with titers ranging from 1:20 to 1:40. Only one sample from a colubrid snake contained measurable antibodies against

Snake AdV-1 with a titer of 1:160. A titer of 1:1280 was measured in a single boid snake against Snake AdV-2 (Table 16).

Table 15: Detection of antibodies against different adenoviruses in reptiles
Percent positive (number positive/total)

AdV / Reptile species (n)	Agamid 1	Helodermatid (Lizard) 1	Helodermatid (Lizard) 2	Snake 1	Snake 2
Snakes (136-141)	44.9% (62/138)	0% (0/136)	10% (14/140)	12.1% (17/141)	3.5% (5/141)
Lizards (263)	33.8% (89/263)	10.3% (27/263)	20.2% (53/263)	1.1% (3/263)	0% (0/263)

Table 16: Results of serological testing (NT) of snake plasma
Percent positive (number positive/total)

AdV / Snake Family (n)	Agamid 1	Helodermatid (Lizard) 1	Helodermatid (Lizard) 2	Snake 1	Snake 2
Viperidae (104-109)	55.7% (59/106)	0% (0/104)	13% (14/108)	14.7% (16/109)	2.8% (3/109)
Pythonidae (20)	15% (3/20)	0% (0/20)	0% (0/20)	5% (1/20)	0% (0/20)
Colubridae (4)	0% (0/4)	0% (0/4)	0% (0/4)	0% (0/4)	25% (1/4)
Boidae (8)	0% (0/8)	0% (0/8)	0% (0/8)	0% (0/8)	12.5% (1/8)

4.3.4 Statistical analysis

For lizard samples, the median titer was 1:40 with a SD of 1:186. For snake samples, the median titer was 1:40 with a SD of 1:192. By statistical analysis no significant effects (α=5%) of families, species, habitat or sex of the tested lizards and snakes were seen. For lizards, a positive correlation was found for detection of antibodies against Agamid AdV-1 and Helodermatid AdV-1 (P=0.0144). For snakes, correlations were found between antibodies against Agamid AdV-1 and Snake AdV-2 (P=0.0148) and antibodies between Helodermatid AdV-2 and Snake AdV-1 (P=0.0011).

5 Discussion

5.1 Pathogenic role of AdVs

Pathogenicity of reptilian AdVs and their ability to cause primary disease is questionable, since AdV infection has been detected in squamates and chelonians with (Jacobson, 2007; Rivera et al., 2009; Papp et al., 2009) and without any clinical signs (Ogawa et al., 1992; Hyndman et al., 2011; Schumacher et al., 2012). A recent report also described AdV detection in clinically healthy bearded dragons in the UK (Kubiak, 2013). Mass mortality in reptiles due to AdV infection has been described in only a few cases (Kim et al., 2002; Rivera et al., 2009). There are also several reported cases in which single animals within a group were infected with AdV (Frye et al., 1994; Moormann et al., 2009). AdV infection has repeatedly been described in association with CNS signs, anorexia, dehydration, lethargy and vomiting in squamates (Kim et al., 2002; Garner et al., 2008; Papp et al., 2009). AdV infection in chelonians has been described in association with nasal and ocular discharge (Rivera et al., 2009).

It is interesting to note that additional viral infections with rana- and invertebrate iridoviruses in several lizards and a paramyxovirus in one bearded dragon (72/3/11), were detected during our study (Table 9). In snakes, additional viral infections with paramyxoviruses were detected in several cases (Table 10). In the AdV positive chelonian samples, on the other hand, no other viruses were detected (Table 11). Ranavirus infections in squamates have been described in association with skin lesions, hepatitis and ulceration of the nasal mucosa (Marschang, 2011; Behncke et al., 2013; Stöhr et al., 2013). Also, skin lesions and pneumonia have been detected in lizards tested positive for the presence of invertebrate iridovirus (Papp et al., 2014). Paramyxoviruses in squamates have been associated with respiratory disease and CNS signs (Jacobson et al., 2001; Jacobson, 2007). Infection without any clinical signs also has been reported for each virus (Marschang, 2011).

AdV was detected in a group of green striped tree dragons (96/11), CNS signs as well as dermatitis were observed in these animals. It is hypothesized that the causative agent for the disease was not a bacterial infection since an initial antibiotic treatment did not reduce the mortality rate of the diseased animals (Behncke et al., 2013). In the case of the blue tree monitor (55/12) CNS signs were seen, and both

AdV and ranavirus were detected in the brain sample. AdV was also found in a group of water monitors (64/12), which demonstrated only a high mortality rate with no specific clinical signs reported prior to death. Other viruses (IIV and ranavirus) were also found in organ samples of these animals. AdV was also detected in green anoles (59/12). These animals demonstrated weakness and skin lesions. In this case it is hypothesized that the skin lesions could be the result of rana- or/and IIV infection, since these two viruses were detected in a skin sample from a diseased animal. AdV and ranavirus were detected in the intestine sample of a green anole.

The only clinical sign observed in the Jackson's chameleon (117/12) was cachexia. In this case, coinfections with different viruses (AdV and IIV) and bacteria (*Proteus spp*.) may have led to the fatal course of disease. The common agama (168/3/10) also had stomatitis and oral abscesses, which are not typical findings in AdV infections. Co-infection of this animal with an AdV, *Proteus spp*., and various endoparasites (*Choleoeimeria sp*. and pinworms) in combination with the stress of transport most likely influenced the fatal course of disease in this case. The oral lesions were most likely caused by the bacterial infection. It was, however, not possible to identify a primary pathogen in these cases. AdV together with PMV were also found in a clinically healthy central bearded dragon (72/3/11) and in several bearded dragons together with IIV (85/08, 57/09), but unfortunately no information on clinical signs or pathology was available in these cases. AdV was also detected in a blue tree monitor (61/13) with crusts on the lips. In this case no other viruses were detected in the crusts. AdVs were also detected in bearded dragons demonstrating CNS signs (92/10), egg binding (47/1/11), obstipation and inappetence (119/11), and bloody diarrhea (127/11). In these cases no other viruses were detected by PCR. AdV infection was also found in clinically healthy bearded dragons during our study (38/12).

In snakes, AdVs were also detected in animals with a variety of clinical signs ranging from clinically healthy through various signs typical and less typical for AdV infection to animals, which died suddenly (Table 10). Thus, an AdV was detected in a group of boa constrictors (57/3/08), which demonstrated vomiting and rapid mortality. In this case other parasites (e.g. *Proteus vulgaris*, *Hypotrihomonas sp*., *Proteromonas sp*.) were also found. AdV was also detected in an eastern corn snake, which died without

preliminary clinical signs. Both PMV and AdV were detected in this animal. Therefore it is not possible to identify the primary pathogen in these cases. However, there are also several cases where AdVs seem to be primary pathogens: e.g. an Indian python (105/16/08), which died after a period of inappetence. Also an eastern corn snake (152/10), which demonstrated clinical signs of respiratory disease. AdV was found in a group of snakes (30/11), which died without any clinical signs. As for lizards, AdVs were also detected in clinically healthy snakes in several cases (105/12/08, 54/1/09, 54/4/09, 54/6/09). AdVs were found in chelonians (Table 11) with clinical signs of pneumonia (106/41/11) as well as without any clinical signs (106/36/11).

Coinfection of lizards, snakes and chelonians with AdVs and other infectious agents have been described previously (Abbas et al., 2011; Kim et al., 2002; Papp et al., 2009; Rivera et al., 2009; Mahapatra et al., 2013). Viruses that have coevolved with their hosts, as is hypothesized for atadenoviruses in reptiles (Harrach, 2000; Benkő et al., 2003) may also be non-pathogenic for their natural hosts, or lead to development of clinical disease only in weakened hosts (May and Anderson, 1983). It is therefore possible that in the presented cases in which the hosts were infected by multiple viruses, the AdVs contributed to the development of clinical disease.

In the cases of the green striped tree dragons (96/11), green anoles (59/12) and a boa constrictor (57/3/08), it is possible that stress from recent importation and adaptation to a new environment and new diet could clinically predispose these reptiles to develop disease. Overcrowding may also have influenced the epidemiology of disease, increasing both the spread of virus and the susceptibility of individuals to infection and disease development.

5.2 AdV sequences analysis

5.2.1 Divergence of reptilian AdVs

Several new reptilian AdVs were detected and analysed during our study. Thus, the comparison of partial DNA polymerase gene sequences of AdVs found in green striped tree dragons, a green anole, a Jackson's chameleon, a common agama, and a Hermann's tortoise demonstrated less than 80% sequence identity to previously described AdVs. The AdV detected in the water monitor had less than 90% sequence identity with a previously described AdV. The detected viruses therefore seem to represent distinct AdVs. AdVs that have been described in snakes (SnAdV-1,

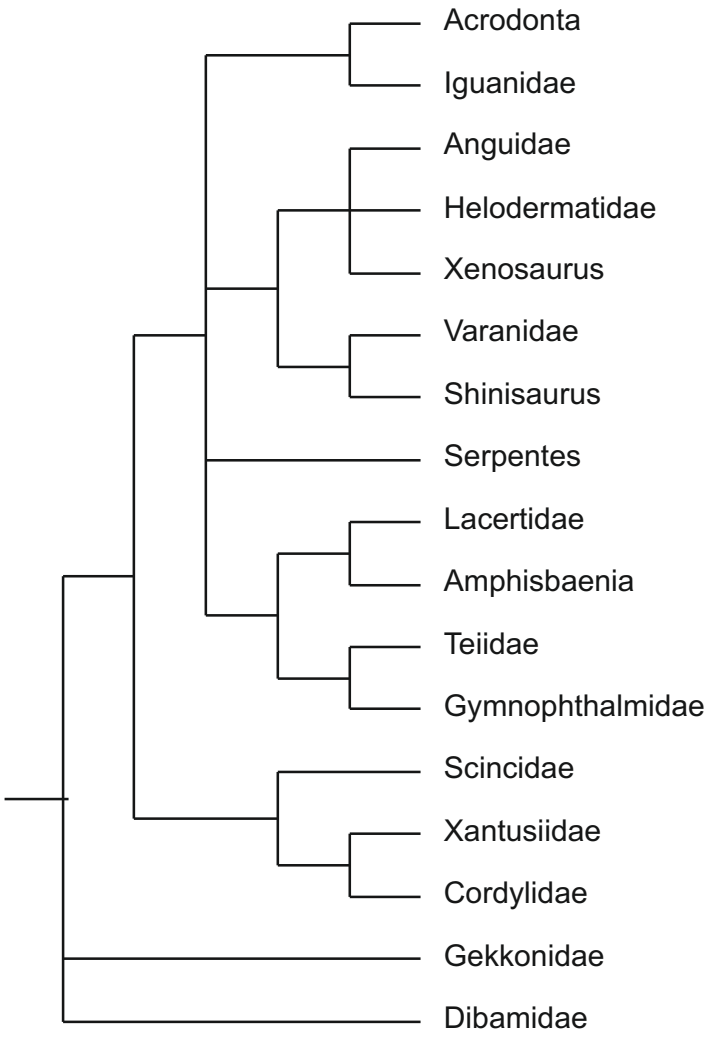
SnAdV-2, and SnAdV-3) have also demonstrated less than 80% identity to one another and with all known atadenoviruses in this portion of the genome (Juhász and Ahne, 1993; Garner et al., 2008). Earlier studies of AdVs of lizards using the same portion of the genome showed that six AdVs detected in seven different lizard species demonstrated less than 90% sequence identity (Wellehan et al., 2004). The finding of these new distinct AdVs also demonstrates the increasing frequency of the detection of genetically distinct atadenoviruses. A number of other viruses that appeared to be closely related to but distinct from previously described AdVs were detected. The AdV detected in a blue tree monitor demonstrated relatively high sequence identity (98.8%) to the previously detected Varanid AdV-1 and may thus represent a new variant of this virus. The Boa constrictor AdV detected in this study demonstrated 95.5% sequence identity to Snake AdV-1 and thereby represents a Snake AdV-1-like virus. The Pond slider AdV also demonstrated 94.5% identity to the previously described Red-eared slider AdV and represents a Red-eared AdV-like virus. The Alabama map turtle AdV, which demonstrated relatively high (91.2%) sequence identity to the Pond slider AdV also falls into this category. These findings are rapidly broadening our understanding of the diversity of AdVs of reptiles.

5.2.2 Phylogeny of reptilian AdVs

The majority of the lizard species in which AdVs were detected during our study (green striped tree dragon, green anole, Jackson's chameleon, common agama, bearded dragons) belong to the infraorder Iguania, which includes, among others, the families Iguanidae, Agamidae and Chamaeleonidae. Monitor lizards belong to the Anguimorpha, which includes the Helodermatidae, Anguidae and Varanidae families. The phylogenetic analysis of squamate reptiles has demonstrated that iguanians are most closely related to the anguimorphs and snakes, all of which have been included in the Toxicofera. They are clearly distinct from the gekkonid lizards, which belong to the Bifurcata. The Scincidae also fall outside the Toxicofera and have been included in the Scinciformata (Townsend et al., 2004; Vidal et al., 2005). Thus, the topology of a phylogenetic tree of squamate reptiles (Figure 5) is not similar to the phylogenetic tree of squamate AdVs (Figure 1) in which AdVs detected previously in lizards belonging to the infraorder Gekkota (Eublepharid AdV-1 and Gekkonid AdV-1) cluster distinctly from each other. Also all Varanid AdVs cluster distinctly from other squamate AdVs. This contradicts the theory that the majority of squamate AdVs described have coevolved with their hosts and indicates that host switches, at least

between different squamate species, are possible and perhaps even common. In that case, Eublepharid AdV-1 might have switched hosts from a member of the Toxicofera to a gekkonid host.

Figure 5: Phylogenetic tree for squamate reptiles (Townsend et al., 2004)



It is interesting to note that the AdV detected in the group of green striped tree dragons (Green striped tree dragon AdV-1), which belong to the family Agamidae, demonstrated the highest identity with Helodermatid AdV-1 and Eublepharid AdV-1. Eublepharid AdV-1 has been described in a leopard gecko (*Eublepharis macularius*) and in fat-tail geckos (*Hemitheconyx caudicinctus*) (Wellehan et al., 2004). An AdV with 99% identity to Helodermatid AdV-2 in a partial DNA-dependent DNA polymerase gene sequence was also detected in a wild-captured western bearded dragon (*Pogona minor minor*) in Australia (Hyndman et al., 2011). Thus, because of

this apparent broader host spectrum, similar to that observed among Snake AdVs (see later), it has been proposed to rename Helodermatid AdVs to Lizard AdVs (Harrach, pers. comm.). Helodermatid AdV-2 was first found in beaded lizards (*Heloderma horridum*) (Papp et al., 2009), and a closely related virus, Helodermatid AdV-1 has been found only in Gila monsters (*Heloderma suspectum*) so far (Wellehan et al., 2004; Papp et al., 2009). Phylogenetic analysis also clustered this virus distinctly from other agamid lizard AdVs. The AdV detected in the Jackson's chameleon (Chameleon AdV-2) demonstrated the highest identity to different Agamid AdV-1 rather than to Chameleon AdV-1, which was detected previously in a mountain chameleon (*Chamaeleo montium*, formerly *Triocerus montium*) (Wellehan et al., 2004). However, Chameleon AdV-2 belongs to the same clade as Chameleon AdV-1, although this clade is not well supported. An AdV infection has been described in a Jackson's chameleon before (Jacobson et al., 1990), but unfortunately no sequence information was available from the virus in that case. The AdV detected in a green anole demonstrated the highest identity with Anolis AdV-2, which was detected in *Anolis distichus ravitergum* (Ascher et al., 2013). The phylogenetic analysis also demonstrated that the Anolis AdV-3 belongs to the same clade as the previously detected Anolis AdVs-1 and 2, but this clade is not well supported. Monitor lizard AdVs, detected together with Varanid AdV-1, also form a separate clade, but this clade clusters outside the genus *Atadenovirus* and forms a paraphyletic cluster with this genus. This paraphyletic cluster is, however, not well supported, although all phylogenetic calculations confirmed the distinctness of Varanid AdVs.

A lack of species specificity has previously been postulated for AdVs from snakes. Snake AdV-1 has been described in both colubrid and boid snakes (Farkas et al., 2002; Marschang et al., 2003) and Snake AdV-2 has been found in viperid and colubrid snakes (Garner et al., 2008; Papp et al., 2009). Snake AdV-3 has been detected in different species of colubrid snakes (Garner et al., 2008). During our study Snake AdV-1 and Snake AdV-1-like viruses (Snake GER09 AdV) were also found in colubrid, viperid and pythonid snakes. Snake AdV-2 and Snake AdV-2-like viruses (Viperid AdV) were also detected in elapid, colubrid, pythonid and boid snakes. Snake AdV-3 was also detected in one case in a colubrid snake. These findings further support the lack of species specificity in snake AdVs. It is interesting to note that although snakes are monophyletic, lizards are not. There is only one

report so far of an AdV originally described in a lizard to be found in a snake. In this case, a Helodermatid AdV-2-like virus was detected in a death adder (Hyndman GenBank submission JN418933). None of the snake AdVs have ever been detected in lizards.

The host animals of novel chelonian AdVs all belong to the superfamily Testudinoidea, and pond sliders, together with red-eared and yellow-bellied sliders, belong to the genus *Trachemus*, subfamily Deirochelyninae, family Emydidae. The two box turtle species in which AdVs have been reported (Farkas et al., 2009; Doszpoly et al., 2013) belong to the genus Terrapene, subfamily Emydinae, also to the family Emydidae. The Hermann's tortoise together with the pancake tortoise belongs to the subfamily Testudininae of the family Testudinidae (Guillon et al., 2012). The previously reported AdVs found in chelonians included Sulawesi tortoise AdV-1, which belongs to the genus *Siadenovirus* and demonstrated a mixed ability to switch hosts (Schumacher et al., 2012) and viruses that have been grouped together in the proposed new genus "Testadenovirus". In this last case, identical AdVs have been found in different turtle subspecies (Doszpoly et al., 2013). In our study, a new variation of Red-eared slider AdV was detected in the pond slider and a virus sharing relatively high identity with the pond slider AdV was detected in an Alabama map turtle. All of these viruses cluster together within the proposed genus *"*Testadenovirus*"*. This clade was well supported by different phylogenetic calculations. Within this clade AdVs from turtles belonging to the family Emydidae form a monophyletic cluster, which is also well supported. Tortoise AdVs from Testudinidae also cluster together, but distinctly from Emydid AdVs. However, the sequences on which all of these findings are based are short (92 amino acids), so that further sequencing is necessary to find out the exact taxonomic position of these viruses.

In conclusion, although apparently newly detected AdVs from lizard hosts belonging to infraorders Iguania and Anguimorpha do not fully support the host-specificity coevolution–cospeciation theory of AdVs, as they do not form a monophyletic subgroup within the cluster of AdVs originating from toxicoferan hosts. But we can find interpretations of these new data supporting the theory, since there is a well supported subgroup of AdVs from chameleons and anolis lizards in close connection

with the Agamid AdV-1 isolates, which might represent the coevolved iguania AdV lineage. The green anole AdV and Jackson's chameleon AdV from this study belong to this subgroup, yet further AdV sequences from other lizards belonging to the infraorder Iguania are needed to test the hypothesis. A sister clade to this presumed lineage contains AdVs from related toxicoferan hosts (except for the Eublepharid AdV), of which two AdVs (from common agama and green striped dragon) also belong to Iguania (family Agamidae).

The AdVs belonging to the *"*Testadenovirus*"* also seem to support this co-evolution theory, since there is a well supported subgroup of AdVs from Emydid turtles in close connection with AdVs from Testudinid tortoises. Chelonian AdVs belonging to this genus, which were described by Doszpoly et al., (2013) had an equilibrated C+G content as well as great sequence variability, which could indicate that these AdVs coevolved with their testudinoid hosts over a long period of time. Finding these AdVs in clinically healthy animals might also support this theory. Sulawesi tortoise AdV-1, on the other hand, caused a systemic infection and killed the majority of infected turtles (Rivera et al., 2009). This virus may therefore represent a host switch from an unknown reservoir into Sulawesi tortoises and other chelonians. Further AdV sequences from more chelonian hosts are necessary to test this theory and to see if any other distinct AdV types may exist.

When interpreting the phylogenetic relationships between AdVs and their hosts it is important to remember that many reptile AdVs have been detected in animals that are pet reptiles and are often captive bred. Also, wild-caught reptiles that are tested for AdV infections may be stationed in holding facilities for variable amounts of time prior to testing, where they may come into contact with large numbers of taxonomically distant animals and other reptiles, carrying a significantly different microbiome. In these cases the spillover of viruses from taxonomically and evolutionarily very distant hosts is also possible. Therefore the AdVs detected in reptiles cannot generally be assumed to exclusively reflect the AdVs found in natural hosts, and opportunities for host switches may be common. Further work is necessary to broaden our understanding of which AdVs can be found in different species of reptiles. In addition, sequence data from larger portions of the genome of the detected viruses are necessary to fully understand the origin of the members of

the genus Atadenovirus. This would also help developing risk analysis tools for AdV infections in reptiles and understanding how infections in individual species might affect other species within a collection.

5.3 Isolation of Agamid AdV-1 in BDE cell culture

Although AdV infections are commonly described in bearded dragons (Jacobson et al., 1996; Kim et al., 2002; Wellehan 2004; Moormann et al., 2009; Papp et al., 2009), previous attempts to isolate the virus in cell culture have been unsuccessful (Papp et al., 2009). Agamid adenovirus 1 seems to be very species specific because the virus has only been detected in bearded dragons to date (Parkin et al., 2009). Based on this fact, it seemed likely that the virus might grow on bearded dragon–specific cells, although it did not grow on other reptilian cell lines tried previously. According to this hypothesis, BDEs were used to create a bearded dragon fibroblast cell line. This cell line provides a tool for the isolation of bearded dragon–specific viruses and was successfully used to isolate AdVs from 11 animals. The isolation of these viruses is important for the study of the biological properties of the virus. Because AdV infection has been described in central bearded dragons with (Marschang, 2011) and without clinical signs (Kübber-Heiss et al., 2006; Kubiak, 2013) the primary pathogenic role of Agamid adenovirus 1 has been questioned in many cases. Isolation of this virus opens up the possibility to carry out experimental transmission studies to help understand how this virus affects its host and what factors might influence the course of infection. Virus isolation also opens up possibilities for the study of ultrastructural properties of this virus. To date, studies have shown clear differences in the number of fibers present on the surfaces of different atadenoviruses (Pénzes et al., 2014). The fiber, hexon and penton proteins that make up the icosahedral capsids of AdVs form the main antigenic components of these viruses (Benkő et al., 2005). Only partial sequence information from the DNA-dependent DNA polymerase gene and the hexon genes are currently available from Agamid adenovirus 1 (http://www.vmri.hu/~harrach/ADENOSEQ.HTM). Further sequence data is necessary to understand the structural differences between various atadenoviruses, and ultrastructural and biological studies will help elucidate the importance of these differences for AdVs. The isolated viruses can also be used for the development of serological tests. Neutralization tests have previously been used for the detection of AdV neutralizing antibodies in serum of infected snakes (Funk et al., 2011), but studies on antibodies against AdVs in lizards have been hindered by

the lack of isolates available. The isolation of Agamid adenovirus 1 allows the performance of serological tests, which are necessary to understand how common Agamid adenovirus infections are among both captive and wild bearded dragons and whether this virus might be able to spread to other reptile species. The isolates can also be used to study serological cross-reactivity between AdV isolates from various reptiles.

5.4 Neutralizing antibodies

5.4.1 Reactivity in squamate plasma

This is the first study to examine the presence of antibodies against a range of AdVs in squamate reptiles. The majority of lizard (133/263, 50.6%) and snake (79/141, 56%) samples were positive for antibodies against at least one of the isolates, with titers ranging from 1:20 to 1:1280, although the median in both groups was 1:40. Since the majority of the plasma samples tested were collected from central bearded dragons, it is not surprising that the majority of positive lizard samples demonstrated the presence of antibodies against Agamid AdV-1. However, when comparing results from bearded dragons with those of other lizards tested, it is interesting to note that a higher percentage of lizards other than bearded dragons (37.5%) had antibodies against this virus than the bearded dragons tested (33% positive). Antibodies against Helodermatid AdV-2 were also detected in a large number of lizards. Low antibody titers against Helodermatid AdV-1 were also detected in central bearded dragons. This may indicate a lack of species specificity for Helodermatid AdV-2 (see above). However, Helodermatid AdV-1 has only been detected in Gila monsters so far (Wellehan et al., 2004; Papp et al., 2009), making the detection of antibodies against this virus in agamid and iguanid lizards surprising. The pattern of reactivity found in snakes and lizards against this range of squamate atadenoviruses shows that these viruses may not be as species specific as previously supposed. Although cross-reactivity between the viruses could also explain this to some degree, the NTs with polyclonal rabbit plasma indicate that although Helodermatid AdV-1 and -2 serologically cross react, cross reaction between these two viruses and the other atadenoviruses used for testing is limited to non existent. A direct comparison of reactivity against Helodermatid AdV-1 and -2 in the lizard plasma tested showed no statistical relationship between antibodies against these two viruses in the animals tested. Statistically, there was a significant relationship between detection of

antibodies against Agamid AdV-1 and Helodermatid AdV-1 in lizards and between antibodies against Helodermatid AdV-2 and Snake AdV-1 as well as between antibodies against Agamid AdV-1 and Snake AdV-2 in snakes. Serological cross reactivity between these isolates is unexpected based on the results of the neutralization tests with rabbit antibodies as well as on the genetic relationships between these viruses. Reasons for these interactions are unknown. It is possible that animals which come into contact with one adenovirus are more likely to come into contact with others. This possibility also supports the hypothesis that squamate AdVs are not always species specific and switch hosts between different reptile species. This hypothesis is also supported by some of the sequencing data obtained in this and other studies (see discussion above).

5.4.2 Cross-reactivity between Helodermatid AdVs

The genome analyses of both helodermatid AdVs have demonstrated that these viruses are closely related to one another (Papp et al., 2009). Results of the neutralization tests with polyclonal antibodies from rabbits clearly demonstrated cross-reactivity between Helodermatid AdV-1 and 2, which also supports the close relationship between these viruses. However, this cross-reactivity was not always reflected in the results of the NT with squamate plasma. Thus, antibodies from squamates did not react equally against both helodermatid AdV isolates. A higher number of lizard samples contained antibodies against Helodermatid AdV-2 and at higher titers than snake samples. In particular, snakes with antibodies against Helodermatid AdV-2 did not have detectable antibodies against Helodermatid AdV-1, indicating a clear serological difference between these viruses. The reasons for the differences in reactivity in rabbit and squamate plasma are unknown. One possible influencing factor is the high antibody titer produced in the rabbits, which might lead to a stronger cross-reactivity than in the lizards tested which had much lower antibody titers against each virus. Future studies are necessary to further compare all five reptilian isolates presented in our study and to determine if infections with other viruses might lead to some cross reactivity with each individual virus used.

5.4.3 NT cut-offs

There are a number of explanations for the detection of relatively low titers of 1:20 or 1:40 in some cases. These titers may reflect the time since infection. Seroconversion against different antigens in reptiles is dependent on a number of factors, including

temperature and body condition (Jacobson, 2007), and generally can be detected as early as 8-10 weeks post infection (Borysenko et al., 1979; Jacobson et al., 1991). No studies are available on seroconversion following AdV infection in reptiles. Following acute infection, titers may drop over time. It is therefore possible that low titers reflect either very recent or older infections. It is also possible that some of the results reflect cross-reactivity between the viruses used for testing and closely related atadenoviruses. Natural antibodies (NA) have also been described as a source of false positive results in serological testing. However, NAs are generally present only at low titers and setting a cut-off for specificity is done in part to rule these out as a source of false positive results. In the present study, predilution of samples at 1:20 is expected to avoid any nonspecific background (Ochsenbein et al., 2000).

There are no standards established for cut-offs to determine specificity of NT reactions against adenoviruses in snakes and lizards. A previous study on the detection of antibodies against viruses in reptiles used a cut-off of ≥ 1:16 (Pees et al., 2010). Nevertheless, these titer cut-offs are not supported by experimental transmission data and so although they make biological sense, they were ultimately arbitrarily set. Establishing such cut-offs could be done using samples from animals in which the presence of AdV infection is known to occur as well as samples from animals which are known to be free of infection (Jacobson, 2007). However, in the present study, insufficient information was available on the health status and case histories of the squamates from which the plasma was collected for NT. Also, the relationships between seroconversion, virus shedding, and clinical signs are unknown.

It was unexpected that a large percentage (62/138, 44.9%) of snake plasma contained antibodies against Agamid AdV-1. Also, 71/106 (67%) of all wild-caught snakes had neutralizing antibodies against different reptilian AdV isolates. In a previous study, neutralizing antibodies were found only in 15/113 (13.3%) of all snake plasma samples tested against Snake AdV-1. That study also included wild-caught snakes, although in that case the snake species (mostly boa constrictors) differed and the animals were caught in Middle America rather than the USA (Marschang et al., 2003). In a recent study of antibodies against Snake AdV-1 in boid

snakes in Germany, these were detected in only 4/86 (4.7%) plasma samples (Pees et al., 2010).

5.4.4 Importance of antibodies

The importance of antibodies in specific animals should be interpreted carefully. Since antibodies were detected in the majority of captive bred lizards, AdVs may be so common in these animals that they have no clinical importance. It is also possible that antibody positive lizards may be protected from AdV infection or disease, so that lack of antibodies in individual animals may represent a disadvantage. The potential for antibody positive animals to be AdV carriers is unknown and requires further study. The use of a combination of virus detection methods, e.g. PCR (Wellehan et al., 2004) and the described neutralization test in a large number and variety of animals over time would be helpful to develop a better understanding of the epidemiology of AdVs in squamate reptiles and to establish an optimal testing regime for the future.

All of these findings demonstrate that AdV infections appear to be common in wild-caught and captive lizards and snakes. It is possible that the relatively high seroprevalence of antibodies against reptilian AdVs in wild-caught animals may suggest asymptomatic, or at least non-lethal, infection of these animals with various virus types. Asymptomatic infections with Agamid AdV-1 have been described previously (Jacobson et al., 1986; Schumacher et al., 1994; Kubiak, 2013). Future investigations are necessary to identify the clinical significance of AdVs in various reptilian hosts and their pathogenic potential in lizards and snakes.

6 Conclusions

Screening of reptiles for the presence of AdV infections during our study demonstrated that 7% of the animals tested were infected with these viruses. Although the pathogenic role of AdVs is not clear, it seems likely that stress, inappropriate husbandry, adaptation to a new environment and new diet could clinically predispose these reptiles to develop disease. A number of new distinct AdVs were detected in our study (Green striped tree dragon AdV-1, Anolis AdV-3, Chameleon AdV-2, Common agama AdV, Hermann's tortoise AdV and Water monitor AdV). These findings demonstrate the increasing frequency of the detection of genetically distinct atadenoviruses. A number of other viruses that appeared to be closely related to but distinct from previously described AdVs were also found (Blue tree monitor AdV, Boa constrictor AdV, Pond slider AdV and Alabama map turtle AdV). These findings are rapidly broadening our understanding of the diversity of AdVs of reptiles. Phylogenetic analysis of these viruses demonstrated that squamate AdVs do not fully support the host-specificity coevolution–cospeciation theory of AdVs. AdV detected in chelonians, on the other hand (Pond slider AdV, Alabama map turtle AdV and Hermann's tortoise AdV), all belonged to the genus "Testadenovirus" and would appear to support a co-evolution theory. Agamid AdV-1, which seems to be very species specific, was isolated from 11 bearded dragons and grown in cell culture using bearded dragon specific cells. This cell line provides a tool for the isolation of bearded dragon–specific viruses. The isolation of these viruses is important for future investigations and serological diagnostics.

Results of NTs using five different squamate AdVs from lizards and snakes demonstrated that antibodies against different AdV isolates could be detected in a large percentage of wild-caught and captive-bred squamates tested, demonstrating that AdV infection is common among these animals. Results of neutralization tests demonstrated clear serological cross-reactivity between Helodermatid AdV-1 and 2. However, squamate plasma did not react equally against these lizard AdV isolates. The results of neutralization tests with snake and lizard plasma against this range of squamate atadenoviruses indicate that all isolates presented in this study may not be as species specific as previously supposed and may be able to spread between various squamate species.

7 Summary

The purpose of this project was to screen reptiles for the presence of adenovirus (AdV) infection, develop serological tests for the detection of antibodies against AdVs in squamate reptiles and to examine the serological relationships between lizard and snake AdVs, helping to ensure the establishment and maintenance of healthy populations. An additional aim of the project was the establishment of an agamid cell line and isolation of adenoviruses from bearded dragons (*Pogona vitticeps*).

A PCR targeting the DNA-dependent DNA polymerase gene was used for screening as described previously (Wellehan et al., 2004; Papp et al., 2009). PCR led to the detection of AdVs in 10.6% (71/668) of lizards, 6.2% (26/420) of snakes, and 4.2% (3/70) of chelonians tested. Thus, AdVs were detected in captive-bred and wild-caught reptiles showing different clinical signs as well as in apparently healthy animals. Also, six new lizard AdVs, one new snake AdV and three new chelonian AdVs were detected during this study. New AdVs were found in green striped tree dragons (*Japalura splendida*), a green anole (*Anolis carolinensis*), a Jackson's chameleon (*Chamaeleo jacksonii*), a common agama (*Agama agama*) and a Hermann's tortoise (*Testudo hermanni*). These demonstrated less than 80% aa sequence identity of a portion of the DNA-polymerase gene to previously described AdVs. An AdV detected in a water monitor (*Varanus salvator*) had less than 90% sequence identity with a previously described AdV. Viruses demonstrating less than 99% sequence identity to previously described viruses were detected in a boa constrictor (*Boa constrictor*), a pond slider (*Trachemys scripta*), an Alabama map turtle (*Graptemys pulchra*) and in a blue tree monitor (*Varanus* (*Euprepiosaurus*) *macraei*). Phylogenetic analysis demonstrated that all of the AdVs detected in squamates, except Varanid AdVs, cluster within the genus *Atadenovirus*. However, all of the Varanid AdVs described so far cluster outside of the currently accepted genera. All chelonian AdVs detected during this study cluster in the proposed genus "Testadenovirus".

For the isolation of AdVs from bearded dragons a cell line was established from whole 6–8 week old central bearded dragon embryos. The established cells grow well at 28°C, are mid-sized and have a polymorphic and elongated form. The cells have been carried in continuous culture for 27 passages. This cell line has been used

with samples from AdV PCR-positive bearded dragons. 11 AdV isolates were obtained during this study on BDE cells. All isolated viruses demonstrated a CPE with rounding and detachment of cells. All isolates have been serially passaged on the established BDE cells. 5-10 additional passages of each virus isolate have been performed to date, following freeze–thaw cycles and low speed centrifugation. The AdVs isolated in BDE cells were identified as AdVs by PCR amplification and sequencing of a portion of the DNA-dependent DNA polymerase gene and showed 99-100% nucleotide identity to the corresponding region of Agamid AdV-1 (DQ077706). Two isolates were also identified as AdVs by electron microscopic examination of the cell culture supernatants. Nonenveloped particles, with a diameter of approximately 80 nm, were detected by negative staining electron microscopy.

During previous diagnostic procedures at the virological lab of the Fachgebiet für Umwelt und Tierhygiene of the University of Hohenheim, AdVs were also isolated from Helodermatid lizards (Helodermatid AdVs-1 and-2) on iguana heart cells (IgH2) and from snakes (Snake AdVs-1 and -2) on viper heart cells (VH-2). These isolations made comparative serological studies of antibodies against AdVs in squamate reptiles possible. Polyclonal antibodies against Helodermatid AdVs-1 and -2 were raised in rabbits and were used to establish neutralization tests with AdVs isolated during this study. Helodermatid AdVs-1 and -2, Snake AdVs-1 and -2 and an Agamid AdV-1 isolated in BDE during this study were used to detect neutralizing antibodies in plasma from a total of 404 reptiles. In lizards and snakes, antibodies were most commonly detected against Agamid AdV-1. The majority of helodermatid lizards tested had antibodies against Helodermatid AdV-2; antibodies against Agamid AdV-1 and Helodermatid AdV-1 were also present. Antibodies against Agamid AdV-1 were most commonly detected in agamid lizards. Three lizards (one helodermatid and two agamid lizards) had antibodies against Snake AdV-1. Antibodies against Helodermatid AdV-2 were also detected in snakes. No antibodies against Helodermatid AdV-1 were found in any of the snakes tested. The majority of viperid snakes tested were wild-caught animals and sent to our laboratory from the USA. The majority of these snakes had antibodies against Agamid AdV-1, while antibodies against the other isolates were less common in these animals. Antibodies against Agamid AdV-1 were also the most commonly detected antibodies in python samples.

The serological studies demonstrate that AdV infections appear to be common in wild-caught and captive lizards and snakes. The pattern of reactivity found in snakes and lizards against this range of squamate atadenoviruses shows that these viruses may not be as species specific as previously supposed. Hence, it is possible that these viruses may be able to spread between various squamate species.

The results of this study show that AdV infections are common among reptiles and increase our understanding of genetic diversity among these viruses. They also further confirm the usefulness of a pan-AdV PCR (Wellehan et al., 2004) for the detection of AdVs in reptiles including both squamates and chelonians. In addition, this is the first report on the detection of antibodies against a wide range of AdVs in lizards and snakes.

8 Zusammenfasung

Ziele dieser Arbeit waren, Reptilien auf Adenoviren (AdV) zu untersuchen, serologische Tests für den Nachweis von Antikörpern gegen Adenoviren in Squamaten zu entwickeln, sowie die serologischen Beziehungen zwischen Adenoviren bei Echsen und Schlangen zu bestimmen. Diese Untersuchungen sollen zum Aufbau und Erhalt von gesunden Beständen beitragen. Weitere Ziele dieser Arbeit waren die Entwicklung einer Agamid-Zelllinie sowie die Isolierung von Adenoviren von Bartagamen (*Pogona vitticeps*).

Die Proben wurden mit einer bereits etablierten PCR, die einen Teil des Gens der DNA-abhängigen DNA-Polymerase nachweist, untersucht. (Wellehan et al., 2004; Papp et al., 2009). Mithilfe dieser PCR wurden in 10,6% (71/668) der untersuchten Echsen, 6,2% (26/420) der Schlangen und 4,2% (3/70) der Schildkröten Adenoviren entdeckt. Es wurden sowohl bei in Gefangenschaft gezüchteten, als auch bei wild - gefangenen Reptilien Adenoviren nachgewiesen. Die betroffenen Tiere zeigten unterschiedliche klinische Symptome, einige waren scheinbar gesund. Im Rahmen dieser Arbeit wurden sechs neue Echsen-AdVn, ein neues Schlangen-AdV und drei Schildkröten-AdVn entdeckt. Viren, deren Genom im untersuchten Abschnitt weniger als 80% Übereinstimmung mit vorher bekannten Adenoviren aufwiesen, wurden bei Chinesischen Bartagamen (*Japalura splendida*), einem Rotkehlanolis (*Anolis carolinensis*), einem Dreihorn-Chamäleon (*Chameleo jacksonii*), einer Siedleragame (*Agama agama*) und einer Griechischen Landschildkröte (*Testudo hermanni*) nachgewiesen. Ein Adenovirus, welches in einem Bindenwaran (*Varanus salvator*) nachgewiesen wurde, wies bei der Sequenzierung weniger als 90% Übereinstimmung mit einem vorher bekannten Adenovirus auf. Vier weitere Viren, welche bei der Sequenzierung weniger als 99% Übereinstimmung mit vorher beschriebenen AdVs aufwiesen, wurden in einer Abgottschlange (*Boa constrictor*), einer Buchstabenschmuckschildkröte (*Trachemys scripta*), einer Alabama-Höckerschildkröte (*Graptemys pulchra*) und in einem Blauen Baumwaran (*Varanus* (*Euprepiosaurus*) *macraei*) nachgewiesen. Die phylogenetische Analyse zeigte, dass alle bei Squamaten nachgewiesenen Adenoviren, außer den Waran-AdVn, in das Genus *Atadenovirus* clustern. Alle bisher bekannten Waran-AdVn clustern außerhalb der bisher anerkannten Genera. Alle entdeckten Schildkröten-AdVn clustern in dem vorgeschlagenen Genus „Testadenovirus“.

Für die Isolierung der Bartagamen-AdVn wurde eine Zelllinie aus sechs bis acht Wochen alten Bartagamenembryos entwickelt (Bearded Dragon Embryo – Zellen; BDE-Zellen). Diese Zellen wurden bisher durchgehend in 27 Passagen gezüchtet. Sie wachsen gut bei einer Temperatur von 28°C, sind mittelgroß und haben eine polymorphe und längliche Form. Diese Zelllinie wurde mit Proben von Bartagamen inokuliert, welche in der PCR vorab AdV-positiv getestet worden waren. Im Rahmen dieser Arbeit konnten elf AdV-Isolate auf BDE-Zellen gezüchtet werden. Alle isolierten Viren führten zu einem CPE mit Abrundungen und Ablösen von Zellen. Bis heute wurde jedes Virusisolat fünf bis zehn Mal durch Einfrieren und Auftauen sowie niedertourige Zentrifugation passagiert. Die auf den BDE-Zellen isolierten Adenoviren wurden durch PCR-Amplifikation und Sequenzierung eines Teiles des DNA-abhängigen DNA-Polymerase Gens identifiziert und zeigten eine 99 bis 100%-ige Nukleotid-Übereinstimmung zum entsprechenden Teil des Agamid AdV-1 (DQ077706). Zwei Isolate wurden außerdem durch eine elektronenmikroskopische Untersuchung des Zellkulturüberstandes als Adenoviren identifiziert. Im Elektronenmikroskop konnten unbehüllte Partikel mit einem Durchmesser von ungefähr 80nm durch Negativkontrastierung nachgewiesen werden.

Im virologischen Labor des Fachgebietes für Umwelt- und Tierhygiene der Universität Hohenheim wurden bei vergangenen Untersuchungen Adenoviren von Krustenechsen (Helodermatid AdV-1 und -2) auf Leguan-Herzzellen (IgH2), sowie von Schlangen (Snake AdV-1 und -2) auf Viper-Herzzellen (VH-2) isoliert. Diese Isolierungen machten vergleichende serologische Untersuchungen von Adenoviren bei Squamaten sowie Antikörpernachweise bei verschiedenen Spezies möglich. In Kaninchen wurden polyklonale Antikörper gegen Helodermatid AdV-1 und -2 hergestellt und kamen zusammen mit den im Rahmen dieser Arbeit isolierten Adenoviren bei der Entwicklung von Neutralisationstests zum Einsatz. Helodermatid AdV-1 und -2, Snake AdV-1 und -2 sowie Agamid AdV-1, welche auf den BDE Zellen isoliert worden waren, wurden eingesetzt, um neutralisierende Antikörper im Plasma von insgesamt 404 Reptilien nachzuweisen. Bei den Echsen wurden hauptsächlich Antikörper gegen Agamid AdV-1 nachgewiesen. Die Mehrheit der untersuchten Krustenechsen wies Antikörper gegen Helodermatid AdV-2 auf. Bei einigen Echsen konnten sowohl Antikörper gegen Helodermatid AdV-1 als auch gegen Agamid AdV-1 nachgewiesen werden. Antikörper gegen Agamid AdV-1 wurden hauptsächlich bei

Agamiden nachgewiesen. Drei Echsen (eine Krustenechse und zwei Agamen) wiesen Antikörper gegen Snake AdV-1 auf. Schlangen wiesen die höchste Seroprävalenz gegen Agamid AdV-1 und gegen Helodermatid AdV-2 auf. Antikörper gegen Helodermatid AdV-1 konnten bei keiner der untersuchten Schlangen festgestellt werden. Die Mehrheit der untersuchten Vipern waren Wildfänge und wurden aus den USA eingeschickt. Von diesen Proben wiesen mehr als die Hälfte Antikörper gegen Agamid AdV-1 auf, während Antikörper gegen andere Viren weniger prävalent waren. Die bei Pythons am häufigsten nachgewiesenen Antikörper waren gegen Agamid AdV-1. Die Ergebnisse der serologischen Untersuchungen belegen, dass Infektionen mit Adenoviren sowohl bei Wildfängen als auch bei in Gefangenschaft gezüchteten Echsen und Schlangen häufig nachweisbar sind. Das Muster der Reaktivität gegen diese Bandbreite an Atadenoviren von Squamaten zeigt, dass diese Viren nicht so artspezifisch sind wie vorher angenommen. Es ist daher möglich, dass diese Viren in der Lage sind, sich zwischen verschiedenen Spezies von Squamaten zu verbreiten.

Die Ergebnisse dieser Arbeit zeigen, dass Adenovirus-Infektionen bei Reptilien häufig vorkommen. Sie vertiefen außerdem unser Verständnis für die genetische Vielfalt dieser Viren. Die Resultate bestätigen weiterhin den Nutzen einer pan-AdV - PCR (Wellehan et al., 2004) für den Nachweis von Adenoviren bei Reptilien wie Squamaten und Schildkröten. Darüber hinaus ist dies der erste Bericht über den Nachweis von Antikörpern gegen eine große Vielfalt von Adenoviren bei Echsen und Schlangen.

9 Literature cited

Abbas, M. D., Marschang, R. E., Schmidt, V., Kasper, A. and Papp, T. (2011) A unique novel reptilian paramyxovirus, four atadenovirus types and a reovirus identified in a concurrent infection of a corn snake (*Pantherophis guttatus*) collection in Germany. *Vet Microbiol,* **150**, 70–79.

Ascher, J. M., Geneva, A. J., Ng, J., Wyatt, J. D. and Glor, R. E. (2013) Phylogenetic analysis of novel squamate adenovirus sequences in wild-caught *anolis* lizards. *PLoS ONE,* **8**, e60977.

Ball, I., Hoferer, M. and Marschang, R. E. (2014) Establishment of an agamid cell line and first isolation of adenoviruses from bearded dragons (*Pogona vitticeps*). Journal of veterinary diagnostic investigation. *J Vet Diagn Invest,* **26**, 221-225.

Ball, I., Stöhr, A. C., Abbas, M. D. and Marschang, R. E. (2012) Detection and partial characterization of an adenovirus in a common agama (*Agama agama*). *J Herpetol Med Surg,* **22**, 12–16.

Barbezange, C., Benkő, M., Dán, Á. and Harrach B. (2000) DNA sequencing and phylogenetic analysis of the protease gene of ovine adenovirus 3 suggest that adenoviruses of sheep belong to two different genera. *Virus Res,* 66, 79–85.

Behncke, H., Stöhr, A. C., Heckers, K., Ball, I., and Marschang, R. E. (2013) Mass-mortality in green striped tree dragons (*Japalura splendida*) associated with multiple viral infections. *Vet Rec*, **173**, 248.

Benkő, M., Élő, P., Ursu, K., Ahne, W., LaPatra, S. E., Thomson, D. and Harrach B. (2002) First molecular evidence for existence of distinct fish and snake adenoviruses. *J Virol*, **76**, 10056 – 10059.

Benkő, M. and Harrach B. (2003) Molecular evolution of adenoviruses. Curr Top *Microbiol Immunol*, **272**, 3–35.

Benkő, M., Harrach, B., Both, G. W., Russel, W.C., Adair, B. M., Ádám, É., de Jong, J. C., Hess, M., Johnson, M., Kajon, A., Kidd, A. H., Lehmkuhl, H. D., Li, O. G., Mautner, V., Pring-Akerblom, P. and Wadell, G. (2005) Adenoviridae. In: Fauquet CM, Mayo MA, Maniloff J, et al.: eds. *Virus taxonomy, eighth report of the international committee on taxonomy of viruses.* Amsterdam, Netherlands: Elsevier Academic Press, 213–228.

Benkő, M. and Harrach B. (1998) A proposal for establishing a new (third) genus within the Adenoviridae family. *Arch Virol* , **143**, 829–837.

Benkő, M., Kaján, G. L., Jánoska, M., Kovács, E. R., Wellehan, J. F. X., Zsivanovits, P., Cough, R. E., Blahak, S., Schirrmeier, H., Bakonyi, T. and Harrach, B. (2006) How to find “new” adenoviruses. In: Leitão, A., and C. Martins (eds.). *Proceedings of the seventh International Congress of Veterinary Virology,* Ministério da Agricultura, Lisboa, Portugal. Pp.103.

Bodewes, R., van de Bildt, M. W., Schapendonk, C. M., van Leeuwen, M., van Boheemen, S., de Jong, A. A., Osterhaus, A. D., Smits, S. L. and Kuiken, T. (2013) Identification and characterization of a novel adenovirus in the cloacal bursa of gulls. *Virology* , **440**, 84-88.

Bonfield, J. K., Smith, K. F. and Staden R. (1995) A new DNA sequence assembly program*. Nucleic Acids Res,* **24**, 4992-4999.

Borysenko, M., and Lewis, S. (1979) The effect of malnutrition on immunocompetence and whole body resistance to infection in Chelydra Serpentina. *Dev Comp Immunol*, **3**, 89-100.

Both, G. W. (2004) Ovine atadenovirus: a review of its biology, biosafety profile and application as a gene delivery vector. *Immunol Cell Biol*, **82**, 189–195.

Dán, Á., Ruzsics, Z., Russell, W.C., Benkő, M. and Harrach, B. (1998) Analysis of the hexon genesequence of bovine adenovirus type 4 provides further support for a new adenovirus genus (Atadenovirus). *J Gen Virol,* **79**, 1453–1460.

Davison, A. J. and Harrach B. (2002) *Siadenovirus*. In: C.A. Tidona, G. Darai (Eds.), *The Springer Index of Viruses*, Springer, New York, pp. 29–33.

de Jong, J. C., Wermenbol, A. G., Verweij-Uijterwaal, M. W., Slaterus, K. W., Dillen, P. W., Van Doornum, G. J. J., Khoo, S. H. and Hierholzer, J. C. (1999) Adenoviruses from human immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51of species B1 and D, respectively. *J Clin Microbiol*, **37**, 3940–3945.

Davison, A. J., Wright, K. M., and Harrach, B. (2000) DNA sequence of frog adenovirus. *J Gen Virol*, **81**, 2431–2439.

Doszpoly, A., Wellehan, J. F. X. Jr., Childress, A. L., Tarján, Z. L., Kovács, E. R., Harrach, B., and Benkő, M. (2013) Partial characterization of a new adenovirus lineage discovered in testudinoid turtles. *Infect Genet Evol,* **17**, 106-112.

Élő, P., Farkas, S. L., Dán, Á., and Kovács, G. M. (2003) The p32K structural protein of the atadenovirus might have bacterial relatives. *J Mol Evol,* **56**, 175–180.

Farkas, S. L., Benkő, M., Elo, P., Ursu, K., Dan, A., Ahne, W., and Harrach, B. (2002) Genomic and phylogenetic analyses of an adenovirus isolated from a corn snake (*Elaphe guttata*) imply a common origin with members of the proposed new genus Atadenovirus. *J Gen Virol,* **83**, 2403-2410.

Farkas, S. and Gál, J. (2008) First Hungarian report of inclusion body hepatitis associated with adenoviruses and secondary parvovirus infection in an Indonesian pit-viper [*Parias* (*Trimeresurus*) *hageni*]. *Hungar Vet J,* **130**, 755-761.

Farkas, S. L. and Gál, J. (2009) Adenovirus and mycoplasma infection in an ornate box turtle (*Terrapene ornata ornata*) in Hungary. *Vet Microbiol*, **138**, 169-173.

Farkas, S. L., Harrach, B., and Benkő, M. (2008) Completion of the genome analysis of snake adenovirus type 1, a representative of the reptilian lineage within the novel genus *Atadenovirus*. *Vir Res,* **132**, 132–139.

Felsenstein, J. (1989) PHYLIP—Phylogeny Inference Package. *Cladistics*, **5**, 64–166.

Fent, G. M., Fulton, R. W., Saliki, J. T., Caseltine, S, L., Lenmkuhl, H. D., Confer, A. W., Purdy, C. W., Briggs, R. E., Loan, R. W. and Duff, G. C. (2002) Bovine adenoviruses serotype 7 infections in postweaning calves. *Am J Vet Res,* **63**, 976-978.

Frye, F. L., Munn, J. R., Gardner, M., Barten, L. S. and Hardfy, B. L. (1994) Adenovirus–like hepatitis in a group of related Rankin's dragon lizards (*Pogona henrylawsoni*). *J Zoo Wildl Med*, **25**, 167-171.

Funk, R. S., Marschang, R. E., Romanova, I. and Degan, M. (2011) Survey of native Arizona rattlesnakes for viral infections. In: *Proceedings of the Biology of the Rattlesnakes symposium*, Tucson, USA, p 31

Garner, M. M., Wellehan, J. F. X., Pearson, M., Koob, M., Boyer, T., Skinner, V., Nordhausen, R. W. and Barr, B. (2008) Pathology and molecular characterization of two novel atadenoviruses in colubrid snakes. *J Herpetol Med Surg,* **18**, 86-94.

Graham, D. A., Calvert, V., Benkő, M., Curran, W., Wylie, M., Snodden, D. A., Moffet, D. A., Papp, T., Adair, B. M. and Smyth, J. A. (2005). Isolation of bovine adenovirus serotype 6 from a calf in the United Kingdom. *Vet Rec,* **156**, 82–86.

Guillon, J-M., Guéry, L., Hulin, V. and Girondot, M. (2012) A large phylogeny of turtles (*Testudines*) using molecular data. *Contrib Zool*, **81**, 147-158.

Hall, T. A. (1999) BioEdit: a user friendly biological sequence alignment editor and analysis for Windows 95/98/NT. *Nucl Acids Symp Ser,* **41**, 95-98.

Harrach, B. (2008) Adenoviruses: general features. In: Mahy, B. W., van Regenmortel, M. H., editors. *Encyclopedia of virology*. Oxford: Elsevier. p. 1–9.

Harrach, B. (2000) Reptile adenoviruses in cattle? *Acta Vet Acad Sci Hung*, **48**, 485–490.

Harrach, B., Benkő, M., Both, G. W., Brown, M., Davison, A. J., Echavarría, M., Hess, M., Jones, M. S., Kajon, A., Lehmkuhl, H. D., Mautner, V., Mittal, S. and Wadell, G. (2011) Family *Adenoviridae*, In: King, A. M. Q., Lefkowitz, E., Adams, M. J., Carstens, E. B. (Eds.), Virus Taxonomy: *IXth Report of the International Committee on Taxonomy of Viruses.* New York: Elsevier, San-Diego, USA, pp 95-111

Hedrick, R. P., Speas, J., Kent, M. L. and McDowell, T. (1985) Adenolike virus associated with a disease of cultured white sturgeon *Acipenser transmontanus*. *Can J Fish Aquat Sci,* **42**, 1231-1235.

Heldstab, A. and Bestetti, G. (1984) Virus associated gastrointestinal diseases in snakes. *J Zoo An Med*, **15**, 118-128.

Hess, M., Blöcker, H. and Brandt, P. (1997) The complete nucleotide sequence of the egg drop syndrome virus: An intermediate between mastadenoviruses and aviadenoviruses. *Virology,* **238**, 145–156.

Hyndman, T. and Shilton, C. M. (2011) Molecular detection of two adenoviruses associated with disease in Australian lizards. *Aust Vet J,* **89**, 232-235.

Jacobson, E. R. (2007) Viruses and viral diseases of reptiles. In: Jacobson, E.R. (ed.). *Infectious Diseases and Pathology of Reptiles*. Taylor and Francis, Boca Raton. pp. 395-460.

Jacobson, E. R. and Gardiner C. H. (1990) Adeno-like virus in esophageal and tracheal mucosa of a Jackson's chameleon (*Chamaeleo jacksonii*). *Vet Path*, **27**, 210-212.

Jacobson, E. R., Gaskin, J. M., Brown, M. B., Harris, R. K., Gardiner, C. H., LaPointe J. L., Adams, H. P. and Reggiardo, C. (1991) Chronic upper respiratory tract disease of free-ranging desert tortoises (*Xerobates agassizii*). *J Wildl Dis*, **27**, 296-316.

Jacobson, E. R., Gaskin, J. M. and Gardiner, C. H. (1985) Adenovirus-like infection in a boa constrictor. *J Am Vet Med Assoc*, **187**, 1226-1227.

Jacobson, E. R. and Kollias, G.V. (1986) Adenovirus-like infection in a Savannah Monitor. *J Zoo Anim Med,* **17**, 149-151.

Jacobson, E. R., Kopit, W., Kennedy, F. A. and Funk, R. S. (1996) Coinfection of a bearded dragon, *Pogona vitticeps*, with adenovirus- and dependovirus-like viruses. *Vet Pathol*, **33**, 343-346.

Jacobson, E. R., Origgi, F., Pessier, A. P., Lamirande, E. W., Walker, I., Whitaker, B., Stalis, I. H., Nordhausen, R., Owens, J. W., Nichols, D. K., Heard, D., Homer, B. (2001) Paramyxovirus infection in caiman lizards (*Draecena guianensis*). *J Vet Diagn Invest,* **13**,143-51.

Juhász, A. and Ahne, W. (1993) Physicochemical properties and cytopathogenicity of an adenovi-rus-like agent isolated from corn snake (*Elaphe guttata*). *Arch Virol* , **130**, 429-439.

Julian and Durham, A. F., and Durham, P. J. (1985) Adenoviral hepatitis in a female bearded dragon (*Amphibolurus barbatus*). *New Zealand Vet J,* **30**, 59-60.

Kajan, G. L., Stefancsik, R., Ursu, K., Palya, V. and Benko, M. (2010) The first complete genome sequence of a non-chicken aviadenovirus proposed to be turkey adenovirus 1. *Virus Res,* **153**, 226-233.

Kajan, G. L., Davison, A. J., Palya, V., Harrach, B. and Benko, M. (2012) Genome sequence of a waterfowl aviadenovirus, goose adenovirus 4. *J Gen Virol,* **93**, 2457-2465.

Katoh, H., Ohya, K., Kubo, M., Murata, K., Yanai, T. and Fukushi, H. (2010) A novel budgerigar-adenovirus belonging to group II avian adenovirus of Siadenovirus. *Virus Res,* **144,** 294-297.

Kim, Y. D., Mitchell, A. M., Bauer, W. R., Poston, R. and Cho, Y. D. (2002) An outbreak of adenoviral infection in inland bearded dragons (*Pogona vitticeps*) coinfected with dependovirus and coccidial protozoa (*Isospora sp.*). *J Vet Diagn Invest,* **14**, 332–334.

Kinsel, M. J., Barbiers, R. B., Manharth, A. and Murnane, R. D. (1997) Small intestinal adeno-like virus in a mountain chameleon (*Chameleo montium*). *J Zoo Wildl Med,* **28**, 498-500.

Kovacs, E. R. and Benko, M. (2009) Confirmation of a novel siadenovirus species detected in raptors: partial sequence and phylogenetic analysis. *Virus Res,* **140**, 64-70.

Kovacs, E. R., Janoska, M., Dan, A., Harrach, B. and Benko, M. (2010) Recognition and partial genome characterization by non-specific DNA amplification and PCR

of a new siadenovirus species in a sample originating from Parus major, a great tit. *J Virol Methods,* **163**, 262-268.

Kovacs, G. M., LaPatra, S. E., D'Halluin, J. C. and Benko, M. (2004) Phylogenetic analysis of the hexon and protease genes of a fish adenovirus isolated from white sturgeon (Acipenser transmontanus) supports the proposal for a new adenovirus genus. *Virus Res,* **98**, 27-34.

Kraft, V., Grund, S. and Monreal, G. (1979). Ultrastructural characterization of isolate 127 of egg drop syndrome 1976 virus as an adenovirus. *Avian Pathol*, **8**, 353–361.

Kübber-Heiss A, Benetka V, Filip T, Benyr, G., Schilcher, F., Pallan, C., Möstl, K. (2006) Erstmaliger Nachweis einer Adenovirus-Infektion bei einer Bartagame (*Pogona vitticeps* AHL, 1926) in Österreich. [First evidence of adenovirus infection in a bearded dragon (*Pogona vitticeps*) in Austria] Veterinary Medicine Austria / *Wien Tierarztl Monat,* **93**, 68–72.

Kubiak, M. (2013) Detection of Agamid Adenovirus-1 in clinically healthy bearded dragons (*Pogona vitticeps*) in the UK. *Vet Rec*, **172**, 475.

Latney, L. V. and Wellehan, J. (2013) Selected emerging infectious diseases of squamata. *Vet Clin North Am Exot Anim Prac,* **6**, 319-38,

Lehmkuhl, H. D., Hobbs, L. A. and Woods, L. W. (2001) Characterization of a new adenovirus isolated from black-tailed deer in California. *EMBL Nucleotide Sequence Database/GenBank*. Accession number AF198354.

Lindl, T. (2002) Zell- und Gewebekultur: Einführung in die Grundlagen sowie ausgewählte Methoden und Anwendungen [Cell and tissue culture: introduction to the basics and selected methods and applications], *Spektrum Akademischer Verlag*, Berlin, Germany, pp. 110–112.

Luschow, D., Prusas, C., Lierz, M., Gerlach, H., Soike, D. and Hafez, H. M. (2007) Adenovirus of psittacine birds: investigations on isolation and development of a real-time polymerase chain reaction for specific detection. *Avian Pathol,* **36**, 487-494.

Mahapatra, D., Reinhard, M. and Naikare, H. K. (2013) Adenovirus and cryptosporidium co-infection in a corn snake (*Elaphae guttata guttata*). *J Zoo Wildl Med,* **44**, 220-224.

Marek, A., Günes, A., Schulz, E. and Hess, M. (2010) Classification of fowl adenoviruses by use of phylogenetic analysis and high-resolution melting-curve analysis of the hexon L1 gene region. *J Virol Methods*, **170**,147-54.

Marschang, R. E. (2011) Viruses infecting reptiles. *Viruses,* **3**, 2087-2126.

Marschang, R. E., Mischling, M., Benkő, M., Papp, T., Harrach, B. and Böhm, R. (2003) Evidence for wide-spread atadenovirus infection among snakes. In: Jestin A, Clement G (eds): Virus persistence and evolution*. Proceedings of the 6th International Congress of Veterinary Virology*. ZOO-POLE development – ISPAIA, Ploufragan, France, 152.

May, R. M. and Anderson, R. M. (1983) Epidemiology and Genetics in the Coevolution of Parasites and Hosts. *Proc R Soc Lond B*, **219**, 281-313.

McFerran, J. B., and Smyth, J. A. (2000) Avian adenoviruses. *Rev sci tech Off int Epiz*, **19**, 589-60.

Milne, I., D. Lindner, M. Bayer, D. Husmeier, G. McGuire, D. F. Marshall and Wright, F. (2008) TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics,* **25,** 126-127.

Moormann, S., Seehusen, F., Reckling, D., Kilwinski, J., Puff, C., Elhensheri, M., Wohlsein, P. and Peters, M. (2009) Systemic adenovirus infection in bearded dragons (*Pogona vitticeps*): histological, ultrastructural and molecular findings. *J Comp Path,* **141**, 78-83.

Ochsenbein, A. F. and Zinkernagel, R.R. (2000) Natural antibodies and complement link innate and acquired immunity. *Immunol Today*, **21**, 624-630.

Ogawa, M., Ahne, W. and Essbauer, S. (1992) Reptilian viruses: adenovirus-like agent isolated from royal python *(Python regius). J Vet Med B Infect Dis Vet Public Health,* **39**, 732-736.

Park, Y. M., Kim, J. H., Gu, S. H., Lee, S. Y., Lee, M. G., Kang, Y. K., Kang, S. H., Kim, H. J. and Song, J. W. (2012) Full genome analysis of a novel adenovirus from the South Polar skua (Catharacta maccormicki) in Antarctica. *Virology,* **422**, 144-150.

Parkin, D. B., Archer, L. L., Childrees, A. L. and Wellehan, J. F. X. (2009) Genotype differentiation of Agamid Adenovirus 1 in bearded dragons *(Pogona vitticeps)* in the USA by hexon gene sequence. *Infect Genet Evol,* **9**, 501-506.

Papp, T., Fledelius, B., Schmidt, V., Kaján, G. L. and Marschang, R. E. (2009) PCR-sequence characterization of new adenovirus found in reptiles and the first successful isolation of a lizard adenovirus. *Vet Microbiol*,**134**, 233-240.

Papp, T., Spann, D. and Marschang, R. E. (2014) Development and use of a real-time PCR for the detection of group II invertebrate iridoviruses in pet lizards and prey insects. *J Zoo Wildl Med*, **45(2)**, 219-227.

Pees, M., Schmidt, V., Marschang R. E., Heckers, K. O. and Krautwald-Junghanns, M-E. (2010) Prevalence of viral infections in a captive collections of boid snakes in Geramany. *Vet Rec,* **166**, 422-425.

Pénzes, J. J., Menéndez-Conejero, R., Ball, I., Papp, T., Doszpoly, A., Paradela, A., Pérez-Berná, A. J., López-Sanz, M., Marschang, R. E., Harrach, B., Benkő, M. and Carmen San Martín, C. (2014) Molecular characterization of a lizard adenovirus reveals the first atadenovirus with two fiber genes, and the first adenovirus with either one short or three long fibers per penton. *J Virol*, **88(19)**, 11304-11314.

Perkins, L. E., Champagnoli, R. P., Harmon, B. G., Gregory, C. R., Steffens, W. L., Latimer, K., Clubb, S., and Crane, M. (2001) Detection and confirmation of reptilian adenovirus infection by *in situ* hybridization. *J Vet Diagn Invest*, **13**, 365-8.

Pitcovski, J., Mualem, M., Rei-Koren, Z., Krispel, S., Shmueli, E., Peretz, Y., Gutter, B., Gallili, G.E., Michael, A., and Goldberg, D. (1998) The complete DNA sequence and genome organization of the avian adenovirus, hemorrhagic enteritis virus. *Virology,* **249**, 307–315.

Pyron, A. R., Burbrink, F. T. and Wiens, J. J. (2013) A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol Biol*, **13**, 93.

Ramis, A., Fernández-Bellon, H., Majó, N., Martínez-Silvestre, A., Latimer, K., and Champagnoli, R. (2000) Adenovirus hepatitis in a boa constrictor *(Boa Constrictor)*. *J Vet Diagn Invest,* **12**, 573.

Raymond, J. T., M. M. Garner, S. Murray, and Nordhausen R. (2002) Oroesophageal adenovirus-like infection in a palm viper, *Bothriechis marchi*, with inclusion body-like disease. *J Herpetol Med Surg*, **12**, 30-32.

Raymond, J. T., Lamm, M., Nordhausen, R., Latimer, K., and Garner, M.M. (2003) Degenerative incephalopathy in a coastal mountain kingsnake (*Lampropeltis zonata multifasciata)* due to adenoviral-like infection. *J Wildl Dis*, **39**, 431-436.

Rivera, S., Wellehan, J. F. Jr., McManamon, R., Innis, C. J., Garner, M. M., Raphael, B. L., Gregory, C. R., Latimer, K. S., Rodriguez, C. E., Diaz-Figueroa, O., Marlar, A. B., Nyaoke, A., Gates, A. E., Gilbert, K., Childress, A. L., Risatti, G. R. and Frasca, S. Jr. (2009) Systemic adenovirus infection in Sulawesi tortoises (*Indotestudo forsteni*) caused by a novel siadenovirus. *J Vet Diagn Invest,* **21**, 415-426.

Romanova, I., Marschang, R. E., Schmidt, V. and Papp, T. (2011) Diagnose und Charakterisierung eines virperid Adenovirus bei einer Kornnatter (*Pantherophis gutattus*). *In: Proceedings of the Annual Meeting der Deutschen Gesellschaft für Zootier-, Wildtier- und Exotenmedizin der DVG*, Stuttgart, Germany, pp. 3-4.

Russell, W.C. (2009) Adenoviruses: update on structure and function. *J Gen Virol,* **90**, 1-20.

Schat, K. A. and Purchase, H. G. (1998) Cell-culture methods. *In*: *A laboratory manual for the isolation and identification of avian pathogens*, 4th ed., ed. Swayne DE, American Association of Avian Pathologists, Kennett Square, PA. pp. 223–234.

Schrenzel, M., Oaks, J. L., Rotstein, D., Maalouf, G., Snook, E., Sandfort, C. and Rideout, B. (2005) Characterization of a new species of adenovirus in falcons. *J Clin Microbiol,* **43**, 3402–3413.

Schumacher, J., Jacobson, E.R., Burns, R. and Tramontin, R. R. (1994) Adenovirus-like infection in two rosy boas *(Lichanura trivirgata)*. *J Zoo Wildlife Med,* **25**, 461-465.

Schumacher, V. L., Innis, C. J., Garner, M. M., Risatti, G. R., Nordhausen, R. W., Gilbert-Marcheterre, K., Wellehan, J. F. X. Jr., Childress, A. L. and Frasca, S. Jr. (2012) Sulawesi tortoise adenovirus-1 in two impressed tortoises (*Manouria impressa*) and a Burmese star tortoise (*Geochelone platynota*). *J Zoo Wildl Med,* **43**, 501-510.

Schwarz, R. and Dayhoff, M. (1979) Matrices for detecting distantrelationships. *In Dayhoff M (ed): Atlas of Protein Sequences.* National Biomedical Research Foundation, Washington, DC. pp. 353–358.

Shivaprasad, H. L. (2008) Adenovirus group II-like infection in chukar partridges

(*Alectoris chukar*). *Avian Dis,* **52**, 353-356.

Stöhr, A. C., Blahak, S., Heckers, K. O., Wiechert, J., Behncke, H., Mathes, K., Günther, P., Zwart, P., Ball, I., Rüschoff, B. and Marschang, R. E. (2013) Ranavirus infections associated with skin lesions in lizards. *Vet Res,* **44**, 84.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, **28**, 2731–2739.

Thomson, D., Meers, J. and Harrach, B. (2002) Molecular confirmation of an adenovirus in brushtail possums (Trichosurus vulpecula). *Virus Res*, **83**, 189–195.

Tomaszewski, E. K. and Phalen, D. N. (2007) Falcon adenovirus in an American kestrel (Falco sparverius). *J Avian Med Surg,* **21**, 135-139.

Townsend, T., Larson, A. Louis, E. and Macey J. (2004) Molecular phylogenetics of squamata: the position of snakes, amphisbaenians, and dibamids, and the root of the squamate tree. *Syst Biol*, **53**, 735–757.

Vidal, N. and Hedges, B. S. (2005) The phylogeny of squamate reptiles (lizards, snakes and amphisbaenians) inferred from nine nuclear protein-coding genes. *C R Biol*, **328**,1000-1008.

Villegas, P. (1998) Titration of biological suspensions. In: *Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M., (Eds.), Isolation and identification of avian pathogens*, 4th ed. American Association of Avian Pathologists, Kennett Square, PA, pp. 248-253.

Wadell, G. (1984) Molecular epidemiology of human adenoviruses. *Curr Top Microbiol Immunol,* **110**, 191–220.

Wellehan, J .F .X., Johnson, A. J., Harrah, B., Benkő, M., Pessier, A. P., Johnson, C. M., Garner, M .M., Childress, A. and Jacobson, E. R. (2004) Detection and analysis of six lizard adenoviruses by consensus primer PCR provides further evidence of a reptilian origin for the atadenoviruses. *J Virol,* **78**, 13366-13369.

Wellehan, J. F. X. Jr., Johnson, A. J., Latimer, K. S., Bischoff, K., Lafortune, M. and Jacobson, E. R. (2005) Initial characterization of an adenovirus associated with fatal hepatic and lymphoid necrosis in a Meyer.s parrot (*Poicephalus meyeri*). *J Avian Med Surg,* **19**, 191-197.

Wellehan, J. F. Jr., Greenacre, C. B., Fleming, G. J., Stetter, M. D., Childress, A. L. and Terrell, S. P. (2009) Siadenovirus infection in two psittacine bird species. *Avian Pathol,* **38**, 413-417.

Wellehan, J. F.X. Jr., Schneider, R. and Childress, A. L. (2012) Identification of Helodermatid adenovirus-2 in a captive central bearded dragon (*Pogona vitticeps*) in the United States. *In: Proceedings of the 19 th Annual Meeting of the Association of Reptilian and Amphibian Veterinarians*, Oakland, USA, p. 5

Wilkinson, R. (2004) Clinical pathology. In: *Medicine and Surgery of Tortoises and Turtles*, McArthur, S., Wilkinson, R., Meyer, J., Eds.; Blackwell Publishing: Chichester, UK, pp. 141–186.

Wozniak, E., DeNardo, D. F., Brewer, A., Wong, V. and Tarara, R. P. (2000) Identification of adenovirus- and dependovirus-like agents in an outbreak of fatal gastroenteritis in captive born California mountain kingsnakes (*Lampropeltis zonata multicincta*). *J Herpetol Med Surg*,**10**, 4-7.

Zakhartchouk, A., Bout, A., Woods, L. W., Lehmkuhl, H. D. and Havenga, M. J. (2002) Odocoileus hemionus deer adenovirus is related to the members of Atadenovirus genus. *Arch Virol,* **147**, 841–847.

Zsivanovits, P., Monks, D. J, Forbes, N. A., Krisztina Ursu, K., Raue, R. and Benkö, M. (2006) Presumptive Identification of a Novel Adenovirus in a Harris Hawk (Parabuteo unicinctus), a Bengal Eagle Owl (Bubo bengalensis), and a Verreaux's Eagle Owl (Bubo lacteus). *J Avian Med Surg,* **20**,105-112.

10 Appendix

Table 17: Reptilian samples tested by PCR during this study
Legend: The number of reptiles/number of positive reptiles (total number of samples/number of positive samples). Reptile species sorted by families, within the families alphabetically by common name

Reptile species	2008	2009	2010	2011	2012	2013	Total
Lizards							
Black-soil bearded dragon (*Pogona henrylawsoni*)	-	-	3/0 (5/0)	1/0 (1/0)	-	-	4/0 (6/0)
Central bearded dragon (*Pogona vitticeps*)	9/1 (12/2)	4/1 (7/2)	63/10 (67/10)	82/14 (92/14)	100/33 (109/34)	40/2 (44/2)	298/61 (331/64)
Common agama (*Agama smithi* formerly *Agama agama*)	1/0 (1/0)	-	1/1 (4/1)	1/0 (2/0)	-	-	3/1 (7/1)
Egyptian spiny-tailed lizard *(Uromastix aegyptia microlepis)*	-	1/0 (6/0)	1/0 (2/0)	2/0 (2/0)	-	-	4/0 (10/0)
Frilled lizard (*Chlamydosaurus kingii pughae*)	-	-	1/0 (1/0)	-	-	-	1/0 (1/0)
Green striped tree dragon (*Japalura splendida*)	-	-	-	5/3 (20/3)	-	-	5/3 (20/3)
Green water dragon (*Physignathus cocincinus*)	-	1/0 (1/0)	4/0 (8/0)	3/0 (3/0)	-	-	8/0 (12/0)
Common wall lizard (*Podarcis muralis nigriventris*)	6/0 (16/0)	18/0 (69/0)	47/0 (183/0)	10/0 (26/0)	-	-	81/0 (294/0)
European green lizard (*Lacerta viridis meridionalis*)	1/0 (1/0)	-	-	-	-	-	1/0 (1/0)
Parelhagedis (*Timon lepidus oteroorum*)	-	1/0 (2/0)	-	-	-	-	1/0 (2/0)
Sand lizard (*Lacerta agilis ioriensis*)	41/0 (82/0)	-	12/0 (49/0)	83/0 (83/0)	16/0 (16/0)	-	152/0 (230/0)
Western green lizard (*Lacerta bilineata fejervaryi*)	-	-	1/0 (1/0)	-	-	-	1/0 (1/0)
Carpet Chameleon (*Furcifer major*)	-	-	1/0 (1/0)	-	-	-	1/0 (1/0)
Flapneck chameleon (*Chamaeleo ruspolii*)	-	-	1/0 (5/0)	-	-	-	1/0 (5/0)
Jackson's chameleon (*Chamaeleo jacksonii xantholophus*)	-	2/0 (4/0)	1/0 (1/0)	1/0 (1/0)	1/1 (1/1)	-	5/1 (7/1)

Reptile species	2008	2009	2010	2011	2012	2013	Total
Lizards							
Malagasy giant chameleon (*Furcifer oustaleti*)	-	-	-	-	1/0 (3/0)	-	1/0 (3/0)
Panther chameleon (*Furcifer pardalis*)	-	-	-	-	2/0 (2/0)	1/0 (1/0)	3/0 (3/0)
Parson's chameleon (*Calumma parsonii*)	1/0 (4/0)	-	-	-	-	-	1/0 (4/0)
Yemen chameleon (*Chamaeleo chamaeleon recticrista*)	-	-	2/0 (5/0)	1/0 (2/0)	-	1/0 (3/0)	4/0 (10/0)
Common green iguana (*Iguana iguana rhinolopha*)	3/0 (6/0)	-	-	2/0 (2/0)	7/0 (8/0)	1/0 (2/0)	13/0 (18/0)
Fiji banded iguana (*Brachylophus fasciatus*)	-	-	-	-	2/0 (2/0)	-	2/0 (2/0)
Beaded lizard (*Heloderma horridum exasperatum*)	-	9/0 (13/0)	7/0 (7/0)	1/0 (1/0)	-	-	17/0 (21/0)
Gila monster (*Heloderma suspectum*)	1/0 (2/0)	-	17/0 (17/0)	1/0 (1/0)	2/0 (4/0)	-	21/0 (24/0)
Common leopard gecko (*Eublepharis macularius*)	-	-	1/0 (2/0)	2/0 (3/0)	-	-	3/0 (5/0)
Iraqi eyelid gecko (*Eublepharis angramainyu*)	-	-	2/0 (2/0)	-	-	-	2/0 (2/0)
Tokay gecko (*Gecko gecko azhari)*	-	-	1/0 (6/0)	-	-	-	1/0 (6/0)
Common bluetongue *(Tiliqua scincoides chimaerea)*	-	-	-	1/0 (1/0)	-	-	1/0 (1/0)
Shingleback lizard (*Tiliqua rugosa palarra*)	-	-	-	-	-	18/0 (18/0)	18/0 (18/0)
Solomon island skink (*Corucia zebrata*)	-	-	-	1/0 (1/0)	-	-	1/0 (1/0)
Black-tailed monitor (*Varanus tristis orientalis*)	-	-	-	1/0 (1/0)	-	-	1/0 (1/0)
Blue tree monitor (*Varanus macraei*)	-	-	-	1/0 (1/0)	1/1 (2/2)	1/1 (1/1)	3/2 (4/3)
Ridgetail monitor (*Varanus acanthurus insulanicus*)	-	-	-	2/0 (3/0)	-	-	2/0 (3/0)

Reptile species	2008	2009	2010	2011	2012	2013	Total
Lizards							
Savannah monitor (*Varanus exanthematicus*)	-	-	1/0 (2/0)	1/0 (1/0)	-	-	2/0 (3/0)
Water monitor (*Varanus salvator ziegleri*)	-	-	-	-	2/2 (11/5)	-	2/2 (11/5)
Green anole (*Anolis carolinensis*)	-	-	-	-	1/1 (3/1)	-	1/1 (3/1)
Collared lizard (*Crotaphytus collaris fuscus*)	-	-	1/0 (2/0)	-	-	-	1/0 (2/0)
Common basilisk (*Basiliscus basiliscus*)	-	-	1/0 (1/0)	1/0 (1/0)	-	-	2/0 (2/0)
Snakes							
Aesculapian snake (*Zamenis longissimus)*	-	1/0 (4/0)	-	-	1/0 (1/0)	-	2/0 (5/0)
Amur ratsnake (*Elaphe schrenckii)*	1/0 (3/0)	-	-	-	-	-	1/0 (3/0)
Beauty snake (*Orthriophis taeniurus yunnanensis*)	-	-	-	3/0 (3/0)	-	-	3/0 (3/0)
Corn snake (*Pantherophis guttatus)*	1/0 (3/0)	33/8 (73/11)	5/1 (9/1)	14/5 (22/5)	9/2 (16/4)	1/1 (3/3)	63/17 (126/24)
Flower snake (*Orthriophis moellendorffi*)	-	-	-	1/0 (1/0)	-	-	1/0 (1/0)
Grey-banded kingsnake (*Lampropeltis alterna*)	-	-	-	-	1/0 (1/0)	-	1/0 (1/0)
Smooth snake (*Coronella austriaca acutirostris)*	2/0 (4/0)	-	-	-	-	-	2/0 (4/0)
Taiwan stink snake (*Elaphe carinata*)	-	-	-	1/0 (1/0)	-	-	1/0 (1/0)
Anaconda (*Eunectes murinus gigas*)	-	-	1/0 (1/0)	-	4/0 (4/0)	3/0 (3/0)	8/0 (8/0)
Boa constrictor (*Boa constrictor ortonii*)	2/1 (6/3)	25/0 (73/0)	9/0 (14/0)	13/1 (13/1)	5/0 (9/0)	10/0 (17/0)	64/2 (132/4)
Garden tree boa (*Corralus hortulanus*)	1/0 (3/0)	2/0 (6/0)	-	-	-	1/0 (7/0)	4/0 (16/0)
Rosy boa (*Lichanura trivirgata*)	-	-	-	-	-	1/0 (2/0)	1/0 (2/0)
Angolan python (*Python anchietae*)	-	1/0 (1/0)	-	-	-	-	1/0 (1/0)

Reptile species	2008	2009	2010	2011	2012	2013	Total
Snakes							
Bismark ringed python (*Bothrochilus boa)*	-	-	1/0 (5/0)	-	-	-	1/0 (5/0)
Carpet python (*Morelia spilota*)	-	5/0 (15/0)	9/0 (13/0)	-	3/0 (3/0)	-	17/0 (31/0)
Children's python (*Antaresia childreni*)	-	1/0 (1/0)	-	-	-	-	1/0 (1/0)
Green tree python (*Morelia viridis*)	-	2/0 (8/0)	3/0 (5/0)	1/0 (1/0)	15/0 (15/0)	-	21/0 (29/0)
Indian python (*Python molurus*)	32/2 (35/2)	16/0 (53/0)	12/0 (29/0)	7/1 (20/1)	13/1 (24/1)	8/0 (11/0)	88/4 (172/4)
Madagascar ground boa (*Acrantophis madagascariensis)*	-	-	1/0 (3/0)	-	-	-	1/0 (3/0)
Red blood python (*Python brongersmai*)	-	-	-	5/0 (11/0)	6/0 (6/0)	-	11/0 (17/0)
Reticulated python (*Python reticulatus saputrai*)	-	-	-	1/0 (1/0)	2/0 (2/0)	1/0 (1/0)	4/0 (4/0)
Royal python (*Python regius*)	7/0 (24/0)	9/0 (36/0)	13/0 (17/0)	39/0 (40/0)	14/0 (34/0)	8/0 (21/0)	90/0 (172/0)
Eyelash viper (Bothriechis schlegelii)	-	-	4/2 (4/2)	-	-	-	4/2 (4/2)
Northern viper (*Vipera berus sachalinensis*)	-	-	-	-	19/0 (19/0)	-	19/0 (19/0)
Nose-horned viper (*Vipera ammodytes montandoni*)	-	-	-	1/0 (3/0)	-	-	1/0 (3/0)
Pigmy rattlesnake (*Sistrurus miliarius*)	-	1/0 (5/0)	-	-	-	-	1/0 (5/0)
Dice snake (*Xenochrophis tessellata*)	-	-	1/0 (1/0)	-	-	-	1/0 (1/0)
European grass snake (*Natrix natrix sicula*)	-	-	-	1/0 (1/0)	3/0 (3/0)		4/0 (4/0)
Common death adder (*Acanthophis schistos*)	-	-	-	-	3/1 (12/1)	-	3/1 (12/1)
Eastern coral snake (*Micrurus fulvius*)	-	-	-	-	1/0 (1/0)	-	1/0 (1/0)
Chelonians							
Hermann's tortoise (*Eurotestudo boettgeri*)	-	-	-	2/1 (2/1)	-	17/0 (27/0)	19/1 (29/1)
Horsfield's tortoise (*Agrionemys horsfieldii rustamovi*)	-	-	-	-	5/0 (5/0)	-	5/0 (5/0)

Reptile species	2008	2009	2010	2011	2012	2013	Total
Chelonians							
Madagascan tortoise (*Astrochelys yniphora*)	-	-	-	-	5/0 (5/0)	-	5/0 (5/0)
Marginated tortoise (*Testudo marginata*)	-	-	-	-	-	1/0 (1/0)	1/0 (1/0)
Mediterranean spur-thighed tortoise (*Testudo graeca zarudnyi*)	-	-	-	-	-	1/0 (1/0)	1/0 (1/0)
Alabama map turtle (*Graptemys pulchra*)	-	-	-	-	1/1 (1/1)	-	1/1 (1/1)
European pond turtle (*Emys orbicularis persica*)	-	-	-	-	24/0 (24/0)	-	24/0 (24/0)
Midland painted turtle (*Chrysemys picta marginata*)	-	-	-	-	1/0 (1/0)	-	1/0 (1/0)
Pond slider (*Trachemys scripta scripta*)				1/1 (1/1)			1/1 (1/1)
Red-eared slider (*Trachemys scripta troostii*)	-	-	-	-	6/0 (10/0)	-	6/0 (10/0)
Yellowbelly slider (*Trachemys scripta elegans*)	-	-	-	-	3/0 (5/0)	-	3/0 (5/0)
Irwin's turtle (*Elseya irwini*)	-	-	-	-	1/0 (1/0)	-	3/0 (5/0)

Table 18: Squamate species from which plasma were collected for NT With country of origin, sex, and age. Total number of animals in parenthesis

Species	Number of animals	Country of origin	Sex	Age	Origin
Lizards					
Central bearded dragon (*Pogona vitticeps*)	221	Germany (140), USA (62), Switzerland (15), Austria (3), Irland (1)	male (75) female (57) unknown (8)	4 months – 10 years	captive-bred (96) unknown (125)
Green iguana (*Iguana iguana*)	3	Germany (1), Switzerland (2)	unknown (3)	3 years (1) unknown (2)	unknown (3)
Mexican beaded lizard (*Heloderma horridum*)	12	Denmark (5), Germany (4), USA (3)	unknown (12)	unknown (12)	wild-caught (1) unknown (11)
Gila monster (*Heloderma suspectum*)	15	USA (15)	unknown (15)	unknown (15)	wild-caught (9) captive-bred (3) unknown (3)

Species	Number of animals	Country of origin	Sex	Age	Origin
Lizards					
Lesser antillean iguana (*Iguana delicatissima)*	3	UK (3)	unknown (3)	unknown (3)	unknown (3)
Green water dragon (*Physignathus cocincinus*)	2	Switzerland (2)	male (1) female(1)	adult (2)	unknown (2)
Solomon island skink (*Corucia zebrata*)	1	Switzerland (1)	unknown (1)	adult (1)	unknown (1)
Common bluetongue *(Tiliqua scincoides chimaerea*)	1	Switzerland (1)	unknown (1)	adult (1)	captive-bred (1)
Savannah monitor (*Varanus exanthematicus*)	1	Switzerland (1)	unknown (1)	juvenile (1)	captive-bred (1)
Ridgetail monitor (*Varanus acanthurus insulanicus*)	2	Germany (2)	male (2)	unknown (2)	unknown (2)
Blue tree monitor (*Varanus macrae*i)	1	Germany (1)	male (1)	unknown (1)	unknown (1)
Black-tailed monitor (*Varanus tristis orientalis)*	1	Germany (1)	male (1)	unknown (1)	unknown (1)
Snakes					
Western diamond-backed rattlesnake *(Hoserea atrox)*	17	USA (17)	male (4) female (4) unknown (9)	6 month – 10 years	wild-caught (16) captive-bred (1)
Mojave (green) rattlesnake *(Crotalus scutulatus salvini)*	2	USA (2)	male (1) unknown (1)	unknown (2)	wild-caught (2)
Tiger rattlesnake *(Matteoea tigris)*	11	USA (11)	male (5) female (3) unknown (3)	unknown (11)	wild-caught (11)
Black tailed rattlesnake *(Crotalus molossus oaxacus)*	4	USA (4)	male (2) female (1) unknown (1)	unknown (4)	wild-caught (4)
Arizona black rattlesnake *(Crotalus cerberus)*	7	USA (7)	male (3) female (1) unknown (3)	unknown (7)	wild-caught (7)
Southwestern speckled rattlesnake (*Crotalus mitchellii pyrrhus)*	16	USA (16)	male (4) female (4) unknown (8)	unknown (16)	wild-caught (16)
Hopi rattlesnake *(Crotalus viridis nuntius)*	21	USA (21)	male (2) female (3) unknown (16)	unknown (21)	wild-caught (21)
Grand canyon rattlesnake (*Crotalus oreganus lutosus*)	3	USA (3)	unknown (3)	unknown (3)	wild-caught (3)
Mjave desert sidewinder *(Crotalus cerastes laterorepens)*	6	USA (6)	male (2) female (1) unknown (3)	unknown (6)	wild-caught (6)
Desert death adder (*Acanthophis pyrrhus)*	1	USA (1)	unknown (1)	unknown (1)	wild-caught (1)

Species	Number of animals	Country of origin	Sex	Age	Origin
Snakes					
Arizona ridgenose rattlesnake *(Crotalus willardi silus)*	7	USA (7)	unknown (7)	unknown (7)	wild-caught (7)
Great basin rattlesnake *(Crotalus oreganus lutosus)*	3	USA (3)	male (3)	unknown (3)	wild-caught (3)
Banded rock rattlesnake *(Crotalus lepidus klauberi)*	3	USA (3)	unknown (3)	unknown (3)	wild-caught (3)
Western twin spotted rattlesnake (Crotalus pricei miquihuanus)	3	USA (3)	unknown (3)	unknown (3)	wild-caught (3)
Lower california rattlesnake (*Crotalus enyo*)	1	USA (1)	female (1)	unknown (1)	wild-caught (1)
Western massasauga (S*istrurus catenatus tergeminus*)	1	USA (1)	unknown (1)	unknown (1)	wild-caught (1)
hybrid Banded rock rattlesnake X Arizona ridgenose rattlesnake (*Crotalus lepidus klauberi* X *Crotalus willardi willardi*)	1	USA (1)	unknown (1)	unknown (1)	wild-caught (1)
Reticulated Python *(Python reticulatus saputrai)*	3	Germany (3)	unknown (3)	unknown (3)	unknown (3)
Indian Python *(Python molurus molurus)*	12	Germany (12)	female (1) unknown (11)	unknown(12)	unknown (12)
Red Blood Python (*Python brongesma*i)	2	Germany (2)	female (1) unknown (1)	5 years (1) unknown (1)	captive-bred (1) unknown (1)
Green tree python (*Morelia viridis*)	1	Italy (1)	unknown (1)	unknown (1)	unknown (1)
Royal python (*Python regius*)	2	Germany (2)	male (2)	unknown (2)	unknown (2)
Puff Adder *(Bitis arietans somalica)*	1	Germany (1)	male (1)	adult (1)	unknown (1)
Nose-horned Viper *(Vipera ammodytes montandoni)*	1	Germany (1)	male (1)	adult (1)	unknown (1)
Beauty snake (*Orthriopis taeniurus yunnanensis*)	3	Germany (3)	unknown (3)	unknown (3)	unknown (3)
Taiwan stink snake (*Elaphe carinata*)	1	Germany (1)	male (1)	unknown (1)	unknown (1)
Boa constrictor (*Boa constrictor*)	4	Germany (4)	female (4)	unknown (4)	unknown (4)
Anaconda *(Eunectes murinus gigas)*	4	Germany (4)	unknown (4)	unknown (4)	unknown (4)

Table 19: Identity matrix
Of the amino acid (under diagonal) and nucleotide (above diagonal) 272 nt and 92 aa sequences of a portion of the DNA-polymerase gene of squamate atadenoviruses. For accession numbers see legend of Figure 1

nt / aa	Ch-1	Agam-1	Sn-1	Hel-2	Hel-1	Eubl	Varan	**BlueTM**	**WaterM**	**Com. a**	**Gr.std**	**Ch-2**	**Anol-3**	Anol-2	Anol-1	**Boa c.**	Box-t	EastBT	Pan. t.	**Herm. T**	RedES	Yell.BS	**Pond s.**	**Map t.**
Chameleon-1		0,613	0,627	0,557	0,549	0,594	0,546	0,590	0,645	0,583	0,621	0,608	0,624	0,558	0,562	0,608	0,432	0,400	0,391	0,402	0,405	0,377	0,405	0,373
Agamid-1	0,655		0,636	0,632	0,639	0,584	0,562	0,610	0,613	0,601	0,608	**0,631**	0,625	0,600	0,600	0,602	0,441	0,434	0,400	0,407	0,432	0,407	0,421	0,393
Snake-1	0,655	0,666		0,701	0,697	0,671	0,557	0,601	0,612	0,637	0,658	0,608	0,591	0,605	0,580	**0,918**	0,464	0,442	0,451	0,377	0,395	0,380	0,380	0,345
Helodermatid-2	0,611	0,711	0,744		0,911	0,667	0,498	0,538	0,564	0,608	0,680	0,564	0,572	0,598	0,572	0,682	0,439	0,410	0,441	0,348	0,384	0,409	0,384	0,341
Helodermatid-1	0,622	0,733	0,744	0,966		0,686	0,501	0,546	0,557	0,612	**0,687**	0,545	0,576	0,620	0,591	0,671	0,439	0,410	0,423	0,345	0,380	0,398	0,377	0,338
Eublepharid	0,611	0,644	0,744	0,800	0,811		0,542	0,594	0,583	**0,655**	0,617	0,549	0,587	0,609	0,558	0,671	0,464	0,396	0,409	0,384	0,370	0,366	0,362	0,327
Varanid	0,633	0,600	0,644	0,600	0,600	0,622		**0,896**	0,690	0,521	0,533	0,545	0,554	0,536	0,583	0,567	0,385	0,385	0,441	0,430	0,370	0,352	0,366	0,392
Blue tree m.	0,622	0,588	0,633	0,588	0,588	0,611	**0,988**		**0,734**	0,561	0,580	0,597	0,609	0,594	0,583	0,592	0,417	0,403	0,459	0,391	0,409	0,391	0,398	0,366
Water mon.	0,655	0,588	0,666	0,611	0,611	0,577	**0,800**	0,788		0,648	0,584	0,604	0,620	0,605	0,594	0,601	0,428	0,403	0,430	0,444	0,427	0,402	0,398	0,370
Common agam.	0,611	0,622	0,722	0,700	0,711	**0,744**	0,644	0,633	0,644		0,613	0,601	0,628	0,557	0,565	0,594	0,451	0,430	0,411	0,464	0,421	0,386	0,393	0,365
Green strip. t. d	0,688	0,644	0,688	0,700	**0,711**	**0,711**	0,622	0,611	0,588	0,700		0,558	**0,653**	0,585	0,581	0,617	0,450	0,417	0,423	0,380	0,387	0,384	0,377	0,341
Chameleon-2	0,688	**0,711**	0,688	0,700	0,700	0,644	0,611	0,600	0,622	0,688	0,633		0,609	0,565	0,572	0,567	0,444	0,412	0,434	0,409	0,416	0,402	0,405	0,373
Anolis-3	0,648	**0,703**	0,626	0,604	0,615	0,604	0,659	0,648	0,626	0,615	0,648	0,692		0,643	0,625	0,558	0,453	0,464	0,366	0,395	0,391	0,373	0,380	0,338
Anolis-2	0,666	0,722	0,655	0,655	0,666	0,633	0,633	0,622	0,622	0,588	0,644	0,688	**0,703**		0,767	0,594	0,467	0,428	0,405	0,355	0,355	0,377	0,352	0,320
Anolis-1	0,622	0,666	0,633	0,577	0,588	0,611	0,633	0,633	0,622	0,577	0,588	0,644	0,637	0,811		0,587	0,417	0,396	0,391	0,375	0,359	0,359	0,348	0,338
Boa constrictor	0,666	0,666	**0,955**	0,744	0,744	0,755	0,644	0,633	0,655	0,711	0,688	0,688	0,637	0,644	0,622		0,453	0,428	0,448	0,387	0,387	0,391	0,373	0,351
Box-turtle	0,391	0,445	0,434	0,467	0,478	0,456	0,402	0,391	0,402	0,434	0,456	0,456	0,445	0,413	0,358	0,434		0,767	0,550	0,469	0,594	0,562	0,602	0,559
Eastern box turt.	0,391	0,434	0,413	0,445	0,456	0,434	0,402	0,391	0,413	0,423	0,434	0,434	0,456	0,402	0,347	0,413	0,945		0,618	0,498	0,663	0,637	0,673	0,580
Pancake tortoise	0,434	0,467	0,445	0,500	0,489	0,467	0,423	0,413	0,445	0,456	0,456	0,478	0,467	0,423	0,380	0,445	0,728	0,750		**0,534**	0,530	0,530	0,559	0,473
Hermann's tort.	0,380	0,380	0,369	0,402	0,413	0,402	0,391	0,391	0,380	0,391	0,380	0,402	0,434	0,358	0,363	0,369	0,576	0,586	**0,641**		0,483	0,476	0,473	0,526
Red eared slider	0,369	0,402	0,434	0,456	0,445	0,456	0,369	0,358	0,380	0,423	0,456	0,423	0,423	0,380	0,326	0,434	0,791	0,758	0,663	0,510		0,840	**0,923**	0,829
Yellow-bellied sl.	0,380	0,402	0,434	0,456	0,445	0,445	0,380	0,369	0,380	0,423	0,434	0,413	0,423	0,380	0,326	0,434	0,725	0,692	0,641	0,532	0,912		0,778	0,680
Pond slider	0,380	0,402	0,423	0,456	0,445	0,456	0,369	0,358	0,369	0,413	0,456	0,434	0,423	0,369	0,315	0,423	0,802	0,769	0,695	0,521	**0,945**	0,857		**0,872**
Al. map turtle	0,326	0,347	0,369	0,402	0,391	0,402	0,315	0,315	0,315	0,358	0,402	0,380	0,369	0,315	0,306	0,369	0,725	0,692	0,619	0,571	0,857	0,769	**0,912**	

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