Faculty of Agricultural Sciences

University of Hohenheim
Institute of Animal Science (460)
Department of Livestock Infectiology and Environmental Hygiene (460e)

Establishment of reverse transcriptase polymerase chain reaction methods for the detection of newly described RNA viruses in reptiles: picornaviruses in tortoises, reptarenaviruses in snakes, and sunshinevirus in snakes.

Dissertation Submitted in fulfilment of the requirements for the degree
“Doktor der Agrarwissenschaften”
(Dr. sc. Agrar./ Ph.D. in Agricultural Sciences)

to the
Faculty of Agricultural Sciences

Presented by

Tara H. Aqrawi
B.Sc. biol., M.Sc. agr.

Stuttgart 2018

Supervisor:

PD Dr. Rachel E. Marschang, Dip ECZM (herpetology), FTÄ
Mikrobiologie, ZB Reptilien
Laboklin GmbH & Co. KG
Steubenstr. 4
97688 Bad Kissingen, Germany

This dissertation has been submitted in fulfilment of the requirements for the degree “Doktor der Agrarwissenschaften” (Dr. sc. Agrar. / Ph.D. in Agricultural Sciences) to the Faculty of Agricultural Sciences

Date of oral exam: 18th July 2018

Examination committee:	PD Dr. Rachel E. Marschang
	PD Dr. Petra Kölle
	Prof. Dr. Ludwig Hölzle
Vice-Dean and Head of	
the examination committee:	Prof. Dr. Andrea Knierim

Publications

Part of the research work described in this doctoral thesis has been published in the following papers:

- Marschang RE, Ihász K, Kugler R, Lengyel G, Fehér E, Marton S, Bányai K, Aqrawi T, Farkas SL. 2016: Development of a consensus reverse transcription PCR assay for the specific detection of tortoise picornaviruses. Journal of Veterinary Diagnostic Investigation, 28(3) 309 –314.
- Aqrawi T, Stöhr AC, Knauf-Witzens T, Krengel A, Heckers KO, Marschang RE. 2015: Identification of snake arenaviruses in live boas and pythons in a zoo in Germany. Tierarztlische Praxis Ausg K, Kleintiere/ Heimtiere, 43(4):239-47
- Marschang RE, Stöhr AC, Aqrawi T, Hyndman TH, Plenz B, Blahak S, Pees M. 2013: First detection of Sunshine virus in pythons (*Python regius*) in Europe. In: Proceedings of the Association of Reptilian and Amphibian Veterinarians Conference. p.15 .Association of Reptilian and Amphibian Veterinarians ARAV.

Table of Contents

List of Tables

List of Figures

Abbreviations

aa	amino acid(s)
AB	antibiotics
BIBDAVs	boid inclusion body disease-associated arenaviruses
bp	base pair(s)
CDS	coding DNA sequence
CPE	cytopathic effect
DEPC	diethylpyrocarbonate
DMEM	Dulbecco´s modified Eagle medium
dNTPs	deoxyribonucleotide triphosphate mixture
EDTA	ethylenediamine-tetraaceticacid
FBS	fetal bovine serum
GPC	glycoprotein precursor gene
IBD	inclusion body disease
ICTV	International Committee on Taxonomy of Viruses
IgH2	iguana heart cells
LB	lysis buffer
ME	minimum evolution method
Milli-Q®	Millipore water
ML	maximum likelihood method
MP	maximum parsimony method
N	nucleocapsid protein
NC	negative control

NEA	non essential amino acids
N-G	glycosylation site N-terminus amino terminal end
NGS	next generation sequencing
nt	nucleotide(s)
ORF	open reading frame
PC	positive control
Rpm	round per minute
RT-PCR	reverse-transcription polymerase chain reaction
VH_2	viper heart cells
WB	washing buffer
Z protein	RING domain-containing zinc binding protein

1. Introduction

In the last decades keeping reptiles as pets has been noticed to be more popular. Reptiles currently represent the second most common group of exotic animals found in the international pet trade after birds. Imports of a wide variety of reptile species to Europe has significantly increased. In 2005, the European Union (EU) was the top global importer of live reptiles for the pet trade; the European Union (EU) member states officially reported the import of 20,788,747 live reptiles between 2004 and 2014 (Auliya et al., 2016).

On the other hand, numerous pathogenic viruses have recently been detected in reptiles, in particular in snakes, tortoises and lizards which are popular as pets all over the world. The international pet trade is capable of spreading infectious diseases across the world, and diagnostic tools need to be developed to screen captive and wild populations for exposure to pathogens. Thus, research focusing on viruses infecting reptiles has increased in recent years. Because there are no effective treatments available for viral infections in reptiles, euthanasia may be the last choice to prevent the spread of pathogens. This emphasizes the need for adequate methods to detect potential pathogens in infected and clinically healthy animals. Many viruses have been proven to be causative agents of disease in reptiles, however, the evidence for many as pathogens is relatively limited (Ariel, 2011). Viruses can led to the loss of entire collections of susceptible animals, as has been described for paramyxoviruses in snakes and herpesviruses and ranaviruses in tortoises (Marschang et al., 1999, Essbauer and Ahne, 2001). Recently, new viruses have been detected and characterized in reptiles. These

new viruses are all potential pathogens, often associated with severe disease, and their characterization allows the development of new and powerful diagnostic tools for direct detection in infected animals. These viruses include new tortoise picornaviruses (TPV1) which have been grouped together in a new genus *Torchivirus* (previously virus “X”) (Zell et al., 2017). The virus has been often found in numerous tortoise species in Europe, mostly in terrestrial tortoise species (Marschang, 2011; Heuser et al., 2010). Another group of viruses that have been recently discovered in reptiles and that appear to be associated with an important disease complex are snake arenaviruses, which have been detected in boid snakes: annulated tree boas (*Corallus annulatus*) and boa constrictors (*Boa constrictor*) suffering from inclusion body disease (IBD) (Stenglein et al., 2012; Bodewes et al., 2013; Hetzel et al., 2013). Inclusion Body disease (IBD) is an infectious and serious fatal disease that affects captive boas and pythons. IBD is the most commonly diagnosed disease of suspected viral origin in these snakes. Previously the definitive diagnosis of IBD relied on histopathological evidence of inclusion bodies in post mortem tissues or biopsies, which are difficult to obtain safely in an already debilitated snake. Infected snakes with IBD often show neurological signs and infection often or always leads to death. The discovery of reptarenaviruses and their association with IBD was a big step to understand an important disease of constrictor snakes. Sunshinevirus was considered in this study as well. This virus was first detected in pythons in Australia. In 2008, an outbreak of neuro-respiratory disease occurred in a collection of Australian pythons in Southeast Queensland, Australia (Hyndman et al., 2012a). A new virus, most

closely related to the described paramyxoviruses, was detected in diseased snakes and has since been detected in additional python collections in Australia. (Hyndman et al., 2012b). *Reptile sunshinevirus 1* is the type species of a new family *Sunviridiae* in the order *Mononegavirales*. Sunshineviruses are commonly found to cause neuro-respiratory and non-specific signs in pythons and they are often associated with a high mortality rate in infected snakes (Hyndman et al., 2012b).

The purpose of this study was to establish methods for the identification of three RNA viruses recently detected in reptiles: Picornaviruses in tortoises, and arenaviruses and sunshinevirus in snakes, and to develop diagnostic reverse-transcription polymerase chain reaction (RT-PCR) assays for their detection and identification in clinical samples as well as to partially characterize the detected viruses in order to optimize diagnostics and determine relationships between RNA viruses found in reptiles in Germany.

2. Literature review

2.1 RNA viruses in reptiles

The study of reptile virology is considered a relatively recent branch of virology. Most of the viruses which have been shown to be important pathogens in reptiles were discovered and described within the past few decades and there is still more to be studied and understood. RNA viruses in reptiles include retroviruses, reoviruses, rhabdoviruses, paramyxoviruses, arenaviruses, sunviruses, bunyaviruses, picornaviruses, caliciviruses, flaviviruses, and togaviruses. These have mostly been described in lizards, snakes and in chelonians (Table 2.1).

Table 2.1 RNA viruses in reptiles (Marschang and Divers, 2014)

virus family	virus properties		reptiles as host species
Retroviruses (*Retroviridae*)	ssRNA	enveloped	lizards, snakes, tuataras, chelonians, crocodilians
Bornaviruses *(Bornaviridae)*	ssRNA	enveloped	snakes
Reoviruses (*Reoviridae*)	dsRNA	non-enveloped	lizards, snakes, chelonians
Rhabdoviruses (*Rhabdoviridae*)	ssRNA	enveloped	lizards, snakes

Paramyxoviruses (*Paramyxoviridae*)	ssRNA	enveloped	snakes, lizards, turtles
Arenaviruses (*Arenaviridae*)	ssRNA	enveloped	snakes
Sunshineviruses (*Sunviridae*)	ssRNA	enveloped	snakes
Bunyaviruses (*Bunyaviridae*)	ssRNA	enveloped	turtles, lizards
Picornaviruses (*Picornaviridae*)	ssRNA	non-enveloped	turtles, snakes,
Caliciviruses (*Caliciviridae*)	ssRNA	non-enveloped	snakes
Flaviviruses (*Flaviviridae*)	ssRNA	enveloped	turtles, snakes, cocodilians
Togaviruses (*Togaviridae*)	ssRNA	enveloped	turtles, snakes, lizards

Research concerning reptile virology and associated diseases is important and interesting mainly because it helps to understand their importance for reptile health as well as virus taxonomy and evolution (Marschang, 2011). In addition, the characterization of these viruses allows the development of diagnostic methods, leading to better prophylaxis and prevention of the spread of infection and disease among these animals. This thesis deals with three different groups of RNA viruses,

all of which have been characterized within the past decade and all of which are associated with important diseases in reptiles:

- Picornaviruses in tortoises
- Arenaviruses in snakes
- Sunshinevirus in pythons

2.2 Picornaviruses

2.2.1 General introduction to picornaviruses

The term “picornavirus” was first coined around 1963 and applied to newly discovered small virus particles (Le Gall et al. 2008). All members of the family *Picornaviridae* show a similar structure and viral life cycle (Knowles et al, 2012). Morphologically, picornaviruses are non-enveloped about 22 to 30 nm in diameter with an icosahedral capsid surrounding the single-stranded RNA genome of positive polarity. The name “Pico” refers to the small size of the viral capsid, so "pico-rna-virus" literally means small RNA virus (Le Gall et al., 2008). The capsid shell has spherical structure and is composed of a densely-packed icosahedral arrangement of 60 copies of four non-identical virion polypeptide chains, VP1, VP2, VP3 and VP4 in icosahedral symmetry (Arnold et al, 1987, Smyth et al., 1995). The fundamental capsid architecture is the same in all members (Smyth and Martin, 2002). The RNA carries a covalently bound non capsid viral protein (VPg) at its 5′ end and a polyadenylated tail at its 3′ end. The 3՝ polyA tail also varies significantly in length between genera and has been shown to be required

for infectivity (Spector and Baltimore, 1974; Racaniello, 2007). The VPg is a small protein that acts as a primer for genome replication. The picornavirus genome consists of a single open reading frame translated as a single polyprotein. Nucleotide sequence analysis of many picornaviral RNAs has revealed a common organizational pattern. The genomes vary in length from 7,209 to 8,450 bases (Racaniello, 2007).

2.2.2 Classification and replication

The family *Picornaviridae* is divided into a number of genera which contain many important human and animal pathogens, including poliovirus, hepatitis A virus, foot-and-mouth disease virus, and rhinoviruses (Liljas et al., 2002). Classification is based on morphology, physicochemical and biologic properties, antigenic structures, genomic sequence and mode of replication. According to the International Committee on Taxonomy of Viruses (ICTV), *Picornaviridae* belongs to the order *Picornavirales* and currently consist of 80 species grouped into 35 genera as of March 2017 (Adams et al., 2017): *Ampivirus, Aphthovirus, Aquamavirus, Avihepatovirus, Avisivirus, Cardiovirus, Cosavirus, Dicipivirus, Enterovirus, Erbovirus, Gallivirus, Harkavirus, Hepatovirus, Hunnivirus, Kobuvirus, Kunsagivirus, Limnipivirus, Megrivirus, Mischivirus, Mosavirus, Oscivirus, Parechovirus, Pasivirus, Passerivirus, Potamipivirus, Rabovirus, Rosavirus, Sakobuvirus, Salivirus, Sapelovirus, Senecavirus, Sicinivirus, Teschovirus*, *Torchivirus* and *Tremovirus*, in addition to a number of unassigned picornaviruses (ICTV 2016). Viruses of the *Picornaviridae* family cause a wider range of illnesses

than most other virus families in humans, plants, insects and even algae (Ng et al., 2015). Infection with various picornaviruses may be asymptomatic or may cause clinical syndromes such as aseptic meningitis (the most common acute viral disease of the CNS), encephalitis, the common cold, febrile rash illnesses (e.g. hand-foot-and-mouth disease), conjunctivitis, herpangina, myositis and myocarditis, and hepatitis.

Picornaviruses multiply in the cytoplasm of the host cell, and their RNA acts as a messenger to synthesize viral macromolecules. Attachment of the virus to a specific cell surface receptor leads to structural changes in the capsid which allow the un-coating of the RNA genome and its subsequent entry into the cell. The genome is first translated to provide copies of the viral proteins required for genome replication. Replication is preceded by negative strand synthesis and this strand is then used as a template for positive strand RNA synthesis. Capsid proteins and newly synthesized viral genomes assemble into mature virions and are then released from the cell. The entire cycle is complete in around 5 to 10 hours, with the exact duration depending on variables such as pH, temperature, cell type, and number of viral particles that infect the cell (Racaniello, 2007).

2.2.3 Picornaviruses in tortoises

Picorna-like-viruses have been frequently detected in a number of different terrestrial tortoise species in Europe, most commonly in spur-thighed tortoises (*Testudo graeca*) (Marschang, 2011). They have also been described in a wide range of other tortoise species in the family *Testudinidae* such as marginated

tortoises (*T.marginata*), Hermann's tortoises (*T.hermanni*), Egyptian tortoises (*T.kleinmanni*) and leopard tortoises (*Stigmochelys pardalis*). They were long identified by isolation in cell culture (Heuser et al., 2014; Marschang, 2011). These viruses have been cultivated in Terrapene heart cells (TH-1; ATCC CCL-50), a chelonian cell line in which they cause cytolysis (Marschang, 2011). Characterization of these viruses was difficult and that is why they have been called virus "X" (Marschang and Ruemenapf, 2002).

Heuser et al. published a study in 2014 which presented the detection of a picorna-like virus (virus"X") in young spur-thighed (*Testudo graeca*) and star tortoises (*Geochelone elegans*) suffering from a sudden softening of the shell. In other studies, virus "X" was detected in tortoises which have shown various disease signs including diphtheroid-necrotizing stomatitis, pharyngitis, rhinitis, pneumonia, enteritis, conjunctivitis, cachexia, sudden weight loss, and/or ascites (Marschang, 2011). However, these viruses have also been isolated from healthy animals (Marschang and Ruemenapf, 2002). Tortoises infected with virus "X" in some cases have also been shown to be infected with other pathogens, especially herpesviruses and *Mycoplasma* spp (Marschang, 2011). Viruses were most frequently isolated from oral/ pharyngeal swabs in live animals, although they were also detected in conjunctival and cloacal swabs. These viruses have also been detected in a wide range of tissues including liver, kidney, heart, brain, and lung (Marschang, 1999). The virus isolated from the juvenile Hermann's and leopard tortoises was named tortoise picornavirus 1 (TPV 1) (Heuser et al., 2014) and 7077 nucleotides of its genome were sequenced. Analysis of the sequences

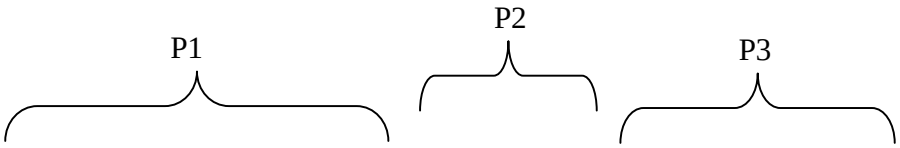
suggested that this virus has some similarities to the family *Picornaviridae*, but also diverges considerably from described picornaviruses. It was therefore proposed that this virus may represent a novel picornavirus genus (Heuser et al., 2010). The sequences from that study were not, however, made public.

Recently, the near complete genome sequences of seven picornaviruses detected in tortoises were determined and analyzed (Farkas et al., 2015). These viruses were isolated from different captive tortoises (*Testudo graeca, T.hermanni, Pyxis arachnoides* and *Centrochelys sulcata*) in Europe. The genome organization of these strains showed a typical picornavirus layout and found to be homologous with the encoding region of other picornaviruses. The study suggested that these tortoise picornaviruses belong to a separate proposed genus in the family *Picornaviridae*, called *"Topivirus"* derived from tortoise picornavirus (GenBank accession numbers KM873611 to KM873617) (Farkas et al., 2015). This group of viruses has now been grouped in a new genus in the family *Picornaviridae*, named *Torchivirus* (from tortoise virus X (Greek chi)), with the typespecies *Torchivirus A*, isolated from a Hermann's tortoise (GenBank accession number KM873611) (Zell et al., 2017).

Another tortoise PV was detected and identified in a Forsten's tortoise (*Indotestudo forstenii*) and named tortoise Rafivirus A1 (GenBank accession No.: ToRaV-A, KJ415177). The complete genome of this new picornavirus was sequenced and the phylogenetic analysis demonstrates that tortoise rafivirus is only distantly related to the torchivirus and belongs to a proposed new genus „Rafivirus" (Ng et al., 2015).

2.2.4 Genome organization of picornaviruses

The following scheme for the structure of the picornavirus genome was proposed based on the predicted protease cleavage sites, which is similar to the typical genome organization of many picornaviruses



VPg+5'UTR[L/1A-1B-1C-1D-2A^{npgp}/2B-2C/3A-3B^{VPg}-3C^{pro}-3D^{pol}]3'UTR-poly(A)

Source: Knowels et al., 2012

In the study by Farkas et al. (2015) nearly complete sequences were obtained from selected virus “X” isolates detected in terrestrial tortoises from Germany and Hungary between 2000 and 2013. The obtained genome sequences were 7065–7079 nucleotides long (without the poly (A) tail). The sequence analyses of these tortoise picornaviruses showed disparate similarity of the amino acids of polyprotein sequences with members of other genera, mostly with *Mosavirus*, *Cardiovirus*, *Cosavirus*, and *Senecavirus.* The sequence of the polyprotein of *Torchivirus* was compared to that of its closest relative, murine mosavirus (accession number: JF973687). The complete capsid-protein-encoding P1 (784 aa) and non-structural-protein-encoding P3 (767 aa) regions showed the highest identity (38.9 % and 34.7 %) to the Mouse Mosavirus A2, while the non-structural-protein-encoding P2 (615 aa) region displayed restricted similarity to genet fecal theilovirus (26.2 %). The G+C content in the coding region was very low

(approximately 35.8–36.8 %) in all strains. The G+C content was slightly higher in the capsid-protein-encoding P1 region (39.3 %) than in the non-structural-protein-encoding P2 and P3 regions (36.1 and 36.2 %, respectively). The 3' untranslated region UTR was 232 nucleotides long and extremely A+T rich (87%), (Knowels et al., 2012, Farkas et al., 2015). Additionally, a 65-nt-long part of the known un-translated region 5´ UTR sequences was most closely related to canine kobuvirus 1 and Aichi virus UTR sequences which belong to the genus *Kobuvirus*. The 3´ UTR did not show any similarities to sequences of picornaviruses in GenBank (Farkas et al., 2015).

2.3 Arenaviruses

2.3.1 General introduction to arenaviruses

Arenavirus particles are spherical to pleomorphic and have an average diameter of 50-300 nanometers, with the average diameter of spherical particles being 110-130 nm (Charell and de Lamballerie X, 2003). Viruses in the family *Arenaviridae* have two segmented single-stranded RNA. All arenaviruses are enveloped in a dense lipid membrane covered with 8-10 nm long club-shaped projections. The name is derived from the Latin word "arenosus" meaning "sandy" which refers to the grainy appearance of virions when viewed in cross section, which represent ribosomes acquired from host cells. Arenaviruses are rapidly inactivated at 56°C, at pH below 5.5 or above 8.5, or by exposure to UV or gamma radiation. Genome replication occurs in the host cytoplasm and involves RNA-dependent RNA

synthesis. The first step is viral binding to the host cell membrane receptor through the viral GP glycoprotein, after which it is endocytosed into vesicles in the host cell. This is followed by fusion of the virus membrane with the vesicle membrane and release of the ribonucleocapsid into the cytoplasm. The next step is sequential transcription in which the viral mRNAs are capped in the cytoplasm. Consequently, replication starts when enough nucleoprotein is present to encapsidate neosynthetized genomes. The ribonucleocapsid interacts with the Z protein under the plasma membrane. Viral particles then bud from the surface of their hosts' cells (Buchmeier et al., 2007).

2.3.2 Classification of family *Arenaviridae*

Until recently, the family *Arenaviridae* contained a single genus, *Arenavirus*, members of which have a worldwide distribution and are known to infect rodents. According to the ICTV, 27 arenavirus species are currently recognized based on morphological, physiochemical, and serological parameters (Radoshitzky et al., 2015; www.ictvonline.org/virustaxonomy.asp?version=2014; Zapata and Salvato, 2013; Coulibaly-N'Golo et al., 2011; Palacios et al., 2010). Ten of these viruses are associated with human diseases causing severe hemorrhagic fever (HF) and zoonotic diseases. Many arenaviruses are endemic among rodents and they are occasionally transmitted to humans (Salazar-Bravo et al, 2002). The first arenavirus isolated was lymphocytic choriomeningitis

virus (LCMV), a common human pathogen discovered in 1933 during the study of samples from a St. Louis encephalitis epidemic (Buchmeier et al., 2007). Arenavirus infections have been detected in Africa, Europe, the U.S. and possibly some parts of Asia (Buchmeier et al., 2001). Historically, two complexes were established within the arenavirus genus through the analysis of the serological and genetic relationships and their distribution in the world: The Old World Arenaviruses (OWAV) and the New World arenaviruses (NWAV), also known as the lassa-lymphocytic choriomeningitis virus (LASV-LCMV) complex and the Tacaribe complex (Bowen et al., 1997). The geographical distribution of the different viral species is related to the natural host's distribution and the specific diseases associated to each virus. In addition, species classification is defined by significant differences of the viral protein sequence compared to other viruses in the genus (Zapata and Salvato, 2013). Recently, several studies have been published concerning the detection and isolation of a divergent group of arenaviruses in captive snakes, and based on phylogenetic analyses these reptilian arenaviruses constitute a sister group to mammalian arenaviruses. As a result, the previous single genus *Arenavirus* has been replaced by two genera, *Mammarenavirus* and *Reptarenavirus*, established to accommodate mammalian and reptilian arenaviruses, respectively, in the same family (Radoshitzky et al., 2015).

2.3.3 Genome organization of arenaviruses

The arenavirus genome consists of two single-stranded RNA segments designated S (small) and L (large) encoding four viral proteins in a unique ambisense manner. The S segment contains two genes; the positive sense 5' portion which encodes the glycoprotein precursor (GPC) which is cleaved into envelope glycoproteins GP1 and GP2, and the negative sense 3' portion which encodes the nucleocapsid protein (NP). The L RNA segment contains two genes that encode two gene products, the viral polymerase (L protein) and the Z protein (Auperin et al, 1984, Buchmeier et al, 2001). GP1 and GP2 have an important role in viral entry in the host cell, uncoating and assembly. In addition, NP contributes to viral assembly, plays an important role in suppressing the innate immune response, and has exonuclease and nucleotide binding activity (Pinschewer et al., 2003; Qi et al., 2010). The L segment (~7.2Kb) encodes the L protein that is an RNA-dependent RNA polymerase (RdRp) and encodes the small zinc-binding protein (Z) which is essential for viral transcription and replication (Zapata and Salvato, 2013).

2.3.4 Inclusion body disease (IBD) in snakes: History, clinical signs and hosts

One of the most important diseases infecting boid snakes in captivity is inclusion body disease (IBD). It has been recognized in the United States, Africa, Australia, and Europe. It was first described in the 1970s in the United States, where it affected captive neonate, juvenile and adult boid snakes in private and zoological collections and was associated with the eradication of entire boid collections

(Schumacher et al., 1994). At the time the disease was described, IBD was most commonly diagnosed in Burmese pythons (*Python molurus bivittatus*) and concern about this fatal disease rose in reptile veterinary medicine. IBD was then reported in captive native carpet pythons (*Morelia spilota variegata*) and diamond pythons (*M. spilota spilota*) in Australia (Carlisle-Nowak et al., 1998), in captive boa constrictors in the Canary Islands, Spain (Oros et al., 19981), and subsequently in Belgium (Vanncraeynes et al., 2006). The early 1990s witnessed cases of IBD which were diagnosed in boa constrictors more than pythons, but the cause of this epidemiologic shift is still unknown (Chang and Jacobson, 2010). IBD is characterized by the formation of intracytoplasmic inclusions found in numerous cells of different body organs (epithelial cells, visceral epithelial cells, and CNS neurons) (Jacobson et al., 2001). The inclusions are made up of a 68-KDa unique protein, which is now recognized as arenavirus nucleoprotein, also known as inclusion body disease protein (IBDP) (Wozniak et al., 2000; Hetzel et al., 2013). In boas, the disease course is variable. Animals either die within weeks or months or may have subclinical infection, thus it is possible that infections persist for long periods of time. In contrast, pythons generally develop severe fatal neurological signs within a few weeks. Although the most common clinical signs in boa constrictors are chronic regurgitation and CNS abnormalities characterized by stargazing, disequilibrium, tremors, and disorientation, stomatitis, pneumonia, and lymph proliferative disorders have also been reported in affected snakes (Schumacher et al., 1994). It is interesting to note that red and white blood cells develop the typical inclusion bodies and immunosuppression is believed to be a

key factor in disease development. Thus affected animals often die from secondary bacterial disease like salmonellosis, fungal (aspergillosis), and protozoal disease (amoebiasis) (Hetzel et al., 2013).

IBD infects a wide range of boid and pythonid species from different families and has been described in Boa constrictors, green anaconda (*Eunectes murinus*), Haitian boa (*Epicrates striatus*), ringed tree boa (*Corallus annulatus*), garden tree boa (*Corallus hortulanus*), the Burmese python (*Python molurus*), reticulated python (*Python reticulatus*), ball python (*Python regius*) and *Morelia spilota variegata* and *Morelia spilota spilota*, which are known as Darwin carpet pythons (or carpet pythons) and diamond pythons. The prevalence of IBD in captive boid and pythonid collections is still unknown and so far there has been no report of IBD in indigenous wild populations. The question is therefore whether boas and /or pythons are the primary host of the IBD agent. Currently, the transmission routes of IBD remain unknown. It has been theorized that droplets in the air or contaminated feeding tools might be factors (Marschang and Divers, 2014); vertical transmission of IBD is also possible in ovoviviparous boids. Because snake mites (*Ophionyssus natricis*) have often been found in many snake collections experiencing IBD outbreaks, mites are thought to be a disease transmission factor (Chang and Jacobson, 2010). Currently no specific treatment is available; a quarantine period of 90 days and up to 6 months for boas has been suggested, coupled with mite control. Euthanasia is recommended for those individuals where there is a transmission risk or presenting with neurological signs.

2.3.5 Arenaviruses in snakes

The etiology of IBD was long unknown, although a viral agent had long been suspected. A virus of the family *Retroviridae* was long suspected (Schumacher et al., 1994). Recent studies presented evidence of previously undescribed viruses with characteristics attributable to arenaviruses in boid snakes with IBD using a next generation sequencing (NGS) and bioinformatics approach (Stenglein et al., 2012). The detected arenaviruses were found in six out of eight snakes diagnosed with IBD. These viruses have been named Golden Gate Virus (GGV) isolated from boa constrictors (*Boa constrictor*) and California Academy of Sciences Virus (CASV) identified in annulated tree boas (*Corallus annulatus*). Snake virus proteins which encoded in the S segment were related to the glycoproteins GP of filoviruses (e.g. Ebola and Marburg viruses), avian retroviruses (e.g., avian leukosis virus) and the cellular syncytin. In phylogenies, the snake virus proteins form an amonophyletic clade separate from those formed by the Old World and New World arenaviruses (Stenglein et al., 2012).

Another group from the Netherlands identified arenaviruses in eight IBD positive snakes, seven boa constrictors and one emerald tree boa (*Corallus caninus*). The near completely sequenced arenavirus was named Boa Av NL B3. Phylogenetic analysis of the nucleotide (nt) of the nucleoprotein gene (NP) and L gene of Boa Av NL B3 virus revealed that the sequences were most closely related to the GGV nucleotides with pairwise sequence identities between 66.6% and 80.9% for all the ORFs (L, NP, Z, and GPC) and pairwise deduced amino acid identities between 66.3% and 90.8%. The virus was more distantly related to CASV with pairwise

nucleotide sequence identities between 42.3% and 60.0% for all the ORFs and pairwise amino acid identities between 51.1% and 58.4% (Bodewes et al., 2013).

An additional study from Europe reported one isolate, University of Helsinki virus (UHV-1) isolated from a boa constrictor with IBD. UHV-1 was completely characterized and represents an addition to the group known as boid inclusion body disease -associated arenaviruses (BIBDAVs). UHV was isolated from cultured cells of an IBD positive boa constrictor and sequenced by combining traditional and NGS techniques (Hetzel et al., 2013). Comparison of sequences of the major open reading frames of boa Av NL B3 with UHV-1 revealed that the pairwise identities of nucleoprotein (NP) was 77%, glycoprotein precursor (GPC) was 67%, RNA-depandent RNA polymerase (RdRp) was 89%, and RING domain-containing zinc binding protein (Z) gene was 79% identical at the nucleotide level, likewise the pairwise identities of the genes respectively were 84%, 62%, 92%, and 89% at the deduced amino acid level (Bodewes et al., 2014). To obtain more evidence of the etiology of IBD, cell lines were established from different tissues of IBD-positive and negative boa constrictors, inclusion bodies were characterized in the positive cells but none were observed in the negative cell culture and the virus has been successfully isolated from cell culture supernatant. Immunofluorescence, immunohistochemistry, and immunoblotting were performed. Polyclonal antibodies were produced against the nucleoprotein of snake arenavirus which recognized the BIBD inclusion bodies in tissue sections of infected snakes (Stenglein et al., 2012; Hetzel et al., 2013). In addition, reverse transcription-PCR (RT-PCR) was

established to reveal the presence of genetically diverse arenaviruses in snakes with IBD, confirming the causative role of arenaviruses (Hetzel et al., 2013).

2.3.6 Diagnosis of arenaviruses and IBD in snakes

Diagnosis of IBD is usually confirmed by microscopic observations of eosinophilic intracytoplasmic inclusion bodies found mainly in neurons of the central and peripheral nervous system and epithelial cells of major organs, as well as in haemopoietic and lymphatic tissues and both leukocytes and erythrocytes may contain intracytoplasmic inclusions (Schumacher et al., 1994; Marschang and Divers, 2014).

Although the presence of these inclusions is a reliable indicator for IBD, their absence does not rule out an infection (Chang and Jacobson, 2010). Characteristic inclusion bodies have been found in affected internal tissues in dead animals including liver, kidney, stomach, spleen, respiratory and uro-genital tracts, pancreas and esophageal tonsils (Marschang and Divers, 2014). For diagnosis in live snakes, currently biopsies of oesophageal tonsils, gastric mucosa, liver, and kidney have been suggested (Jacobson et al., 1999). Direct blood smears can be useful for the detection of inclusions in blood cells, but may be less sensitive (Marschang and Divers, 2014). Since the identification of arenaviruses as the probable etiological agent of IBD, detection of arenaviruses has been performed through polymerase chain reaction (PCR) using samples from circulating blood cells, tissue biopsies and swabs (Hellebuyck et al, 2015; Hepojoki et al., 2015).

2.4 Sunshinevirus

Sunshinevirus is a newly described virus that has been found in Australian pythons. It has been identified as a novel virus most closely related to viruses of the family *Paramyxoviridae* (Hyndman et al., 2012b) and recently been placed in a new family, *Sunviridae* in the order *Mononegavirales* (ICTV, 2016). Members of the order *Mononegavirales* are enveloped viruses, have linear, non-segmented single stranded negative-sense RNA genomes, and replicate by synthesizing complete anti-genome, and the virions bud off from the host cell, gaining their envelopes from the cellular membrane they bud from. The order currently includes eight virus families: *Bornaviridae*, *Mymonaviridae*, *Filoviridae*, *Nyamiviridae*, *Paramyxoviridae*, *Pneumoviridae*, *Rhabdoviridae*, and *Sunviridae*. The family *Sunviridae* includes a single genus (*Sunshinevirus*) with a single species (*Reptile sunshinevirus 1*) (Afonso et al., 2016). The family was established in 2016 to taxonomically accommodate Sunshine Coast virus (SunCV), previously referred to as "Sunshine virus" (Hyndman et al., 2012a).

The first documented outbreak of sunshinevirus was in 2008 as neuro-respiratory disease spread in a collection of 70 Australian pythons in particular *Antaresia* sp., *Morelia* sp. and *Aspidites* sp. (Hyndman et al, 2012a). Histopathological examinations of the snakes that had died indicated the presence of a viral infection. The entire collection was euthanized and samples were used for virus isolation. A virus was isolated from the lung suspension of a black-headed python *(Aspidites melanocephalus*) and named BHP1-Lung. The genome of the isolate was obtained using NGS techniques and the analysis of the genome showed it to

be a novel virus. 17,187 nucleotides of the genome of sunshinevirus were assembled from RNA extracts from infected viper heart cells (VH2) displaying widespread cytopathic effects in the form of multinucleate giant cells. The viral sequence contains open reading frames (ORFs) identified as nucleocapsid (N), matrix (M), fusion (F) and polymerase (L) ORFs (Hyndman et al., 2012a). The virus was named Sunshinevirus after the geographical origin on the Sunshine Coast of Queensland, Australia.

Sunshinevirus has been found only in pythons including carpet python (*Morelia spilota*), diamond python (*Morelia spilota spilota*), black-headed python (*Aspidites melanocephalus*), woma python (*Aspidites ramsayi*), and spotted python (*Antaresia maculosa*), the virus has not been detected in other snake species and boas. However this host range is likely to expand as further testing is performed (Hyndman et al., 2014).

2.4.1 Clinical signs and virus diagnosis

Sunshinevirus has been found in pythons with a variety of clinical signs. Some infected animals displayed no overt signs of disease while for others the clinical signs were neurological, neurorespiratory or non-specific. Neurological signs included head tremors, opisthotonus, incoordination, diminished righting response, uncoordinated movement of the cranial and caudal body and erratic mouth gaping. Respiratory signs included a mild discharge of clear viscous fluid from the mouth and dyspnoea. Non-specific signs included anorexia, stomatitis, dermatitis, weakness, lethargy, regurgitation, inappetence and weight loss (Hyndman et al.,

2012b). However, several sunshinevirus positive snakes appeared healthy and showed no clinical signs of disease; infection in the snakes was confirmed by PCR (Hyndman et al., 2014).

Diagnosis of sunshinevirus has been performed usually by PCR in both oral and cloacal swabs, fresh samples of brain, lung, liver and kidney, and formalin-fixed paraffin-embedded tissues (Hyndman et al., 2012b). Thus, it is assumed that the virus could be taken up via the oral cavity or the trachea-lung and possible routes of transmission would be fecal-oral, air-borne, droplet, or direct contact. Sunshinevirus was most frequently detected in the brain in infected snakes, and most of the pathology in diseased snakes is seen in the hindbrain (Hyndman et al., 2014). Sunshine virus has also been detected in blood by PCR (Hyndman unpublished data); this result has raised the prospect of blood-sucking ectoparasites (e.g. mites) as transmission factors of sunshinevirus between snakes (Hyndman et al., 2014). The presence of sunshinevirus in snakes was also confirmed by microscopic examination and virus isolation on VH2 cell cultures (Hyndman et al, 2012 a).

3. Aims of the study

Based on the description of a number of new RNA viruses infecting reptiles and their apparent clinical relevance in reptile medicine, the aim of this study was to generally improve and test molecular diagnostic tools for the detection of several newly described reptile viruses in clinical samples in a diagnostic laboratory. Specifically, the aims were:

1. To establish a molecular diagnostic test for the detection of tortoise picornavirus (*Torchivirus*) in diagnostic samples for use on historical virus isolates as well as clinical samples, and to characterize the detected viruses by partial sequencing.
2. To gain sequences and deduced phylogenetic information about picornaviruses occurring in tortoises.
3. To screen RNA from clinical samples from boas and pythons for the presence of reptarenaviruses using an RT-PCR with primers described by Stenglein et al. (2012), and to characterize the detected viruses by partial sequencing.
4. To identify sunshinevirus in snake samples from captive pythons in Europe using the RT-PCR method described by Hyndman et al. (2012).

4. Materials and methods:

4.1 Laboratory equipments and utensils

for processing diagnostic samples

Branson-250 Sonifier	Ausrüstung 707, Schwäbisch Gmünd
Varifuge 3.2 RS, Centrifuge	Heraeus Osterode
BD FalconTM 15 ml polypropylene Conical tubes	Biosciences Discovery Lab ware, Heidelberg

for cell culture and virus isolation

Biosafety sterile cabinet, Heraeus	Kendro Laboratory Products, Hanau.
Sterile Laminar flow cabinet	Prettl, Bempflingen
Fireboy S1000, bunsen burner	Tecnorama, Fernwald
CO2 Incubator	Binder Tuttlingen
Inverted light microscope	Leitz, Wetzlar
30mm diameter Cellstar®	Greiner Bio-One GmbH, Frickenhausen
BD Falcon BD Falcon™ with vented cap (75 cm3)	Becton Dickinson GmbH, Heidelberg

for the extraction of viral RNA/ RT-PCR

Table centrifuge:	
Centrifuge 5415 R	Eppendorf, Wesseling Berzdorf

Centrifuge 5415 D	Eppendorf, Wesseling Berzdorf
Vortex Genie 2™	Bender & Hohlbein AG, Zurich (CH)
Dry Bath incubator	Kisker, Steinfurt
(Heating block)	
Thermal cyclers:	
Peqlab Primus 35 Adv.	Peqlab, Erlangen
Doppio thermocycler	VWR, Darmstadt
Mastercycler Gradient	Eppendorf, Wesseling Berzdorf
Pipettes 1-10 µl, 10-100 µl	Biozyme scientific GmbH, Oldendorf
and 20-200 µl	
0.5 ml PCR tubes	Biozyme scientific GmbH, Oldendorf
1.5 ml reaction tubes	Eppendorf, Hamburg.

for gel electrophoresis/ gel extraction

Sterile surgical blades	Aesculap Ag, Tuttlinge
Power supply:	
Consort 443 electrophoresis	Keutz, Reiskirchen
EC-105 electrophoresis	Biometra, Gottingen
Agagel Mini G45/2- gel chamber	Biometra, Gottingen
UV-transilluminator, Infinity 1000 Vilber	Lourmat,Eberhardzell

4.2 Cell lines used in experimental trials

Terrapene heart cells, TH1: established from the heart of the box turtle *Terrapene carolina*. Obtained from American Type Culture Collection Rockville, MD, ATCC-CCL 50

Viper heart cells, VH2: established from the heart of a female Russells Viper *Vipera russelli*. Obtained from American Type Culture Collection USA, ATCC CCL-140.

4.3 Chemicals, reagents and solutions

4.3.1 Cell culture and virus isolation

Water, deionised and filtered:

Milli-Q® water (Millipore GmbH, Eschborn)

Dulbecco´s modified Eagle medium (DMEM)

13.4 g/L DMEM medium, 4.5 g/L D-glucose, with L-glutamine, were all dissolved in 1 L Milli-Q® water. 2.2 g/L NaHCO3 (Merck KGaA, Darmstadt) was added and the solution was stirred thoroughly until the ingredients were dissolved completely. After that, the medium was sterile filtered and kept at 4°C for future use.

Amphotericin B

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

Gentamicin sulfate solution

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

Penicillin-G solution

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

Streptomycin sulfate solution

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

FBS

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

NEA

Non essential amino acids (Biochrom AG, Berlin, Germany)

Growth medium: Dulbecco´s modified Eagle medium (DMEM) for cell culture and virus isolation contains the following substances in ml /500 ml DMEM

	Final concentration	
50 ml	10% (v/v)	FBS
5 ml	1% (v/v)	NEA
2 ml	200 U/ml	Penicillin-G-solution
2 ml	6.4 U/ml	Gentamycin sulfate-solution
2 ml	380 U/ml	Streptomycin sulfate-solution
4 ml	0.5 µg/ml	Amphotericin B-solution

Trypsin-versen (TV) solution 0.05 %

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

DMEM supplemented with 2x antibiotic concentration: amount added to 500 ml (2x AB DMEM) used for processing diagnostic samples

	Final concentration
4 ml	400 U/ml Penicillin-G-solution
4 ml	12.8 U/ml Gentamycin sulfate-solution
4 ml	760 U/ml Streptomycin sulfate solution
8 ml	1 µg/ml Amphotericin B-solution

4.3.2 Viral RNA extraction

Diethylpyrocarbonate (DEPC) as nuclease free water

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

Silica-matrix

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

solution was adjusted to 2.00 by the addition of 600 µl of 25 % HCl (6.85 M). The solution was shaken to re-suspend the silica, and then aliquoted into 1.5 ml nuclease free tubes in 1 ml portions, autoclaved at 121 °C for 15 min and kept at room temperature in the dark.

Tris-HCl

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

Ethylenediamine-tetraaceticacid (EDTA)

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

Lysis buffer (LB)

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

Washing buffer (WB)

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

70 % Ethanol

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

RNase inhibitor

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

Acetone

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

Nuclease-free water

(QIAGEN Hilden, Germany)

4.3.3 Reverse transcription polymerase chain reaction (RT-PCR)

All chemicals and reagents used for RT-PCR were obtained from Fermentas, St. Leon-Roth and Genaxxon bioscience GmbH, Ulm

10x *Taq* buffer (with KCl)

10x buffer E with $(NH_4)_2SO_4$

25 mM $MgCl_2$

40 U/µl RiboLock™ RNase Inhibitor

200 U/µl RevertAid™ Reverse Transcriptase RT-Enzyme

5 U/µl Taq DNA Polymerase

5x transcriptor reaction buffer (250 mM Tris/HCl, 150 mM KCl, 40 mM $MgCl_2$)

1 mM DTT (Dithiothreitol)

Primers

Primers were ordered from biomers.net (Ulm, Germany). The lyophilized primer was first centrifuged at maximum speed for 10 seconds to be settled as sediment in the bottom of the tube, then a stock solution (100 pmol/µl) was prepared by dissolving the primer with the appropriate amount of Milli-Q® water (calculated by the provider) into 1.5 ml RNase- free centrifuge tubes. The tube was then vortexed for five seconds and allowed to stand on ice for 15 min. Subsequently, the tube was again shortly vortexed to ensure the proper mixing of the primer components then was stored at -20°C until use. The stock solution was diluted in Milli-Q® water to achieve working solutions of 10 pmol/µl and 50 pmol/µl.

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

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

4.3.4 Gel electrophoresis

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

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

Ethidium bromide solution (Roth, Karlsruhe,Germany)

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

Agarose Roth (Karlsruhe)/Germany

6x Loading Dye Fermentas (St. Leon-Roth),Germany

GeneRuler™100 bp DNA Ladder Fermentas (St. Leon-Roth),Germany

GeneRuler™ DNA Ladder Mix Fermentas (St. Leon-Roth),Germany

4.3.5 Gel extraction

PeqGOLD Gel-Extraction Kit (PEQLAB, Erlangen). Detailed protocol: see methods 4.9.

4.4 Samples and isolates:

4.4.1 Samples for picornavirus studies

A total of 37 virus isolates from TH1 cell culture were investigated for the presence of picornaviruses using RT-PCR techniques. The isolates were collected between

1997 and 2012 under the supervision of PD Dr. Rachel Marscahng. In addition, screening was performed on 75 clinical samples from a total of 51 different tortoises collected between 2012 and 2013 (Table 11.2 in appendix). The samples were obtained from different tortoises and oral/ cloacal and conjunctival swabs, mostly from private collections from Germany, the United Kingdom, Italy, and Switzerland.

Table 4.1 Picornavirus isolates

Virus isolates provided by PD Dr. Marschang		
	Lab No. Isolates	**Host species**
1.	TGT1a/96	Spur-thighed tortoise *Testudo graeca*
2.	374/96	unknown
3.	33/97	Spur-thighed tortoise *Testudo graeca*
4.	2442/97 TGT8	Spur-thighed tortoise *Testudo graeca*
5.	907/97 TGT10	Spur-thighed tortoise *Testudo graeca*
6.	2443/97	Spur-thighed tortoise *Testudo.graeca*
7.	2445/97	Egyptian tortoise *Testudo kleinmanni*
8.	1791/98	Spur-thighed tortoise *Testudo graeca*
9.	2281/7/98	*Testudo spp.*
10.	2691/98	Leopard tortoise

		Stigmochelys pardalis
11.	1167/5/99	Tortoise
12.	1167/9/99	Tortoise
13.	659/00	Hermann's tortoise *Testudo hermanni*
14.	2041/00	Russian tortoise *Testudo horsfieldii*
15.	5_03	Spider tortoise *Pyxis arachnoids*
16.	16_03	*Testudo* sp.
17.	26_03	Marginated tortoise *Testudo marginata*
18.	31_03	Hermann's tortoise *Testudo hermanni*
19.	2_4_04	Tortoise
20.	5_04	Spur-thighed tortoise *Testudo graeca*
21.	14_04	Hermann's tortoise *Testudo hermanni*
22.	15_04	Spur-thighed tortoise *Testudo graeca*
23.	16_04	Hermann's tortoise *Testudo hermanni boettgeri*
24.	17_04	Hermann's tortoise *Testudo hermanni boettgeri*
25.	19_1_04	Hermann's tortoise *Testudo hermanni*
26.	19_2_04	Hermann's tortoise *Testudo hermanni*
27.	9_05	African spurred tortoise

		Centrochelys sulcata
28.	25_09	Marginated tortoise *Testudo marginata*
29.	19_10	Russian tortoise *Testudo horsfieldii*
30.	124_1_10	Spur-thighed tortoise *Testudo graeca*
31.	124_2_10	Spur-thighed tortoise *Testudo graeca*
32.	124_4-10	Spur-thighed tortoise *Testudo graeca*
33.	124_5_10	Spur-thighed tortoise *Testudo graeca*
34.	124_6_10	Spur-thighed tortoise *Testudo graeca*
35.	124_7_10	Spur-thighed tortoise *Testudo graeca*
36.	36_10	Marginated tortoise *Testudo marginata*
37.	144_10	Spur-thighed tortoise *Testudo graeca*

4.4.2 Samples for arenavirus studies

In order to obtain a positive control for the proposed diagnostic RT-PCR, RNA from four organ samples: lung, liver, kidney and intestine from a boa constrictor with histologically diagnosed IBD were tested with RT-PCR (4.7.2). All four samples resulted in strong PCR signals at 300 bp. Sequence analyses revealed 97% similarity to the recently described snake arenavirus: Golden Gate Virus

Alethinophid 1 reptarenavirus, (Genbank accession number: NC_018483). These four samples were used as positive controls during the screening process. Over a period of nine months (2012 - 2013), 450 different diagnostic samples (swabs, tracheal-lung lavage, serum and whole blood, fresh and paraffin embedded tissues) were screened for the detection of arenaviral RNA using RT-PCR and sequence analysis. These samples were drawn from 170 snakes (boid, pythonid). From dead snakes, organ samples (brain, kidney, spleen, gut, skin, lung, liver, testis and pancreas) were sent to be tested. The samples tested came from various species and included animals that tested IBD positive on histology or cytology as well as snakes that appeared to be IBD negative. The animals and samples tested are shown in Table 11.4 in the appendix.

4.4.3 Samples for sunshinevirus studies

Two cell culture isolates (PMV P8 and PMV P10) of sunshinevirus were kindly provided by Dr. Tim Hyndman, Murdoch University, Perth, Australia, as positive controls for developing an RT-PCR for the detection of sunshinevirus. Both viruses had been isolated on VH2 cells. For the presence of sunshinevirus, screening was performed for a total of 38 different samples including tissues (brain, lung, kidney, skin, liver, and pancreas), and lung lavages from 12 snakes (Table 5.2).

4.4.4 Processing of diagnostic samples:

Swabs, blood, and organ samples of examined animals were placed in 15 ml tubes, immersed in 3 ml growth medium DMEM supplemented with 2x concentration of antibiotics (4.3.1) and sonicated at an output level of 30 for 3

impulses for the destruction of cell membranes. Subsequently, samples were centrifuged at 3000xg for 15 min for removal of bacteria and cell debris and then stored at 4 ºC.

4.5 Subculture and preparation of tissue culture dishes for virus isolation:

After discarding the DMEM from a complete cell monolayer flask (75 cm^3) and washing with 5 ml trypsin, the cells were incubated with 1 ml trypsin at room temperature for a few minutes. Subsequently, the flask was shaken by hand until the cells were detached. 4 ml of growth medium was added to the subcultured cells. A glass pipette was used to separate the cells by pipetting them up and down several times. The cells were diluted at a ratio of 1:10, 1:5, or 1:2 during subcultivation (depending on the density of the cells) by transferring 0.5, 1, or 2.5 ml of the cell suspension respectively into 17 ml DMEM. To prepare tissue culture dishes the cell suspension was mixed 1:10 with DMEM in a cell reservoir to prepare 30 mm tissue culture dishes. 2 ml of this diluted suspension was added to each dish. One day old dishes were used for virus isolation. All flasks and dishes were incubated at 28 °C with 5 % CO_2.

4.5.1 Virus isolation

Virus isolation was attempted from samples or isolates on Russell's viper heart cells (VH-2, ATCC: CCL-140) and/or Terrapene heart cells (TH-1, ATCC: CCL-50) depending on the origin of the sample from snakes or tortoises. 200 µl of the

sample supernatant were inoculated onto one day old, medium-free tissue culture dishes, with about 70% confluent cell monolayers. Dishes were then incubated for 2 hours at 28 ºC with 5% CO2. After incubation, 2 ml DMEM supplemented with 2% FBS, 1% NEA and antibiotics (see 4.3.1) were added to each dish. One dish was always left un-inoculated as a cell control. Inoculated cells were examined for cytopathic effects (CPE) approximately every 2-3 days with an inverted light microscope (Wilovet, Wetzlar, Germany), and dishes were frozen when extensive CPE was seen.

4.6 Extraction of viral RNA

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

1. 300 µl of the cell culture supernatant or of the homogenous supernatant after processing the diagnostic samples (see 4.4.4), was mixed with 900 µl lysis buffer and 40 µl silica matrix in 2 ml tubes. The suspension was then incubated at room temperature for 10 minutes and vortexed every 3 minutes.

2. The suspension was centrifuged with Varifuge 3.2 RS, 14000 rpm at 4°C for 1 minute and the supernatant was discarded.

3. The pellet was washed with 1000 µl washing buffer twice then was washed with 1000 µl 70% ethanol twice respectively. After each washing step, the pellet was vortexed, centrifuged with Varifuge 3.2 RS, 14000 rpm at 4°C for 0.5 minute and the supernatant was discarded.

4. The pellet was washed a final time with 1000 µl acetone, followed by centrifugation with Varifuge 3.2 RS, 14000 rpm at 4°C for 3 minutes. The acetone was then decanted and the pellet dried (10 min. 56 °C) on a heating block.

5. Following complete drying, 60 µl of nuclease-free water and 1 µl RNase-inhibitor were added to the pellet, vortexed and incubated at 56°C for 15 minutes. During that the tubes were vortexed every 5 minutes. After incubation the suspension was centrifuged with the Varifuge 3.2 RS, 14000 rpm at 4°C for 3 min.

6. The supernatant was carefully removed with a micropipette (without any contact to the silica matrix) and transferred to a new 0.5 ml tube. This process was repeated once again for a total of approximately 100 µl of prepared RNA suspension and the supernatant stored immediately at -80 °C.

4.7 Reverse transcription – polymerase chain reaction

4.7.1 RT-PCR protocol used to identify picronaviruses from the isolates:

The reaction was performed in two rounds (Table 4.3). 2 µl of the prepared viral RNA was added to a 9.4µl mixture containing the reverse primer then incubated at 95°C for 5 min and immediately transferred to liquid nitrogen and placed on ice to thaw. In the meantime, a master mix was prepared. 8.6 µl from this mix was transferred to the first reaction tube for a final volume of 20 µl. The reaction mixture was then incubated 30 min at 50°C, 5 min at 85°C and stored at 4°C. In this step reverse transcription was carried out. For the amplification step, 2.5 µl of the first round RT-PCR product was added to a 47.5 µl reaction mixture (Table 4.3). The

mixtures were amplified with an initial denaturation at 95°C for 2 min followed by 40 cycles at 95ºC for 20 s, 45ºC for 20 s and 70ºC for 20 s. The extension time at 70°C was 1 min followed by hold step at 4°C.

4.7.1.1 Primers:

Table 4.2 Description of the primer sets used in the RT-PCR for the detection of picornavirus virus in tortoise.

sets	Primers		Expected size	Source of the sequences
1	Oligo dT^{18}	TTTTTTTTTTTTTTTT	900 bp	Dr. Günther Keil, FLI Riems, personal communication
	Kei+4343	CTTCAGTATAGAAAACGGATTTGATG		
2	RKei2	AAGCCAATCCTGCAACACT	400 bp	New primers designed for RT-PCR amplification
	FKei2	CTACCATCAGGATGCAGTT		
3	RVX1	TGCACATGGCGGTTCCAGTG	1120bp	
	FVX1	GCCATGGCACCCCTCCTTCT		
4	RVX3	GATGCATTCATTTGAACTT	600-800 bp	
	FVX3	TTTCATCTAGTGCTGATGCT		
5	Rtgt1	TGCCATTTACTGCTCTTGGA	238bp	
	Ftgt1	TGACCAGTGGGTTCTACAACTTC		

Table 4.3 Chemicals and reagents used in the RT-PCR for the detection of picornavirus in tortoise

Reagent	1st round RT-PCR	Final concentration	Reagent	2nd round RT-PCR	Final concentration
	volume in 20 µl total amount			volume in 50 µl total amount	
Millipore water	8.4 µl		Millipore water	32.25 µl	
50 mM Reverse primer	1 µl	2.5 mM	10x buffer + (NH4)2SO4	5 µl	1x
Template	2 µl of extracted viral RNA sample		Template	2.5 µl of the 1st round RT-PCR products	
5x Transcriptor reaction buffer	4 µl	1x	25 mM MgCl2	3 µl	1.5 mM
2 mM dNTPs	2µl	0.2 mM	2 mM dNTPs	5 µl	0.2 mM
40 U/µl Ribolock RNase inhibitor	0.5 µl	1 U/µl	50 mM forward primer	0.625 µl	0.625 mM
1 mM DTT (Dithiothreitol)	1 µl	0.05 mM	50 mM reverse primer	0.625 µl	0.625 mM
200 U/µl RT (reverse transcriptase)	1.1 µl	11 U/µl	5 U/µl Taq DNA-polymerase	1 µl	0.1 U/µl

4.7.2 RT-PCR protocol used for the detection of Arenaviruses:

The primer combinations MDS-400 (5'-TTCATTTCTTCATGRACTTTRTCAATC-3') and MDS-402 (5'-GGSATAACAAAYTCACTTCAAATATC-3') targeting part of the glycoprotein gene (expected size 300 bp) (Stenglein et al., 2012) were used for reptarenavirus screening.

25 µl of master mix was prepared with the necessary reagents for the RT-PC reaction (Table 4.4). After the RT reaction at 42°C for 60 min, amplification was carried out with an initial denaturation at 95°C for 2 min, followed by 30 cycles with cycling conditions: 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min. There was a final extension at 72°C for 5 min followed by cooling at 4°C until further processing on gel.

Table 4.4 Chemicals and reagents used in the RT-PCR for the detection of reptarenavirus

Reagent	One round RT-PCR	Final concentration
	volume in 25 µl total amount	
Millipore water	9.25 µL	
10x buffer + KCL	2.5 µL	1x
2 mM dNTPs	2.5 µL	0.2 mM
40 U/µl Ribolock RNase inhibitor	0.15 µL	0.24 U/µl
200 U/µl RT (reverse transcriptase)	0.35 µL	2.8 U/µl
25 mM $MgCl_2$	2.5 µL	2.5 mM
5 U/µl *Taq* DNA-	0.25 µL	0.05 U/µl

polymerase		
10 mM forward primer MDS-400	2.5 µL	1 mM
10 mM reverse primer MDS-402	2.5 µL	1 mM
Template	2.5 µl of extracted viral RNA sample	

4.7.3 RT-PCR protocol used for the detection of sunshinevirus:

Optimization of an RT-PCR protocol was carried out based on a previously published protocol provided kindly from Dr. Tim Hyndman (2012a). 13.5 µl from the total extracted viral RNA was added to 1 µl of reverse primer named Sunshine AS1: 5'ATTCAACATC-TGGGGTC (Hyndman et al., 2012a) and 1 µl of dNTPs and incubated at 65°C for 5 minutes. 0.5 µl of RT enzyme and 4 µl 5 x Puffer were then added to make a final volume of 20 µl and incubated at 25°C for 5 minutes, 24°C for 45 minutes and 70°C for 15 minutes. For PCR amplification, a 20 µl master mix was prepared (Table 4.5) containg reverse primer Sunshine AS1 and Sunshine S2 5'TTCAAGGAGAT-AACCAGG (Hyndman et al., 2012a). Cycling descriptions initiated with denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 20 sec., 45°C for 45 sec., 72°C for 30 sec ended with cooling at 4°C. PCR products were visualised using agarose gel electrophoresis. The expected product size was approximately 357 nucleotides.

Table 4.5 Chemicals and reagents used in the RT-PCR for the detection of sunshinevirus in snakes

Reagent	1st round RT-PCR	Final concentration	Reagent	2nd round RT-PCR	Final concentration
	volume in 20 µl total amount			volume in 20 µl total amount	
10 mM dNTPs	1 µl	0.5 mM	Millipore water	7.1 µl	
1 mM Reverse primer	1 µl	0.05 mM	10x buffer + KCL	2 µl	1x
Template	13.5 µl of extracted viral RNA sample		Template	1µl of the 1st round RT-PCR products	
5x Transcriptor reaction buffer	4µl	1x	25 mM $MgCl_2$	3 µl	3.75 mM
200 U/µl RT (reverse transcriptase)	0.5 µl	5U/ µl	2 mM dNTPs	2.5 µl	0.25 mM
			10 mM forward primer	2 µl	1 mM
			10 mM reverse primer	2 µl	1 mM
			5 U/µl *Taq* DNA-polymerase	0.25 µl	0.0625 U/µl
			40 U/µl Ribolock RNase inhibitor	0.15 µl	0.3 U/ µl

4.7.4 RT-PCR positive controls

Table 4.6 The positive controls used in the RT-PCRs

	Lab. No.	**Source**
Picornavirus virus (X)	144-10, 36-10	Dr. R.E. Marschang (Hohenheim University,Germany)
Arenaviruses	128-1,2,3,4-12	Dr. Kim Heckers (Laboklin, GmbH & Co. KG, Germany)
Sunshinevirus	P8, P10	Dr. T. Hyndman (Murdoch University, Perth, Western Australia)

4.8 Gel electrophoresis

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

4.9 Gel extraction

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

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

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

2. 700 µl of the solution was then pipetted onto Perfect Bind DNA columns with a membrane for adsorption of DNA, which were plugged into collection tubes. The tubes were centrifuged with Varifuge 3.2 RS at 10,000 rpm for 1 min and the flow-through was discarded. If the solution was more than 700 µl, the process was repeated using the same collection tubes and columns.

3. Columns were washed once with 300 µl Binding Buffer and twice with 300 µl Wash Buffer supplemented with EtOH. Prior to centrifugation (1 min at 10,000 rpm), the columns were incubated for about 2 min at room temperature.

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

5. For elution of the DNA from the membrane, 15 µl Elution Buffer was added (exactly on the membrane), the tubes were then incubated for about 2 min at room temperature. After that, the tubes and the membrane were centrifuged for 1 min at 5,000 rpm. This elution step was repeated once to get a final volume of about 30 µl of the extracted DNA which was stored at 4ºC until sequencing.

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

4.10 Sequencing

Gel purified PCR amplicons were diluted (1:2-1:20), based on the strength of the band visualized on the UV-transilluminator. 15 µl of the diluted samples and 20 µl of the corresponding PCR primer were submitted to a commercial service for sanger sequencing (Eurofins, MWG Operon, Ebersberg, Germany).

4.11 Analyses of sequences

Initial sequences were first downloaded from the website of the company http://www.eurofinsdna.com/de/home.html and processed by ABI Sequence Analysis Programme 5.1.1 (Applied Biosystems, Foster City, USA). For processing DNA sequences several software programs were used:

1. Staden Package version:1.7.0 (2003.0) Pregap4 and Gap4 programme (Bonfield et al., 1995) for sequence assembly, editing, correcting and comparon. The sequences were compared to the data in GenBank (National Center for Biotechnology

Information, Bethesda, USA) online (www.ncbi.nih.gov) using the BLASTN and BLASTX homology search programs.

2. BioEdit 7.0.5.3 (2005). Multiple alignments of sequences were performed with ClustalW algorithm of the BioEdit Sequence Alignment Editor programme (Hall, 1999) using default settings. Identity matrices were generated as well.
3. PHYLIP program Package, Versions 3.6 (University of Washington, Felsenstein, 1989). Alignments were used for phylogenetic calculations applying DNAdist and Fitch programs to obtain an optimal tree.
4. TOPALi v2.5 program (Biomathematics and Statistics Scotland) used for Bayesian analysis which was carried out from deduced amino acid (aa) and nucleotide (nt) alignments with their homologues derived from GenBank, using CPRev+G and HKY+G substitution models in MrBayes (Huelsenbeck & Ronquist, 2001) assuming gamma distribution with 100 generations, sample frequency 10 and burn in ratio 25% to configure an optimal tree showing the posterior probability and maximum likelihood values. Bootstrapping was carried out with 100 replicates to estimate the validity of trees.
5. FigTree v1.4.2. is a graphical viewer program of phylogenetic trees, which was used to optimize the graphic visualization of tree produced using the above named programs.

5. Results

5.1 Picornavirus detection

5.1.1 Cell culture and RT-PCR Trials

5.1.1.1 Picornavirus (previously known as virus “X”) isolates

37 virus “X” isolates from different years (see Table 4.1) were used in the study to test a variety of RT-PCRs. The isolates had been identified as possible virus “X” candidates based on their growth in TH1 causing a characteristic cell lysis and lack of sensitivity to chloroform (Marschang, 1999). Each isolate was inoculated onto TH1 using the methods described earlier (section 4.4) to yield more virus stock utilized for the study. After two days, virus growth and CPE were observed in all of the inoculated cell cultures. In all cases, CPE was characterized by cell lysis of the TH1 cells (Fig. 5.1). Cell cultures were frozen at -20°C to harvest the viruses and then collected in tubes to be used for RNA extraction and RT-PCR.

Five primer sets were tested in RT-PCRs for their specificity and sensitivity (Table 4.2). Not all of the primer sets produced PCR products. In some trials, no PCR products or unspecific PCR products were produced (Table 11.1 in appendix). Among all primers, primer pair (FKei2 - RKei2) was the most widely reactive as it produced the highest number of positive PCR products (26 isolates were positive, 70%) from the 37 virus “X” isolates tested and amplicons with the expected size (400 bp) were all specific for picornaviruses in all cases in which amplicons were obtained. The other primer sets (Kei+4343, FVX1-RVX1, FVX2-RVX2, Ftgt1-Rtgt1) were only tested based on their performance on the isolates, no diagnostic samples were screened with these primers.

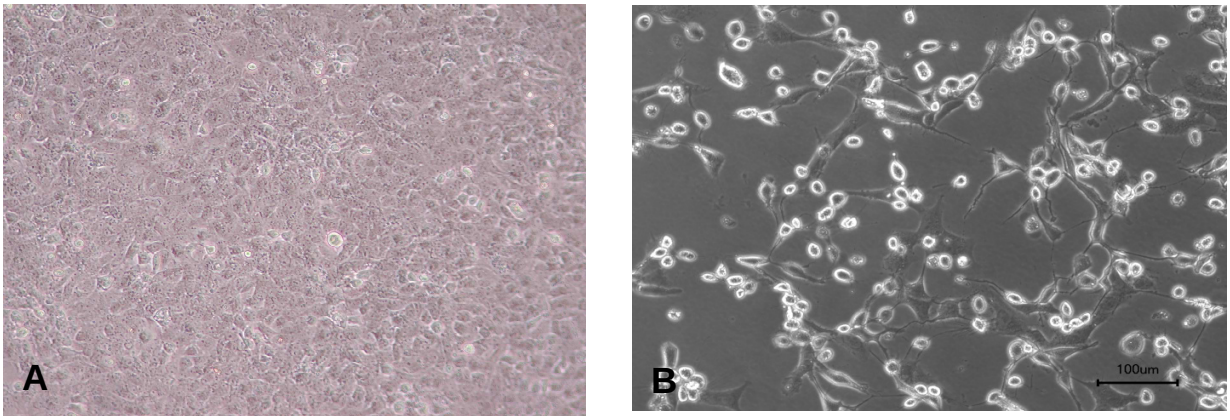


Figure 5.1 microscopic views of cytopathic effects (CPE) of a virus "X" isolate (A): uninoculated TH1 cells (B): 2nd passage of a virus "X" isolate (lab. no. 9_05), 2 days post inoculation (magnification: 400 x). CPE was characterized by significant cell lysis with multiple small round cells in the supernatant.

The primer pair FVX1-RVX1 produced seven positive products from 37 virus isolates (18.9%), while primer set FVX2- RVX2 produced 12 positive products from 24 virus isolates (50%). Only two positive products from 23 virus isolates were found in the RT-PCR using the Kei+4343 primers (8.3%) and four positive products resulted from 37 virus isolates (10.8%) using the Ftgt1 - Rtgt1 primer pair (Table 11.1 in appendix). All resulting amplicons were purified and sent to be sequenced. No host specificity was observed, viruses were founded in several different tortoise species.

5.1.1.2 Diagnostic samples

RT-PCR was performed for the detection of picornaviruses using the FKei2-RKei2 primer set to screen 74 different samples and swabs from tortoises which were submitted to the laboratory between 2012 and 2013 for diagnostic purposes. Performing RT-PCR using the primer set Kei2 led to the detection of picornaviruses in six samples (8% of 74 diagnostic samples tested) originating from three tortoises (5.8% of 51

tortoises screened). From diagnostic samples collected in 2012, one picornavirus was isolated on TH1 cells from the head of a *Stigmochelys pardalis* sent from Italy. In 2013, two picornaviruses were isolated on TH1 cells from samples of organs (liver, kidney, heart, and lung) and an oral swab from two tortoises from Germany (see Table 11.1 in appendix). The RT-PCR successfully detected picornaviruses in RNA from tissues from these cases. Unfortunately, there were no clinical reports available for these tortoises, only the tortoise from sample 36_13 was infected with pinworms (*Oxyuris* spp.) and had high uric acid values. All three tortoises were negative for ranaviruses, herpesviruses and adenoviruses by PCR.

5.1.2 Sequence analysis

All amplicons were sequenced and the sequences identified using BLAST on the NCBI web site (www.ncbi.nlm.nih.gov/BLAST). The BLASTn results showed 93% identity with Saffoldvirus complete genome, while BLASTx results showed 57% identity with polyprotein of Mouse Mosavirus. Partial sequences of 26 picornavirus isolates were obtained from the RT-PCR using the primer pair FKei2 and RKei2 which targets 400bp of the polyprotein gene. Multiple alignments of the acquired nucleotide sequences of the isolates were successfully performed and an identity matrix was constructed with identity values of 79.2 – 100% (Table 11.3 in appendix). Multiple alignments of the obtained isolate sequences were performed comparing these with the corresponding nucleotide sequences of a portion of the polyprotein gene at nucleotide positions 6400–7700 from four picornaviruses, members of the family *Picornaviridae*, which were used in this study for the comparison: Canine picornavirus (CanPV: JN831356) which is listed

as an unassigned picornavirus, Human enterovirus 109 (HEV: GQ865517), Human rhinovirus C (HRV: HM485529), and Human coxsackievirus A13 (HCV: DQ995642) which belong to the genus *Enterovirus*. All appeared to have relative similarities with the obtained sequences and the identity values ranged between 38.5-50.9 %.

A phylogenetic distance tree was constructed based on the multiple alignments of 308 nucleotides (Figure 5.2) and showed a close genetic relationship among the detected picornavirus isolates and the tree illustrates two main clusters, which together form a single monophyletic cluster.

Sequence analysis was also done on the amplicons obtained using the other primer sets (Kei+4343, FVX1-RVX1, FVX2-RVX2, Ftgt1-Rtgt1). Blast N of the sequences confirmed that each of the PCRs was specific. Similarity values of the obtained viral sequences of the polyprotein gene vary, and relative similarity was observed in the corresponding gene sequences of picornavirus members including: Human cosavirus D. Theiler's-like virus, Saffold virus, Cardiovirus, Rhinitis B virus and Mouse Mosavirus.

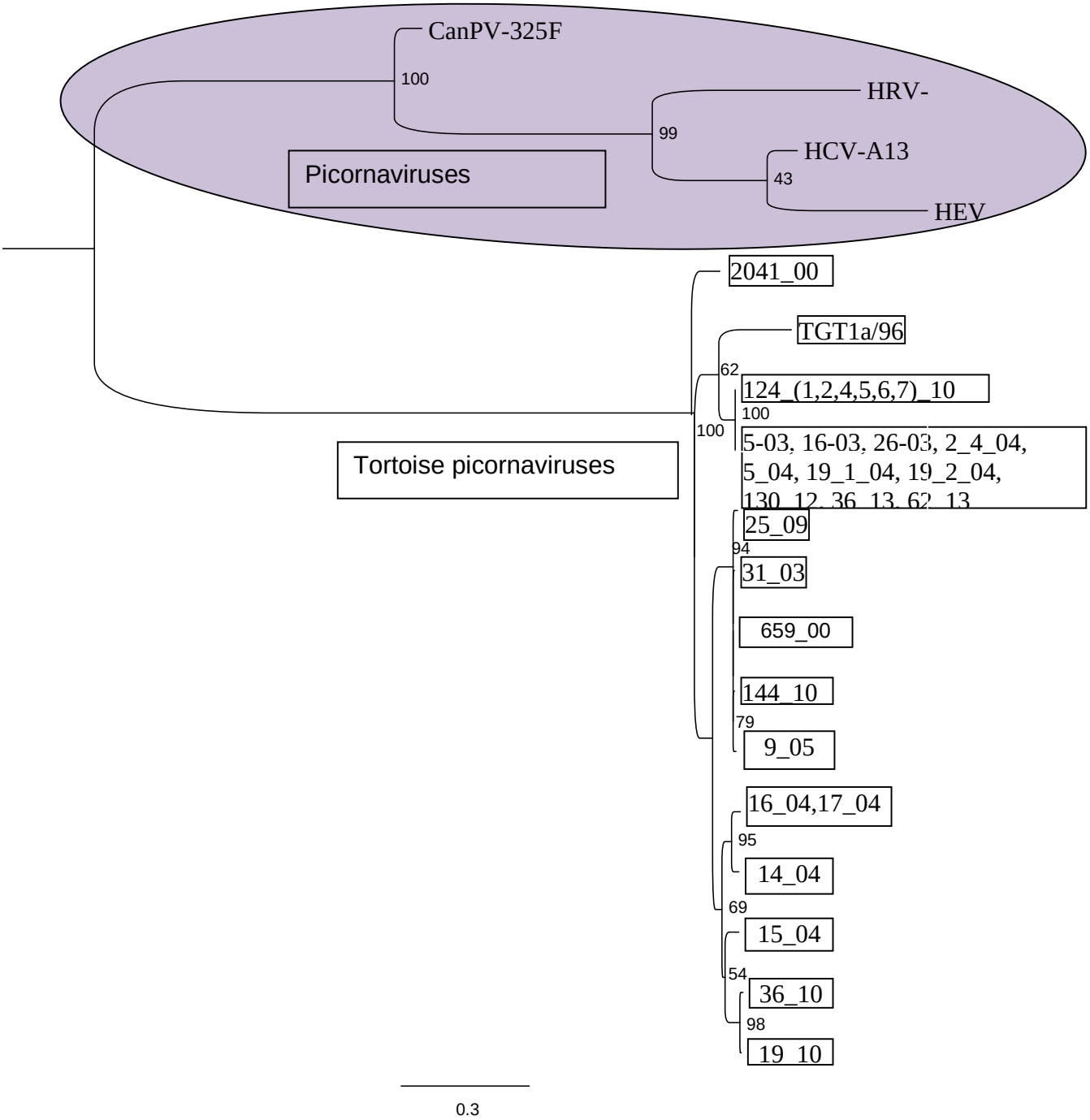


Figure 5.2 DNA distant tree based on 308 bp of the partial polyprotein gene of virus “X” isolates. Four picornaviruses were used as outgroups by applying DNAdist and Fitch programs of the PHYLIP program Package version 3.6. GenBank accession numbers: Canine picornavirus (CanPV: JN831356), Human enterovirus 109 (HEV: GQ865517), Human rhinovirus C (HRV: HM485529) and Human coxsackievirus A13 (HCV: DQ995642).

5.2 Arenavirus screening in snakes

In this study, 450 swabs, tissues and organ samples from 170 snakes all from the families *Boidae* and *Pythonidae* (Table 11.5 in appendix) were screened for the detection of reptarenaviruses. 129 samples (28.6%) appeared positive with specific sized amplicons (300 bp) in the RT-PCR. Positive samples originated from 49 snakes (28.8%), 40 boa constrictor (81.6%) and 9 pythons (18.3%). A history of histological findings consistent with inclusion body disease (IBD) was reported for a number of cases (Table 11.4 in Appendix). 17 of 20 IBD positive animals (85%) (in addition to two samples in which IBD was diagnosed in the animal collection) were arenavirus positive by RT-PCR. 16 of 43 IBD negative animals were arenavirus positive (39.5 %), in 8 of these the reptarenavirus was found in brain samples, in 3 in oral swabs and in 6 in whole blood samples but not in other tissues. 14 of 93 animals with unknown IBD status were arenavirus positive (15 %). Most of the samples were submitted to the veterinarians to determine and examine the IBD status by cytology and/or histology. No host specificity was observed, arenavirus was found in both boas and pythonsbut different prevelances.

Statistical data concerning snake screening, arenavirus detection, IBD status in tested snakes as follows (details shown in Table 11.4 in appendix):

- 170 snakes, 450 samples subjected to RT-PCR
- 129 arenavirus positive samples
- 321 arenavirus negative samples
- IBD pos.: 20 of which arenavirus pos: 17 neg: 3
- IBD neg.: 43 of which arenavirus pos: 16 neg: 27

- IBD unknown.: 90 of which arenavirus pos: 13 neg: 79
- IBD ?: 3 of which arenavirus pos: 1 neg: 2
- IBD in collection: 12 of which arenavirus pos: 2 neg: 10

5.2.1 Sequence analysis

187 bp of the glycoprotein gene of the detected arenaviruses were obtained from arenavirus positive RT-PCR products. Multiple alignments of the acquired sequences showed high identity values between 72.7-100% among the detected viruses, while the nucleotide identity values of the detected arenaviruses with reptarenaviruses were as follows: 75.9-98.3% identical with the corresponding portion of Golden Gate virus (GGV) (*Alethinophid 1 reptarenavirus*) genome (GenBank accession number: JQ717264), 77-100% identical with the corresponding portion of the Boa Arenavirus Netherlands (Boa AV NLB3) (*Alethinophid 3 reptarenavirus*) genome (GenBank accession number: KC508669), 71.6- 80.7% with the corresponding portion of the University of Helsinki Virus (UHV) (*Alethinophid 3 reptarenavirus*) genome (GenBank accession number: KF297880), and 71.1- 78.6% identical with the corresponding portion of the California Academy of Sciences (CASV) (*Alethinophid 2 reptarenavirus*) genome (GenBank accession number: JQ717262) (Table 11.6 in appendix). A phylogenetic tree was calculated based on NT sequences to demonstrate the distance and clustering between the detected viruses and the snake arenaviruses GGV, Boa AV NLB3, UHV and CASV (Figure 5.3). In the tree, a majority of the detected reptarenavirus strains clearly clustered with GGV, Boa AV NL and UHV. None of the detected virus clustered with CASV.

Snake Arenaviruses

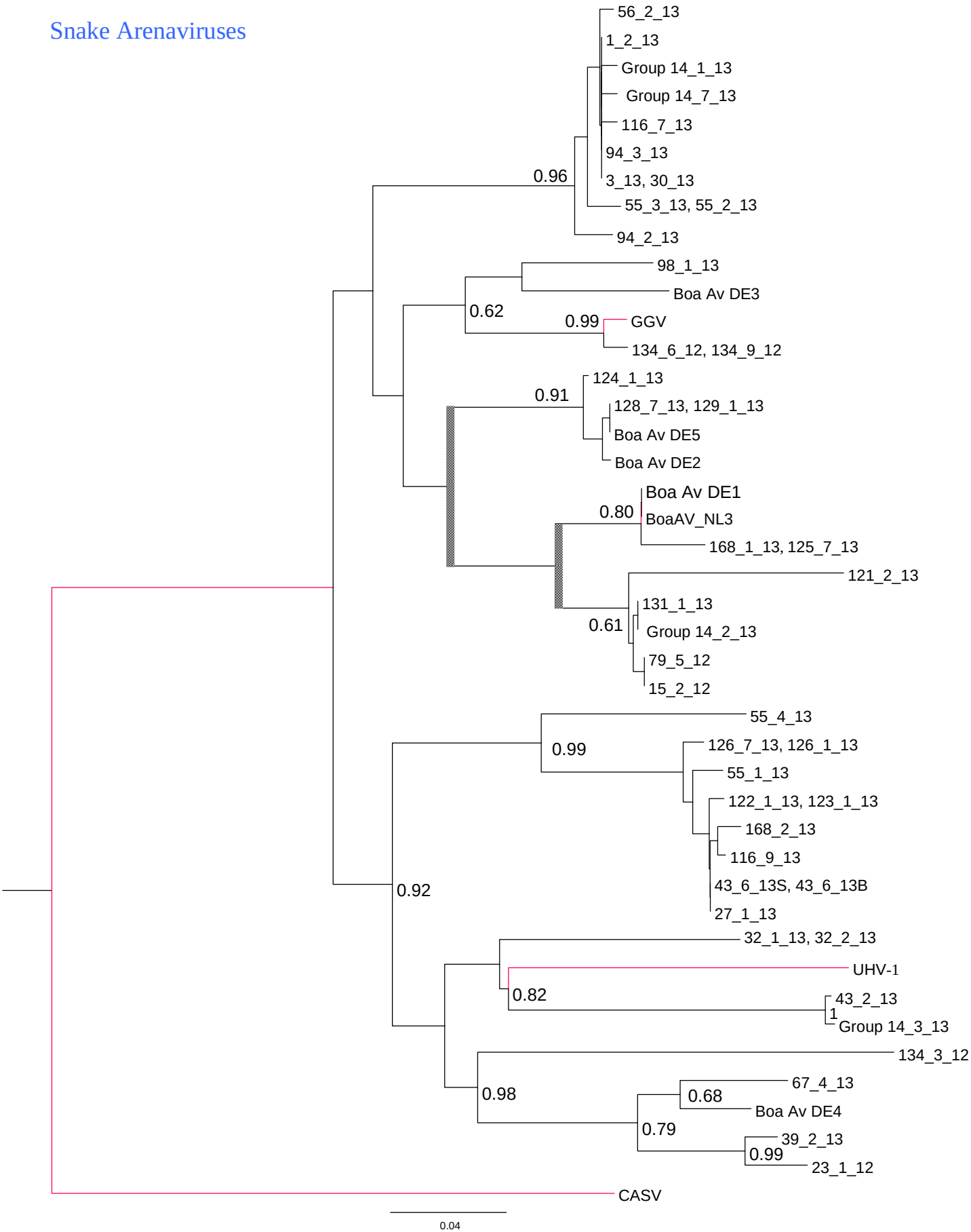


Figure 5.3 DNA distance tree of the detected arenaviruses in snakes (Boas and Pythons) based on a 187 bp portion of the glycoprotein gene (GP2). Posterior probability values over 60 of 100 replicates are indicated beside the node. Branches with lower values are drawn with checkerboard lines. The maximum likelihood and the Bayesian analysis resulted in the same topology tree as the FITCH analysis.

GenBank accession numbers: Golden Gate Virus (GGV JQ717264), Boa Arenavirus Netherlands (Boa AV NLB3 KC508669), California Academy of Sciences (CASV JQ717262) and University of Helsinki Virus (UHV-1 KF297880)

Boa Av DE includes a group of samples with lab numbers as follows:

Boa Av DE1 = 11_1_13S

Boa Av DE2 = 11_11_13S, 11_12_13S, 11_13_13S, 11_14_13S, 11_16_13S, 11_11_13B, 11_13_13B, 11_14_13B, 11_16_13B

Boa Av DE3 = 11_25_13S, 11_25_13B

Boa Av DE4 = 44_21_13

Boa Av DE5 = 11_23_13S

Abbreviation S: pharyngeal swab B: whole blood

Group 14_1_13 includes arenaviruses detected in different organs: gut, liver, lung, pancreas, spleen and kidney.

Group 14_2_13 includes arenaviruses detected in different organs: gut, liver, lung, pancreas, and kidney.

Group 14_3_13 includes arenaviruses detected in different organs: lung, skin and brain.

Group 14_7_13 includes arenaviruses detected in different organs: liver, spleen, trachea and kidney.

5.3 Detection of Sunshinevirus in snakes

Multiple RT-PCR trials were done to optimize the PCR for the detection of sunshinevirus using previously described primer sets and cycling conditions (Hyndman et al., 2012a). Positive bands from PCR products were consistantly weak. However, four of 38 samples (10 %) originating from three animals (25% of 12 animals tested) clearly displayed positive signals at the specific size (357 bp) and were sent for sequencing. Sequencing results of the detected viruses were subjected to BLASTN analysis and were all 95- 98 % identical to the corresponding portion of the *Reptile sunshinevirus 1* genome (Genbank accession number: JN192445). RNA samples P4 and P8, which were the 4th and the 8th passage on VH2 cell lines of an original sunshinevirus isolate, in addition to RNA samples of Mia and Tia (original sunshinevirus isolates provided by Dr. Hyndman, Murdoch University, Perth, Australia), were all used successfully as positive controls in the RT-PCR. Multiple alignments of the obtained sequences were performed to compare the detected sunshineviruses with each other and with the complete genome of the original sunshinevirus as well (Fig.11.3 in appendix). The obtained sequences matched nucleotide positions 12578-12933 of the original sunshinevirus. The detected viruses are highly similar to the original sunshinevirus (Table 5.1). The viruses were found in skin sample and oral-cloacal swabs of pythons, all boas tested were negative. Clinical signs reported in the animals infected with sunshinevirus vary significantly, but mostly respiratory problems were reported.

Table 5.1 Identity values (%) of the detected sunshinevirus in snakes in Germany
(16_13H: 16_13 Haut): sunshinevirus was found in skin. The upper diagonal refers to the values for 148 nucleotides. The lower diagonal shows values for 49 deduced amino acids of the detected viruses.

Seq.	81_1_12,81_2_12, 89_12, Milla,P4,P8	16_13H	Tia
81_1_12, 81_2_12, 89_12, Milla, P4, P8		89.1	99.1
16_13H	80		88.5
Tia	100	80	
Sunshinevirus	100	80	100

Table 5.2 Data collected for sunshinevirus RT-PCR from diagnostic samples 2012-2013

	Species	**Sample**	**PCR**	**Clinical signs**	**Lab no.**
1.	Ball python *Python regius*	lung lavage oral/tracheal swab	**pos.** **pos.**	dyspnea, weight loss, flagellates	81_1-2_12
2.	Ball python *Python regius*	oral/ cloacal swab	**pos.**	dyspnea, stomatitis	89_12
3.	Indian python *Python molurus*	n.a.	neg.	weak, nasal discharge	38_1_13

4.	*Boa constrictor*	pancreas kidney brain lung heart	Unspecific sized bands	weak	38_2_13
5.	Garden tree boa *Corallus hortulanus*	organs	neg.	dead, strangled by partner boa	34_1-7_13
6.	Ball python *Python regius*	lung lavage	neg.	were in contact with snakes infected with sunshinevirus	26_1-2_13
7.	Anaconda *Eunectes murinus*	n.a	unspecific sized band	n.a	24_9_13
8.	Ball python *Python regius*	brain lung kidney skin liver	neg. neg. neg. neg. neg.	n.a	23_1-5_13
9.	Ball python *Python regius*	Lung lavage	unspecific sized band	pneumonia, was in contact with infected snake (137_12)	9_13
10.	Ball python *Python regius*	kidney(isolated) lung liver	unspecific sized bands	n.a	8_5_13

11.	Ball python *Python regius*	lung liver brain gut	unspecific sized bands	pneumonia	137_1-4_12
12.	Ball python *Python regius*	lung liver brain skin kidney gut pancreas	unspecific sized bands skin: **pos.**	catarrhal – purulent pneumonia	16_13

6. Discussion and conclusion

6.1 Picornaviruses in Tortoises

Since reptile picornaviruses have only recently been characterized, there are limited references available about picornavirus in tortoises. It is commonly known that picornaviruses caused diseases in mammals, but they have also been found in reptiles, birds, fish, plants, insects, and even algae (Ng et al., 2015). The family *Picornaviridae* has been expanded greatly in recent years, and now includes at least 35 genera with a rapidly expanding number of new picornavirus genera (ICTV 2017). Sequence analyses of tortoise picornavirus genomes showed that they did not belong to any previously recognized genera. An initial proposal was to create a new genus with the name *Topivirus* (Farkas et al., 2015, Marschang et al., 2016). The genus finally established by the ICTV and is now named *Torchivirus* (Zell et al., 2017). At the same time, a genetically distinct picornavirus has been identified in Forsten's or Sulawesi tortoises (*Indotestudo forsteni*). This virus also appears to belong to a novel genus with the proposed name *Rafivirus A* (Ng et al., 2015).

The purpose of this study was to develop a convenient diagnostic technique using reverse transcription polymerase chain reaction for the detection and identification of picornaviruses in terrestrial tortoise species. Virus diagnostic procedures using cell culture are limited by the expertise of the diagnostic laboratory and the time required obtaining a result. As such, viral culture has a limited place in routine diagnostic microbiology, although it is an effective diagnostic method and was previously the only reliable tool for the detection of

picornaviruses (Farkas et al., 2015). It is, however, difficult to visualize virions in infected cell cultures via electron microscopy, making virus identification dependent on the typical cytopathic effect (CPE) and resistance to chloroform (Heuser et al., 2010). Therefore, the development of a diagnostic (RT)-PCR assay was considered necessary for the detection and preliminary characterization of these viruses as a useful laboratory diagnostic tool. Sequencing portions of viruses has contributed to taxonomic classification of new viruses in this new genus. Future work might also be able to widen our understanding of the possible factors involved in viral pathogenicity. Sequence analysis can also provide the potential for understanding the genetic basis for presence or lack of species specificity.

Although all isolates appeared positive in the TH1 cell culture, not all of them produced RT-PCR positive results. This could be due to the use of primer sets that did not match the genomic sequences of all viruses. The reaction of the PCR using the primer set Kei +4343 was low. The other primer sets tested were also unable to detect all of the isolates included in the screening. There are several possible reasons for this. Since the preliminary identification of the isolates used in this study was based on relatively unspecific criteria (type of CPE and resistance to chloroform), it is possible that some of the virus isolates tested were not picornaviruses. It is also possible that these virsues are highly variable, especially since they are ssRNA viruses, and that the primers designed during the course of this study did not bind to the specific sites in some cases. Sequencing of the PCR products produced using the primers (FKei2 and RKei2)

showed the highest sequence similarity values (33-85%) with the corresponding polyprotein gene of members of the genera *Cardiovirus* (includes serotypes Saffold virus and Theiler-like virus), *Mosavirus* and *Cosavirus.* These findings agree totally with those reported by Farkas et al. (2015) for a similar set of viruses.

This RT-PCR protocol using the primer set Kei2 targeting a small portion of the polyprotein gene was useful for the detection of picornaviruses in terrestrial tortoises mainly in tortoises belonging to the *Testudinidae* family. Sequencing of the PCR products indicate that this protocol is very specific, because there were no unspecific products or unexpected bands produced by the RT-PCR. Marschang et al. (2016) also described the development of a RT-PCR protocol in which the picornavirus specific primer sets amplified 2 regions of the genome (containing a portion of the 5′-untranslated region, the leader peptide, and the VP4 capsid protein, and a portion of the picornaviral RNA-dependent RNA polymerase (3D), respectively). Both methods described there and methods developed in this study appear to be highly specific for torchiviruses, although further study is necessary to directly compare the naturally occurring torchivrus genotypes. In all cases, sequencing of each RT-PCR positive amplicon is highly recommended for a final diagnosis.

According to phylogenic calculations, the viruses showed close genetic relationships between all of the isolates studied, and beside the typical CPE characteristic for these viruses, analysis of the obtained partial sequence data also confirmed that all strains belong to the genus *Torchivirus.* The viruses

appeared to be divided into two main lineages, with most of the strains belonging to the first lineage which contains four groups (Fig 5.2).

Tortoise picornaviruses have been isolated from infected animals exhibiting various clinical signs (Table 11.1 in appendix). Consequently, there are no fixed signs indicating the presence of picornaviruses in tortoises and none of the previous studies have provided evidence relating to the pathogenicity of tortoise PVs. In addition, the detection of picornaviruses in samples from healthy animals could indicate that these viruses may lead to undetectable transmission by asymptomatic animals in animal collections. It is important to mention that previous studies indicate that these viruses might be involved in kidney disease and softening of the carapace in juvenile tortoises and possibly in various disease syndromes in adults including upper respiratory disease and sudden death (Heuser et al., 2014; Marschang and Ruemenapf, 2002).

In order to prevent the spread of picornaviruses in a collection, a quarantine period would be necessary to observe and test newly acquired animals. Based on the data collected on the picornavirus isolates in this study, there was no strict host specificity and no association could be observed between the geographic distribution of the hosts and the genetic sequences, although the origin of the tested animals were all from within Europe including Germany, Italy, England and Switzerland. However, the pet trade may influence the distribution of pathogens in pet tortoises in these countries (Marschang et al, 2016). In any case, no significant relationship was noted between the geographic distributions of the

host animals. Therefore, further study is necessary to understand the global distribution of these viruses.

6.2 Arenaviruses in snakes

Arenaviruses were originally known as a group of mainly rodent-borne viruses which infect mammals and can cause a variety of serious diseases (Buchmeier et al., 2007). Recently, four novel arenavirus genomes were sequence from tissues of snakes diagnosed with Inclusion body disease (IBD) (Bodewes et al., 2013; Stenglein et al., 2012; Hetzel et al., 2013). IBD is considered one of the most important diseases of snakes mainly because of its very high rate of mortality, and the lack of treatment and prevention options. The disease is infectious and commonly affects captive snakes of the families Boidae (boas and anaconda) and Pythonidae (pythons) (Schumacher et al., 1994; Wozniak et al., 2000; Jacobson et al., 2001).

The identification of arenaviruses from various snake samples in the present study is the first report in live snakes in Germany. This is also the first description of the detection of arenaviruses in pythons including reticulated python, green tree python, ball python and Indian python (Table 11.4 in appendix). 28.6% of the screened samples were positive using the described RT-PCR and all of the viruses detected were closely related to recently described viruses in the new genus *Reptarenavirus* which contains three species: *Alethinophid 1 reptarenavirus* (member virus: Golden Gate virus), *Alethinophid 2 reptarenavirus* (CASV), and *Alethinophid 3 reptarenavirus* (University of Helsinki virus 1

[UHV1], boaAVNLB3 virus). The detected arenaviruses in this study all grouped together with GGV and BoaAVB3 and UHV. Moreover, Hepojoki et al. (2015) presented a study which demonstrates the identification of four novel representatives of *Reptarenavirus* from boa constrictors. This indicates that the recently established genus is likely to expand in the near future. Studying genetic diversity of arenaviruses infecting captive snakes is very important for the future to understand viral evolution as well as the development of IBD in infected snakes. Stenglein et al. (2015) documented viral genetic complexity of naturally infected snakes and pointed out that the majority of snakes were infected with multiple reptarenavirus genotypes and a spectacular amount of viral diversity was reported as well as viral recombination and re-assortment.

Most of the positive results in the present study were found in apparently healthy snakes. It is possible that (as is often the case in rodents) arenavirus infection of snakes is chronic and sub-clinical (Stenglein et al., 2012). Besides the IBD history reports of the arenavirus positive snakes, clinical signs including CNS signs, respiratory signs, inappetence and skin problems were reported in a few cases in our study. Moreover, other studies suggest that reptarenaviruses may establish chronic infections in snakes, and that co-infections or super-infection with other reptarenaviruses in chronically infected snakes might be relevant for the pathogenesis of IBD, due to immune-suppression (Hepojoki et al., 2015). The absence of clinical signs in some animals with IBD has been described. However, a method for early detection of viral infection is necessary in order to develop effective screening and quarantine procedures as well as to increase

understanding of virus pathogenicity. Virus strain, host factors, and infection time may all also play a role in virus shedding and disease development (Aqrawi et al., 2015). Two of 12 snakes which were found to be in contact with IBD infected snakes were arenavirus positive, indicating possible transmission within collections.

The identification of protein inclusions in tissues or blood films has been the standard diagnostic method for IBD for decades. While many snakes with IBD may have many inclusions in different tissues (Jacobson, 2007), some snakes may have few, which are limited to certain organs and are easily overlooked. Furthermore, inclusions resembling those seen in IBD have been seen in snakes with other viral diseases, and distinguishing them from IBD inclusions can be challenging (Bodewes et al., 2013; Raymond et al., 2001; Jacobson, 2007). In our study we used microscopic examination of blood smears or tissue sections to identify inclusions as a common IBD diagnostic method (done by Dr. K. Heckers in Laboklin GmbH & Co. KG, Bad Kissingen, Germany). Since 39.5 % of the IBD negative animals were arenavirus positive, further study is needed to determine if arenavirus infection always eventually leads to the development of IBD or if some animals may be able to clear infection and not develop disease. On the other hand, three of 20 snakes diagnosed as IBD positive in this study tested negative for arenavirus infection. This may indicate infection with other divergent arenaviruses not detected by the methods used. Other explanations are testing of inappropriate samples in which no virus was present or degradation of viral RNA during transport and/or preparation. The development of sensitive methods

for the detection of arenaviruses in live snakes will facilitate the identification of infection prior to the development of clinical disease and lower the chances for the introduction of these viruses into new collections.

6.3 Sunshinevirus in snakes

When it was first described, sunshinevirus was hypothesized to be a novel paramyxovirus and was shown to be distantly related to the ferlaviruses (Hyndman et al., 2012a). Ferlaviruses are enveloped RNA viruses which commonly cause infections associated with neurologic, respiratory and immunosuppressive diseases and have caused significant mortality in snake collections worldwide (Fry, 2015). In addition, Nidoviruses have recently been described as a cause of severe respiratory diseases in captive ball pythons and boas in the USA and Europe (Stenglein et al., 2014; Uccellini et al., 2014; Bodewes et al., 2014; Marschang and Kolesnik, 2016). The nidoviruses present a diverse order that includes important human and veterinary pathogens.

Respiratory diseases in snakes are common in captivity. It can be very difficult to detect illness in live snakes due to the ability of these animals to hide clinical signs of disease. Therefore, some animals may appear clinically healthy despite suffering from e.g. pneumonia. In one study, acute catarrhal pneumonia was diagnosed more often in snakes without respiratory signs than in snakes with respiratory signs, and respiratory signs and pneumonia were detected more often in pythons than in boas (Schmidt et al, 2013). Establishment of effective diagnostic tools for the detection and differentiation of various causes of

neurological and respiratory disease in snakes is therefore of particular importance.

In 2016, the genetic differences between sunshinevirus and other paramyxoviruses led to the establishment of a new family "*Sunviridae"* within the order *Mononegavirales*. The family includes a single genus *Sunshinevirus* with a single species (*Reptile sunshinevirus 1*) (Alfonso et al., 2016). The initial detection of sunshinevirus was described in 2008 from a collection of 70 Australian pythons which was experiencing an outbreak of neuro-respiratory disease on the Sunshine Coast of Queensland, Australia (Hyndman et al., 2012b). The present study includes the first detection of sunshinevirus in snakes outside Australia. Affected species have mostly included Australian pythons mainly *Antaresia sp*., *Morelia sp.* and *Aspidites sp.* (Hyndman et al., 2012a). In Germany in this study sunshinevirus was found in three ball pythons (*Python regius*). Clinically, sunshinevirus has generally been associated with neuro-respiratory disease, although other clinical signs have been reported as well including anorexia, stomatitis, weakness, regurgitation, skin problems like blisters and dermatitis have also been reported (Hyndman et al., 2014). Sunshinevirus was detected in four samples (lung lavage, oral swab, cloacal swab and in skin) from three ball pythons, each from a different private owner, which were presented for medical examination in southern Germany because they were suffering from dyspnea, weakness, stomatitis and pneumonia, two of which were still alive at the time of sampling (Anke Stöhr, personal communication). In a previous study, sunshinevirus was found most often in samples of brain, and

specific neuro-histopathological changes seem to serve as a reliable indicator of infection with sunshinevirus (Hyndman et al., 2014).

Despite studies and reports focusing on detection of sunshinevirus in snakes which have been published in recent years, there are still important gaps in the knowledge concerning these viruses and their pathogenesis. For example, the incubation periods, propagation of the virus within reptilian hosts and transmission in animal collections, all are poorly understood and as a consequence, it is difficult to advise on the prevention and control of sunshinevirus infections. Because snakes are popular pets and their trade has increased in the last few decades, owners need to be aware of infectious diseases in these animals and how to deal with them. Until the epidemiology of these viruses is better understood, it is necessary to observe and test newly acquired animals during a quarantine period to avoid introducing potential pathogens into snake collections.

In Conclusion:

Tortoises and snakes are popular pets and interest in infectious diseases of these animals has grown in recent years. Reliable, fast and inexpensive diagnostic methods are important for improving animal health in captivity. This study has led to the establishment of conventional PCRs to detect new viruses in these animals (picornaviruses in tortoises, reptarenaviruses in snakes and sunshineviruses in snakes). Detection of these viruses in live animals can be of interest both in diseased animals as well as in clinically healthy animals. These viruses appear to be relatively common among tortoises and snakes in captivity

in Europe. Animal screening for these viruses should be considered, particularly for those animals in quarantine situations which would allow control of these infections in zoos and private collections.

7. Summary

The purpose of this study was to establish conventional reverse-transcriptase PCRs for the detection of recently described RNA viruses in reptiles. The viruses studied included picornaviruses of tortoises (torchivirus or virus “X”), reptarenaviruses and sunviruses in snakes.

Picornaviruses are detected frequently in tortoises of various species in Europe. Until recently, tortoise picornaviruses (previously designated as virus “X”) could only be detected by isolation in Terrapene heart (TH1) cells in which they cause cell lysis, and nothing was known about the relationships of various isolates to one another. Clinical signs that have been described in picornavirus infected tortoises include softening of the shell in juvenile tortoises, rhinitis, conjunctivitis, kidney failure, and sudden death, but these viruses have also been detected in clinically healthy animals. This group of picornaviruses is able to infect a wide range of species in the family *Testudinidae* and has been detected in tortoises in many different European countries.

In this study, a conventional RT-PCR was developed and established for the detection and identification of picornaviruses in clinical samples. To test the reliability of this RT-PCR as a diagnostic method and the several primer sets which were designed for this purpose (Table 4.7), 37 picornaviruses isolated

from swabs and tissue samples collected in Germany and Italy between 1997 and 2012 were screened. The primer pair FKei2 (CTACCATCAGGATGCAGTT) - RKei2 (AAGCCAATCCTGCAACACT) gave the highest number of positive results from the chosen isolates (70%). 308 nucleotide long sequences of the amplified products of 26 picornavirus isolates were obtained which represent a small part of the viral polyprotein gene. Alignment of the obtained sequences from the amplified products revealed a close genetic relationship among the detected tortoise picornavirus isolates confirmed by the high identity values between 79.2 – 100% (Table 11.3 in appendix). Phylogenetic analysis clearly shows two main clusters, which together form a single monophyletic cluster. The obtained viral sequences of the polyprotein gene were compared with previously described picornaviruses and the highest similarities were observed with the corresponding gene sequences of picornavirus family members including: Canine picornavirus, Human enterovirus 109, Human rhinovirus C, and Human coxsackievirus A13. Based on sequence analysis, no association was observed between the geographic distribution and genetic relatedness. BLASTn analysis of the sequences confirmed that each of the PCRs with the different primer sets was specific. Furthermore, no strict host specificity was indicated. The PCR-based diagnosis may provide a time-saving and sensitive method to detect tortoise picornaviruses and to help prevent viral spread among animal collections.

A conventional RT-PCR was also established for the detection of arenaviruses in snakes and used to screen clinical samples from live and dead animals for these

viruses. The reptarenaviruses are considered to be the causative agent of inclusion body disease (IBD) which is a chronic progressive disease affecting captive boas and pythons worldwide. Samples from animals screened for virus detection were also screened for the presence of IBD typical inclusions in blood smears or histological preparations of 63 organs. The primer combinations MDS-400 (5'-TTCATTTCTTCATGRACTTTRTCAATC-3') and MDS-402 (5'-GGSATAACAAAYTCACTTCAAATATC-3') targeting part of the glycoprotein gene were used for the detection of reptarenavirus. 49 of 170 snakes tested (28.8%) were arenavirus positive. While 17 of 20 IBD positive snakes (85%) were arenavirus positive by RT-PCR, 17 of 43 IBD negative animals (39.5%) were arenavirus positive. Arenavirus was found in both boas (*Boa constrictor*) and pythons (*Python regius*, *Malayopython reticulatus*, *Python molurus*, and *Morelia viridis)*. Alignment of the obtained sequences of a small portion of the glycoprotein gene from the detected arenaviruses showed high identity values between 71- 100% with previously described reptarenaviruses. Phylogenetic analysis indicated that a majority of the detected reptarenavirus strains clearly clustered with GGV, Boa AV NL B3 and UHV. None of the detected viruses clustered with CASV.

Furthermore, this work includes a study concerning the first detection of sunshinevirus in snakes in Europe using RT-PCR as a diagnostic method. The first description of sunshinevirus was in 2008 after an outbreak of neuro-respiratory disease in an Australian collection of 70 pythons.

The RT-PCR used in this study resulted in the detection of sunviruses in three out of 12 snakes tested (25%). The obtained sequences were compared with the corresponding portion of the sunshinevirus genome and the identity values were between 95- 98 %. The viruses were found in oral/ cloacal swabs, lung lavage and skin sample of ball pythons, all other tested snakes were negative (two boas, one anaconda and one Indian python). Clinical signs reported in the animals infected with sunshinevirus vary significantly, but mostly respiratory problems were reported.

8. Zusammenfassung

Ziel der vorliegenden Arbeit war Etablierung von konventionellen Reverse-Transkriptase-PCRs für den Nachweis von den bisher bekanten RNA-Viren in Reptilien. Zu den untersuchten Viren gehörten Picornaviren in Schildkröten (*Torchiviren* oder virus "X"), Reptarenaviren und Sunviren in Schlangen.

Picornaviren wurden bisher bei verschiedenen Schildkrötenspezies nachgewiesen im Europa am häufigsten werden sie bei *Testudinidae* gefunden. Bis vor kurzem, Picornaviren aus verschiedenen Proben von Landschildkröten wurde nur auf die Zelllinie Terrapene Heart cells (TH1) nachweisen und isoliert, dabei einen lytischen zytopathischen Effekt verursacht und nichts war bekannt über die Beziehung zwischen Vieren Isolaten. Zu den klinischen Zeichen gehören Panzererweichung bei Jungtieren, Rhinitis, Konjunktivitis, Nierenausfall, sowie plötzlichen Todesfällen, allerdings Picornaviren können gelegentlich auch bei scheinbaren gesunden Schildkröten nachweisen werden.

In dieser Arbeit wurde eine konventionelle RT-PCR entwickelt und festgestellt für die Diagnose und Identifizierung von Piconaviren in klinischen Tierproben. 37 Picornaviren wurde isoliert vom Rachentupfer und von den verschiedenen Gewebeproben, diese wurden in Deutschland und Italien zwischen 1997 – 2012 gesammelt. Alle Isolate wurden abgeschirmt für den Zweck überprüfen die Zuverlässigkeit dieser RT-PCR als Diagnostische Methode und überprüfen die verschiedenen Primer-Sets, die zu diesem Zweck entworfen wurden (Table 4.7). Das Primer paar FKei2 (CTACCATCAGGATGCAGTT) - RKei2 (AAGCCAATCCTGCAACACT) hat die Mehrheit (70%) von der positiven Ergebnisse aus den ausgewählten Isolaten vorgelegt. Von 26 Picornaviren Isolaten Nukleotidsequenzen der amplifizierten Produkte wurde erhalten mit einer Länge von 308, und die einen kleinen Teil des viralen Polyprotein-Gens darstellen. Die Ausrichtung der erhaltenen Sequenzen aus den amplifizierten Produkten ergab eine enge genetische Beziehung zwischen den detektierten Schildkrötenpikornavirus-Isolaten, die durch die hohen Identitätswerte zwischen 79,2 und 100% (Table 11.3 in Appendix). Die phylogenetische Analyse zeigt deutlich zwei Hauptcluster, die zusammen einen einzigen monophyletischen Cluster bilden. Die erhaltenen viralen Sequenzen des Polyprotein-Gens wurden mit den zuvor beschriebenen Picornaviren verglichen und die höchsten Ähnlichkeiten wurden mit den entsprechenden Gensequenzen von Picornavirus-Mitgliedern beobachtet, einschließlich: Human cosavirus D. Theiler's-like virus, Saffold virus, Cardiovirus, Rhinitis B virus and Mouse Mosavirus. Nach der Sequenzanalyse, es wurde keine Verbindung zwischen der geographischen

Verteilung und der genetischen Verwandtschaft beobachtet. Die BLASTn-Analyse der Sequenzen bestätigte, dass jede der PCRs mit den verschiedenen Primersätzen spezifisch war. Weiterhin es wurde keine strenge Wirtstierspezifität angegeben. Die PCR-basierte Diagnose kann eine zeitsparende und empfindliche Methode zur Erkennung von Schildkröten-torchivirusinfectionen und zur Vermeidung von Virusausbreitung unter Tierkollektionen bieten.

Eine konventionelle RT-PCR wurde auch für den Nachweis von Arenaviren in Schlangen etabliert und verwendet, um klinische Proben von lebenden und toten Tieren für diese Viren zu untersuchen. Die Reptarenaviren gelten als Erreger der Inclusion Body Disease (IBD), die eine chronische bedeutende Erkrankung ist, die die Gefangenen Boas und die Pythons weltweit betrifft. Proben von Tieren, die auf Virenerkennung untersucht wurden, wurden ebenfalls auf die Anwesenheit von IBD-typischen Einschlüssen in Blutabstrichen oder histologischen Präparationen von 63 Organen untersucht. Die Primer-Kombination MDS-400 (5'-TTCATTTCTTCATGRACTTTRTCAATC-3 ') und MDS-402 (5'-GGSATAACAAAYTCACTTCAAATATC-3'), die einen Teil des Glykoprotein-Gens ausrichten, wurden für den Nachweis von Reptarenavirus verwendet. 49 von 170 getestet Schlangen (28,8%) waren Arenavirus positiv. Während 17 von 20 IBD-positiven Schlangen (85%) waren Arenavirus positiv, waren 17 von 43 IBD-negativen Tieren (39,5%) Arenavirus positiv durch RT-PCR arenavirus. Arenaviren wurde in beiden Boas und Pythons gefunden, besonders in *Python regius*, *Malayopython reticulatus*, *Python molurus* und *Morelia viridis*. Die Ausrichtung der erhaltenen Sequenzen eines kleinen Teils des Glykoprotein-

Gens aus den nachweisenden Arenaviren zeigte hohe Identitätswerte zwischen 71- 100% mit zuvor beschriebenen Reptarenaviren. Die phylogenetische Analyse zeigte, dass eine Mehrheit der detektierten Reptarenavirus-Stämme eindeutig mit GGV, Boa AV NL B3 und UHV gruppiert war. Keines der erkannten Viren mit CASV gruppiert.

Weiterhin, umfasst diese Arbeit eine Studie über die erste Erkennung von Sunsheinviren in Schlangen in Europa mit RT-PCR als diagnostische Methode. Die erste Beschreibung von Sunshinevirus war im Jahr 2008 nach einem Ausbruch der Neuro-Atemwegserkrankung in einer australischen Sammlung von 70 Pythons. Die in dieser Studie verwendete RT-PCR führte zum Nachweis von Suniviren in drei von 13 getesteten Schlangen (23,1%). Die erhaltenen Sequenzen wurden mit dem entsprechenden Teil des Sunsheinvirus-Genoms verglichen und die Identitätswerte waren zwischen 95- 98%. Die Viren wurden in oralen / kloakalen Tupfern, Lungenspülung und Hautprobe von Ball python gefunden, alle anderen getestete Schlangen waren negativ (zwei Boas, eine Anaconda und eine indische Python). Klinische symptome, die bei den mit Sunshinevirus infizierten Tieren gemeldet wurden, deutlich variieren, aber meistens wurden Atemwegserkrankungen gemeldet.

9. References:

- Adams, M.J., Lefkowitz, E.J., King, A.M.Q., Harrach, B., Harrison, R.L., Knowles, N.J., Kropinski, A.M., Krupovic, M., Kuhn, J.H., Mushegian, A.R., Nibert, M., Sabanadzovic, S., Sanfaçon, H., Siddell, S.G., Simmonds, P., Varsani, A., Zerbini, FM., Gorbalenya, A.E., Davison, A.J. 2017: Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2017). Archives of Virology, doi: 10.1007/s00705-017-3358-5.
- Afonso, C.L., Amarasinghe, G.K., Bányai, K., Bào, Y., Basler, Ch.F., Bavari, S., Bejerman, N., Blasdell, K.R., Briand, F. 2016: Taxonomy of the order *Mononegavirales*: update 2016. Archives of Virology, 161 (8): 2351 – 2360.
- Ariel, E. 2011: Viruses in reptiles. Veterinary Research, 42(1): 100.
- Arnold, E., Luo, M., Vriend, G., Rossmann, M.G., Palmenberg, A.C., Parks, G.D., Nicklin, M.J., Wimmer, E. 1987: Implications of the picornavirus capsid structure for polyprotein processing. Proceeding of the National Academy of Sciences of the United States of America, 84 (1): 21 – 25.
- Atmar, R.L., Piedra, P.A., Patel, S.M., Greenberg, S.B., Couch, R.B., Glezen, W.P. 2011: Picornavirus - the Most Common Respiratory Virus Infection among Patients of All Ages Hospitalized with Acute Respiratory Illness. Journal of Clinical Microbiology, 50(2): 506 – 508.

- Aqrawi, T., Stöhr, A.C., Knauf-Witzens, T., Krengel, A., Heckers, K.O., Marschang, R.E. 2015: Identification of snake arenaviruses in live boas and pythons in a zoo in Germany. Tieärztliche Praxis Ausgabe K Kleintiere Heimtiere, 43(4): 239 –47.
- Auliya, M., Altherr, S., Ariano-Sanchez, D., Baard, E.H., Brown, C., Brown R.M., Cantu, J.C., Gentile, G., Gildenhuys, P., Henningheim, E., Hintzmann, J., Kanari, K., Krvavac, M., Lettink, M., Lippert, J., Luiselli, L., Nilson, G., Nguyen, T.Q., Nijman, V., Parham, J.F., Pasachnik, S.A., Pedrono, M., Rauhaus, A., Córdova, D.R., Sanchez, M.E., Schepp, U., van Schingen, M., Schneeweiss, N., Segniagbeto, G.H., Somaweera, R., Sy Emerson, Y., Türkozan, O., Vinke, S., Vinke, T., Vyas, R., Williamson, S., Ziegler, T. 2016: Trade in live reptiles, its impact on wild populations, and the role of the European market. Biological Conservation, 204: 103 – 119.
- Auperin, D.D., Romanowski, V., Galinski, M., Bishop, D.H. 1984: Sequencing studies of pichinde arenavirus S RNA indicate a novel coding strategy, an ambisense viral S RNA. Journal of Virology, 52: 897 – 904.
- Bowen, M.D., Peters, C.J., Nichol, S.T. 1997: Phylogenetic analysis of the *Arenaviridae*: patterns of virus evolution and evidence for cospeciation between arenaviruses and their rodent hosts. Molecular Phylogenetics and Evolution, 8(3):301 – 316.
- Bodewes, R., Kik, M., Raj, V.S., Schapendonk, C., Haagmans, B., Smits, S.L., Osterhaus, A. 2013: Detection of novel divergent arenaviruses in

boid snakes with inclusion body disease in The Netherlands. Journal of General Virology, 94 (6):1206 – 1210.

- Bodewes, R., Raj, V.S., Kik M.J.L., Schapendonk, C.M., Haagmans, B.L., Smits, S.L., Osterhaus, A. D.M.E. 2014: Updated phylogenetic analysis of arenaviruses detected in boid snakes. Journal of Virology, 88 (2): 1399 – 1400.
- Bonfield, J.K., Smith, K.F., Staden, R. 1995: A new DNA sequence assembly program. Nucleic Acids Researches, 24: 4992 – 4999.
- Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-Van Dillen, P.M.E., Van Der Noordaa. J., 1990: Rapid and simple Method for Purification of Nucleic Acid, Journal of Clinical Microbiology, 28: 495-503.
- Buchmeier, M.J., Bowen, M.D., Peters, C.J. 2001: Arenaviridae: The viruses and their replication. In: Knipe, D.M., Howley, P.M. (eds) Fields virology, 4th edn., Lippincott Williams & Wilkins. pp. 1635 – 1668.
- Buchmeier, M.J., Peters, C.J., de la Torre, J.C. 2007: Arenaviridae: the virus and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M. (eds) Fields Virology. 5th (ed), Lippincott Williams & Wilkins; USA. pp. 1792 – 1827.
- Carlisle-Nowak, M.S., Sullivan, N., Carrigan, M., Knight, C., Ryan, C., Jacobson, E.R. 1998: Inclusion body disease in two captive Australian pythons (*Morelia spilota variegata* and *Morelia spilota spilota*). Australian Veterinary Journal, 76 (2): 98 – 100.

- Chang, L.W., Jacobson, E.R. 2010: Inclusion body disease, a worldwide infectious disease of boid snakes: a review. Journal of Exotic Pet Medicine, 19 (3): 216 – 225.
- Chang, L.W., Fu, A., Wozniak E., Chow M., Duke, D.G., Green L., Kelley, K., Hernandez, J.A., Jacobson, E.R. 2013: Immunohistochemical Detection of a Unique Protein within Cells of Snakes Having Inclusion Body Disease, a World-Wide Disease Seen in Members of the Families *Boidae* and *Pythonidae*. PLoS One, 8 (12): e82916.
- Charrel, R.N., de Lamballerie, X. 2003: Arenaviruses other than Lassa virus. Antiviral Research, 57(1-2): 89 – 100.
- Charrel, R.N., Coutard, B., Baronti, C., Canard, B., Nougairede, A., Frangeul, A., Morin, B., Jamal, S., Schmidt, C.L., Hilgenfeld, R., Klempa, B., de Lamballerie, X. 2011: Arenaviruses and hantaviruses: from epidemiology and genomics to antivirals. Antiviral Research, 90 (2): 102 – 114.
- Coulibaly-N'Golo, D., Allali, B., Kouassi, S.K., Fichet-Calvet, E., Becker-Ziaja, B., Rieger, T., Olschläger, S., Dosso, H., Denys, C., Ter, M.J, Akoua-Koffi, C., Günther, S. 2011: Novel arenavirus sequences in Hylomyscus sp. and Mus (Nannomys) setulosus from Côte d'Ivoire: implications for evolution of arenaviruses in Africa. PLoS One, 6(6):e20893.

- Essbauer, S., Ahne, W. 2001: Viruses of lower vertebrates. Journal of Veterinary Medicine Series B-infectious Diseases and Veterinary Public Health, 48 (6): 403 – 475.
- Farkas, S.L., Ihász, K., Fehér, E., Bartha, D., Jakab, F., Gál, J., Bányai, K., Marschang, R.E. 2015: Sequencing and phylogenetic analysis identifies candidate members of a new picornavirus genus in terrestrial tortoise species. Archives of Virology, 160 (3): 811–816.
- Felsenstein, J. 1989: PHYLIP- phylogeny inference package. Cladistics, 5: 164 – 166.
- Fields, B.N., Knipe, D.M., Howley, P.M. 2007: Fields' Virology. 5th edition. Wolters Kluwer Health /Lippincott Williams and Wilkins. USA.
- Fry, B. 2015: Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery. Oxford University Press, Science. pp. 148 – 149.
- Hall, T.A. 1999: BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series, 41: 95– 98.
- Hellebuyck, T., Pasmans, F., Ducatelle, R., Saey, V., Martel, A. 2015: Detection of arenavirus in a peripheral odontogenic fibromyxoma in a red tail boa (*Boa constrictor constrictor*) with inclusion body disease.Journal of Veterinary and Diagnostic Investigation, 27(2): 245 – 248.

- Hepojoki, J., Salmenperä, P., Sironen, T., Hetzel, U., Korzykov, Y., Kipar, A., Vapalahti, O. 2015: Arenavirus co-infections are common in snakes with boid inclusion body disease. Journal of Virology, 89 (16): 8657–8660.
- Hetzel, U., Sironen, T., Laurinmäki, P., Liljeroos, L., Patjas, A., Henttonen, H., Vaheri, A., Artelt, A., Kipar, A., Butcher, S.J. 2013: Isolation, identification, and characterization of novel arenaviruses, the etiological agents of boid inclusion body disease. Journal of Virology, 87 (20): 10918-10935.
- Heuser, W., Kaleta, E., Giesow, K., Keil, G.M., Knowles, N.J. 2010: Genome sequence of virus “X”, a picornavirus isolated from a spur-thighed tortoise (*Testudo graeca*). In Proceedings EUROPIC 2010: XVI Meeting of the European Study Group on the Molecular Biology of Picornaviruses, St. Andrews, UK, Abstract H15, 147.
- Heuser, W., Pendl, H., Knowles, N.J., Kei,l G., Herbst, W., Lierz, M., Kaleta, E.F. 2014: Soft plastron, soft carapace with skeletal abnormality in juvenile tortoises. Histopathology and isolation of a novel picornavirus from *Testudo graeca* and *Geochelone elegans*. Tierärztliche Praxis Ausgabe K Kleintiere Heimtiere, 42 (5): 310 – 320.
- Huelsenbeck, J.P., Ronquist, F. 2001: MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 17: 754 – 755.
- Hyndman, T.H., Marschang, R.E., Wellehan, J.F.X., Nicholls, P.K. 2012a: Isolation and molecular identification of Sunshine virus, a novel

paramyxovirus found in Australian snakes. Infection, Genetics and Evolution, 12 (7): 1436 – 1446.

- Hyndman, T.H., Shilton, C.M., Doneley R.J.T., Nicholls, P.K. 2012b: Sunshine virus in Australian pythons. Veterinary Microbiology, 161 (1-2): 77–87.
- Hyndman, T.H., Johnson, R., Shilton, C. 2014: An update on Australian reptile viruses. Proceedings of the Australian Veterinary Association (AVA) Annual Conferences, 2014 AVA Annual Conference, Perth, Unusual Pets and Avian / Conservation Biology. Australian Veterinary Association.
- Hyndman, T.H., Johnson, R.S. 2015: Evidence for the vertical transmission of Sunshine virus. Veterinary Microbiology, 175 (2-4):179 – 184
- International Committee on Taxonomy of Viruses. www.ictvonline.org
- Jacobson, E.R., Klingenberg, R.J., Homer, B.L., Mader, D.R. 1999: Roundtable: Inclusion body disease. Bulletin of the Association of Reptilian and Amphibian Veterinarians 9(2): 18–25.
- Jacobson, E.R., Orós, J., Tucker, S.J., Pollock, D.P., Kelley, K.L., Munn, R.J., Lock, B.A., Mergia, A., Yamamoto, J.K. 2001: Partial characterization of retroviruses from boid snakes with inclusion body disease. American Journal of Veterinary Research, 62(2): 217 – 224.
- Jacobson, R. 2007: Viruses and Viral Diseases of Reptiles. Infectious Diseases and Pathology of Reptiles. Taylor & Francis Group, LLC, Florida, pp. 395-460.

- Knowles, N.J. 2011: Tortiose picornavirus. http://www.picornaviridae.com/unassigned/reptile/tpv.htm
- Knowles, N.J., Hovi, T., Hyypia, T., King, A.M.Q., Lindberg, A.M., Pallansch, M.A., Palmenberg, A.C., Simmonds, P., Skern, T., Stanway, G., Yamashita, T., Zell, R. 2012: Family *Picornaviridae*. Virus taxonomy: classification and nomenclature of viruses. Ninth report of the International Committee on Taxonomy of Viruses. Elselvier, San Diego, pp. 855 – 880.
- Le Gall, O., Christian, P., Fauquet, C.M., King, A.M., Knowles, N.J., Nakashima, N., Stanway, G., Gorbalenya, A.E. 2008: *Picornavirales*, a proposed order of positive-sense single-stranded RNA viruses with a pseudo-T = 3 virion architecture. Archives of Virology, 153(4): 715 – 27.
- Liljas, L., Tate, J., Lin, T., Christian. P., Johnson, J.E. 2002: Evolutionary and taxonomic implications of conserved structural motifs between picornaviruses and insect picorna-like viruses. Archives of Virology, 147(1):59 – 84.
- Marschang, R.E., Becher, P., Posthaus, H., Wild, P., Thiel, H.J., Müller-Doblies, U., Kalet, E.F., Bacciarini, L.N. 1999: Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudohermanni*). Archives of Virology, 144(10):1909 –1922.
- Marschang, R.E., Ruemenapf, T.H. 2002: Virus ‘‘X’’: Characterizing a new viral pathogen in tortoises. In Proceedings Association of Reptilian and Amphibian Veterinarians ARAV, Reno, NV, 101 – 102.

- Marschang, R.E., 2011: Viruses Infecting Reptiles. Viruses, 3(11): 2087 – 2126.
- Marschang, R.E., Stöhr, A.C., Aqrawi, T., Hyndman, T.H., Plenz, B., Blahak, S., Pees, M. 2013: First detection of Sunshine virus in pythons (*Python regius*) in Europe. In Proceedings of the Association of Reptilian and Amphibian Veterinarians Conference. pp. 15. Association of Reptilian and Amphibian Veterinarians ARAV.
- Marschang, R.E., Divers, S.J. 2014: Appendix 2: Reptile Viruses. In: Maders, D.R., Divers, S.J. (eds.), Current Therapy in Reptile Medicine and Surgery. Saunders, St. Louis, MO, pp.368 – 381.
- Marschang, R.E., Kolesnik, E. 2016: Detection of nidoviruses in live pythons and boas. Tierärztliche Praxis Ausgabe K Kleintiere Heimtiere, 45(1): 22 – 26.
- Marschang, R.E., Ihász, K., Kugler, R., Lengyel, G., Fehér, E., Marton, S., Bányai, K., Aqrawi, T., Farkas, S.L. 2016: Development of a consensus reverse transcription PCR assay for the specific detection of tortoise picornaviruses. Journal of Veterinary and DiagnosticInvestigation, 28(3): 309 –314.
- Ng, T.F.F., Wellehan, J.F.X., Coleman, J.K., Kondov, N.O., Deng, X., Waltzek, T.B., Reuter, G.,Knowles, N.J., Delwart, E.2015: A tortoise-infecting picornavirus expands the host range of the family *Picornaviridae.* Archives of Virology, 160(5):1319– 1323.

- Oros, J., Tucker, S., Jacobson, E.R. 1998: Inclusion body disease in two captive boas in the Canary Islands. Veterinary Record, 143(10): 283 – 285.
- Palacios, G., Savji, N., Hui, J., Travassos da Rosa, A., Popov, V., Briese, T., Tesh, R., Lipkin, W.I. 2010:Genomic and phylogenetic characterization of Merino Walk virus, a novel arenavirus isolated in South Africa. Journal of General Virology, 91(5):1315 – 1324.
- Pinschewer, D.D., Perez, M., de la Torre, J.C. 2003: Role of the virus nucleoprotein in the regulation of lymphocytic choriomeningitis virus transcription and RNA replication. Journal of Virology, 77(6): 3882–3887.
- Qi, X., Lan, S., Wang, W., Schelde, L.M., Dong, H., Wallat, G.D., Ly, H., Liang, Y., Dong, C. 2010: Cap binding and immune evasion revealed by Lassa nucleoprotein structure. Nature 468 (7325): 779 – 783.
- Racaniello, V.R. 2007: Picornaviridae: The viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M. (eds), Fields Virology. 5th ed Lippincott Williams & Wilkins, USA. pp. 795 – 838.
- Radoshitzky, S.R., Bào, Y., Buchmeier, M.J., Charrel, R.N., Clawson, A.N., Clegg, C.S., DeRisi, J.L., Emonet, S., Gonzalez, J.P., Kuhn, J.H., Lukashevich, I.S., Peters, C.J., Romanowski, V., Salvato, M.S., Stenglein, M.D., de la Torre, J.C. 2015: Past, present, and future of arenavirus taxonomy. Archives of Virology, 160(7):1851 – 1874.
- Raymond, J. T., Garner, M. M., Nordhausen, R. W., Jacobson, E. R. 2001: A disease resembling inclusion body disease of boid snakes in captive

palm vipers (*Bothriechis marchi*). Journal of Veterinary Diagnostic Investigation, 13 (1):82 – 86.

- Salazar-Bravo, J., Ruedas, L. A., Yates, T. L. 2002:: Chapter Arenaviruses In: Mammalian Reservoirs of Arenaviruses. pp. 25-63.
- Schmidt, V., Marschang, R.E., Abbas, M.D., Ball, I., Szabo, I., Helmuth, R., Plenz, B., Spergser, J., Pees, M. 2013: Detection of pathogens in Boidae and Pythonidae with and without respiratory disease. Veterinary Record, 172(9): 236.
- Schumacher, J., Jacobson, E.R., Homer, B.L., Gaskin, J.M. 1994: Inclusion body disease of boid snakes. Journal of Zoo and Wildlife Medicine, 25(4): 511– 524.
- Smyth, M., Tate, J., Hoey, E., Lyons, C., Martin, S., Stuart, D. 1995: Implications for viral uncoating from the structure of bovine enterovirus. Nature Structural Biology, 2 (3): 224–231.
- Smyth, M.S., Martin, J.H. 2002: Picornavirus uncoating. Molecular Patholology, 55(4): 214 – 219.
- Spector, D.H., Baltimore, D. 1974: Requirement of 3'-terminal poly(adenylic acid) for the infectivity of poliovirus RNA. Proceeding of the Natlional Academy of Sciences of the United States of America, 71(8): 2983–2987
- Stenglein, M.D., Sanders, C., Kistler, A.L., Ruby, J.G., Franco, J.Y., Reavill, D.R., Dunker, F. DeRisi, J.L. 2012: Identification, characterization, and in vitro culture of highly divergent arenaviruses from boa constrictors

and annulated tree boas: candidate etiological agents for snake inclusion body disease. Molecular Biology, 3: e00180 .

- Stenglein, M.D., Jacobson, E.R., Wozniak, E.J., Wellehan, J.F., Kincaid, A., Gordon, M., Porter, B.F., Baumgartner, W., Stahl, S., Kelley, K., Towner, J.S., DeRisi, J.L. 2014: Ball python nidovirus: a candidate etiologic agent for severe respiratory disease in *Python regius*. Molecular Biology, 5(5): e01484-14.
- Stenglein, M.D., Jacobson, E.R., Chang, L.W., Sanders, C., Hawkins, M.G., Guzman, D.S., Drazenovich, T., Dunker, F., Kamaka, E.K., Fisher, D., Reavill, D.R., Meola, L.F., Levens, G., DeRisi, J.L. 2015: Widespread recombination, reassortment, and transmission of unbalanced compound viral genotypes in natural arenavirus infections. PLoS Pathologens,11(5): e1004900.
- Uccellini, L., Ossiboff, R.J., de Matos, R.E., Morrisey, J.K., Petrosov, A., Navarrete-Macias, I., Jain, K., Hicks, A.L., Buckles, E.L., Tokarz, R., McAloose, D., Lipkin, W.I. 2014: Identification of a novel nidovirus in an outbreak of fatal respiratory disease in ball pythons (*Python regius*).Virology Journal, 8(11): 144.
- Vancraeynest, D., Pasmans, F., Martel, A., Chiers, K., Meulemans, G., Mast, J., Zwart, P., Ducatelle, R. 2006: Inclusion body disease in snakes: a review and description of three cases in boa constrictors in Belgium. The Veterinary Record, 158 (22): 757 – 761.

- Wozniak, E., McBride, J., DeNardo, D., Tarara, R., Wong, V., Osburn, B. 2000: Isolation and characterization of an antigenically distinct 68-kd protein from nonviral intracytoplasmic inclusions in Boa constrictors chronically infected with the inclusion body disease virus (IBDV: Retroviridae). Veterinary Pathology, 37 (5): 449 – 459.
- Zapata, J.C., Salvato, M.S. 2013: Review. Arenavirus variations due to host-specific adaptation. Viruses, 5 (1): 241 – 278.
- Zell, R., Delwart, E., Gorbalenya, A.E., Hovi, T., King, A.M.Q., Knowles, N.J., Lindberg, A.M., Pallansch, M.A., Palmenberg, A.C., Reuter, G., Simmonds, P., Skern, T., Stanway, G., Yamashita, T. and ICTV Report Consortium. 2017.ICTV Virus Taxonomy Profile: *Picornaviridae*, Journal of General Virology, 98: 2421-2422.

10. Acknowledgements

I would like to thank my supervisor, PD Dr. Rachel Marschang, warmly and most sincere and I am really very grateful for the patient guidance, encouragement and advice she has provided throughout my time as her student. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. I would also like to thank all the members of staff at the virology lab/ department of Livestock Infectiology and Environmental Hygiene (460e) (previously known as institute of environmental and animal hygiene) - Hohenheim University/ Stuttgart, who supported me in my research work. In particular I would like to thank my friend Dr. Maha Abbas for her kind cooperation especially for solving complex problems in respect to analyses of sequences and for the suggestions she made in this work. I warmly thank my friends (Inna Ball and Anke Stöhr) for their friendly help. I owe my sincere gratitude to Silvia Speck and Christa Schäfer for supporting my work.

I would like to express my gratitude to Sattar, my beloved husband, for his continued support and encouragement. I was continually amazed by his patience and his willingness to finish this work and to be proud of me. My special gratitude is to my mother, father and sisters who experienced all of the ups and downs of my study. Completing this work would have been all the more difficult were it not for the support and friendship provided by my supervisor. I am indebted to them for their help.

11. Appendix

Table 11.1 Data collected for tortoise picornaviruses (virus "X"), RT-PCR results from RNA isolates and from diagnostic samples. All PCR positive products were sequenced.

n.d: not done n.a: not available

	Lab No. Isolates	**Host species**	**Samples**	**PCR with primer sets**					**Origin**	**Source/Case history**
				Oligo dT18 Kei+4343	Rvx_1 Fvx_1	Rvx_2 Fvx_2	$Rtgt_1$ $Ftgt_1$	$Rkei_2$ $Fkei_2$		
1.	TGT1a/96	Spur-thighed tortoise *Testudo graeca*	n.a	neg.	neg.	**pos.**	**pos.**	**pos.**	n.a	Isolate from Dr. Marschang
2.	374/96	n.a	? (TH1 P6)	n.d.	neg.	n.d.	neg.	neg.	n.a	Isolate from Dr. Marschang
3.	33/97	Spur-thighed tortoise *Testudo graeca*	oesophagus	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
4.	2442/97 TGT8	Spur-thighed tortoise *Testudo graeca*	liver	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
5.	907/97 TGT10	Spur-thighed tortoise *Testudo graeca*	heart	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
6.	2443/97	Spur-thighed tortoise *Testudo.graeca*	intestine	n.d.	neg.	n.d.	neg.	neg.	Switzerland	Isolate from Dr. Marschang
7.	2445/97	Egyptian tortoise	trachea	n.d.	neg.	n.d.	neg.	neg.	Switzerland	Isolate from Dr.

		Testudo kleinmanni								Marschang
8.	1791/98	Spur-thighed tortoise *Testudo graeca*	brain	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
9.	2281/7/98	*Testudo spp.*	throat swab	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
10.	2691/98	Leopard tortoise *Stigmochelys pardalis*	tongue	n.d.	neg.	n.d.	neg.	neg.	Germany	Isolate from Dr. Marschang
11.	1167/5/99	Tortoise	tongue	n.d.	neg.	n.d.	neg.	neg.	n.a	Isolate from Dr. Marschang
12.	1167/9/99	Tortoise	colon	n.d.	neg.	n.d.	neg.	neg.	n.a	Isolate from Dr. Marschang
13.	659/00	Hermann's tortoise *Testudo hermanni*	tongue	n.d.	neg.	n.d.	neg.	**pos.**	n.a	Isolate from Dr. Marschang Herpesvirus PCR postive
14.	2041/00	Russian tortoise *Testudo horsfieldi*	n.a	n.d.	neg.	n.d.	neg.	**pos.**	n.a	Isolate from Dr. Marschang Herpesvirus found in same group of tortoises
15.	5_03	Spider tortoise *Pyxis arachnoids*	lung	neg.	neg.	neg.	**pos.**	**pos.**	n.a	Isolate from Dr. Marschang
16.	16_03	*Testudo spp.*	kidney	neg.	neg.	neg.	neg.	**pos.**	Italy	Isolate from Dr. Marschang
17.	26_03	Marginated tortoise *Testudo marginata*	pharyngeal swabs	neg.	neg.	**pos.**	neg.	**pos.**	Italy	Isolate from Dr. Marschang

										Death in collection following introduction of Indian star tortoises
18.	31_03	Hermann's tortoise *Testudo hermanni*	tongue	neg.	**pos.**	**pos.**	neg.	**pos.**	Germany	Isolate from Dr. Marschang study
19.	2_4_04	Tortoise	-	neg.	neg.	neg.	neg.	**pos.**	Germany	Isolate from Dr. Marschang Sudden death, fatty liver, diffuse mixed vacuolar hepatocellular fatty degeneration *Mycoplasma agassizii* PCR positive
20.	5_04	Spur-thighed tortoise *Testudo graeca*	-	neg.	**pos.**	neg.	neg.	**pos.**	Germany	Isolate from Dr. Marschang
21.	14_04	Hermann's tortoise *Testudo hermanni*	cloacal swab	neg.	neg.	**pos.**	neg.	**pos.**	Germany	Isolate from Dr. Marschang Animals were clinically healthy, , but animals sold from this collection died in their new home Herpesvirus PCR negative
22.	15_04	Spur-thighed tortoise	tongue	neg.	neg.	neg.	neg.	**pos.**	Germany	Isolate from Dr.

		Testudo graeca								Marschang study Sudden death, shell softening, ascites, lung tissue thickened Herpesvirus PCR negative
23.	16_04	Hermann's tortoise *Testudo hermanni boettgeri*	liver, small intestines, heart, lung	neg.	neg.	neg.	**pos.**	**pos.**	Germany	Isolate from Dr. Marschang Shell softening, liver light and friable
24.	17_04	Hermann's tortoise *Testudo hermanni boettgeri*	heart	neg.	neg.	neg.	neg.	**pos.**	Germany	Isolate from Dr. Marschang Shell softening, Herpesvirus PCR negative
25.	19_1_04	Hermann's tortoise *Testudo hermanni*	-	neg.	neg.	neg.	neg.	**pos.**	England	Isolate from Dr. Marschang Virus isolated from heart, liver and gonad.
26.	19_2_04	Hermann's tortoise *Testudo hermanni*	-	neg.	neg.	neg.	neg.	**pos.**	England	Isolate from Dr. Marschang study Virus isolated from stomach , backbone, head, lung

										and liver. both suffered from necrotising stomatitis, Multifocal-bronchitis /pneumonia
27.	9_05	African spurred tortoise *Centrochelys sulcata*	oral swab	neg.	**pos.**	neg.	neg.	**pos.**	Italy	Isolate from Dr. Marschang Stomatitis
28.	25_09	Marginated tortoise *Testudo marginata*	-	neg.	**pos.**	neg.	neg.	**pos.**	Germany	Isolate from Dr. Marschang Clinically healthy
29.	19_10	Russian tortoise *Testudo horsfieldii*	tongue swab	neg.	**pos.**	neg.	**pos.**	**pos.**	Germany	Isolate from Dr. Marschang Red soft-shell, yellow plaque coating the mouth cavity
30.	124_1_10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal swabs	neg.	neg.	**pos.**	neg.	**pos.**	Germany	Isolate from Dr. Marschang All were clinically healthy
31.	124_2_10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal swabs	neg.	neg.	**pos.**	neg.	**pos.**		
32.	124_4-10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal	neg.	neg.	**pos.**	neg.	**pos.**		

			swabs							
33.	124_5_10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal swabs	neg.	neg.	**pos.**	neg.	**pos.**		
34.	124_6_10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal swabs	neg.	neg.	**pos.**	neg.	**pos.**		
35.	124_7_10	Spur-thighed tortoise *Testudo graeca*	oral& cloacal swabs	neg.	neg.	**pos.**	neg.	**pos.**		
36.	36_10	Marginated tortoise *Testudo marginata*	oral& cloacal swabs	**pos.**	**pos.**	**pos.**	neg.	**pos.**	Germany	Isolate from Dr. Marschang Obstipation, gastirits
37.	144_10	Spur-thighed tortoise *Testudo graeca*	oral swab	**pos.**	**pos.**	**pos.**	neg.	**pos.**	Italy	Isolate from Dr. Marschang Suffered closed eyes for 25 days. Blepharoconjunctivitis and glossitis
38.	130_12	Leopard tortoise *Stigmochelys pardalis*	head	n.d.	n.d.	n.d.	neg.	**pos.**	Italy	From diagnostic samples 2012 Herpesvirus PCR negative

39.	36_13	Tortoise	liver kidney heart lung	n.d.	n.d.	n.d.	neg.	**pos.** **pos.** **pos.** **pos.**	Germany	From diagnostic samples 2013
40.	62_13	Tortoise	oral swab	n.d.	n.d.	n.d.	neg.	**pos.**	n.a	From diagnostic samples 2013

Table 11.2 Data from all tortoise samples received in 2012-2013 and screened for the detection of picornavirus virus “X” using RT-PCR with the primer set Kei2 with negative results

	Lab.no.	Species	Sample	Source
1.	36_12	Spur-thighed tortoise *Testudo graeca*	n.a	Uni Leipzig Germany
2.	37_2_12	Russian tortoise *Testudo horsfieldii*	n.a	-
3.	37_3_12	Tortoise	n.a	-
4.	37_4_12	Tortoise	n.a	-
5.	40_12	Hermann’s tortoise *Testudo hermanni*	n.a	-
6.	42_12	Russian tortoise *Testudo horsfieldii*	n.a	-
7.	43_1_12	Hermann’s tortoise *Testudo hermanni*	n.a	-
8.	43_2_12	Tortoise	n.a	-
9.	44_12	Leopard tortoise *Stigmochelys pardalis*	n.a	-
10.	46_12	Spur-thighed tortoise *Testudo graeca*	n.a	Italy
11.	49_1_12	Russian tortoise	n.a	-

		Testudo horsfieldii		
12.	49_2_12	Tortoise	n.a	-
13.	53_1_12	Indian star tortoise *Geochelone elegans*	n.a	-
14.	53_2_12	Indian star tortoise *Geochelone elegans*	n.a	-
15.	53_3_12	Leopard tortoise *Stigmochelys pardalis*	n.a	-
16.	53_4_12	Spur-thighed tortoise *Testudo graeca*	n.a	-
17.	56_12	Hermann's tortoise *Testudo hermanni*	n.a	Italy
18.	58_1_12	Elongated Tortoise *Indotestudo elongata*	n.a	-
19.	58_2_12	Tortoise	n.a	-
20.	69_12	Russian tortoise *Testudo horsfieldii*	n.a	-
21.	72_12	Hermann's tortoise *Testudo hermanni*	n.a	-
22.	76_12	Hermann's tortoise *Testudo hermanni*	n.a	Tübingen Germany
23.	82-12	Russian tortoise	n.a	Achen

		Testudo horsfieldii		Germany
24.	84_1_12	Aquatic turtule	tracheal swab	-
25.	84_2_12		carapacial swab	-
26.	84_3_12		cloacal swab	-
27.	84_4_12	Aquatic turtule	trachea swab	-
28.	84_5_12		carapacial swab	-
29.	84_6_12		cloacal swab	-
30.	84_7_12	Aquatic turtule	trachea swab	-
31.	84_8_12		carapacial swab	-
32.	84_9_12		cloacal swab	-
33.	85_1_12	Hermann's tortoise	n.a	-
34.	85_2_12	*Testudo hermanni*	n.a	-
35.	86_1_12	Tortoise	colon lung small intestine liver spleen kidney skin	-
36.	86_2_12	Tortoise	colon lung small intestine	-

			liver kidney	
37.	90_12	Hermann's tortoise *Testudo hermanni*	n.a	-
38.	93_3_12	Tortoise *Astrochelys yniphora*	n.a	-
39.	99_12	Russian tortoise *Testudo horsfieldii*	n.a	Uni Leipzig Germany
40.	104_12	Marginated tortoise *Testudo marginata*	n.a	-
41.	10_13	Red-footed tortoises *Chelonoidis carbonaria*	swab	Wilhelma Germany
42.	47_1_13	Hermann's tortoise *Testudo hermanni*	pharyngeal swab	-
43.	47_2_13		cloacal swab	-
44.	42_13		spleen	Uni Leipzig Germany
45.	64_2_13	Tortoise	liver	Germany
46.	64_3_13		brain	Germany
47.	64_4_13		lung	Germany
48.	64_5_13		kidney	Germany
49.	64_6_13		gut	Germany
50.	65_1_13	Hermann's tortoise	intestine	Germany

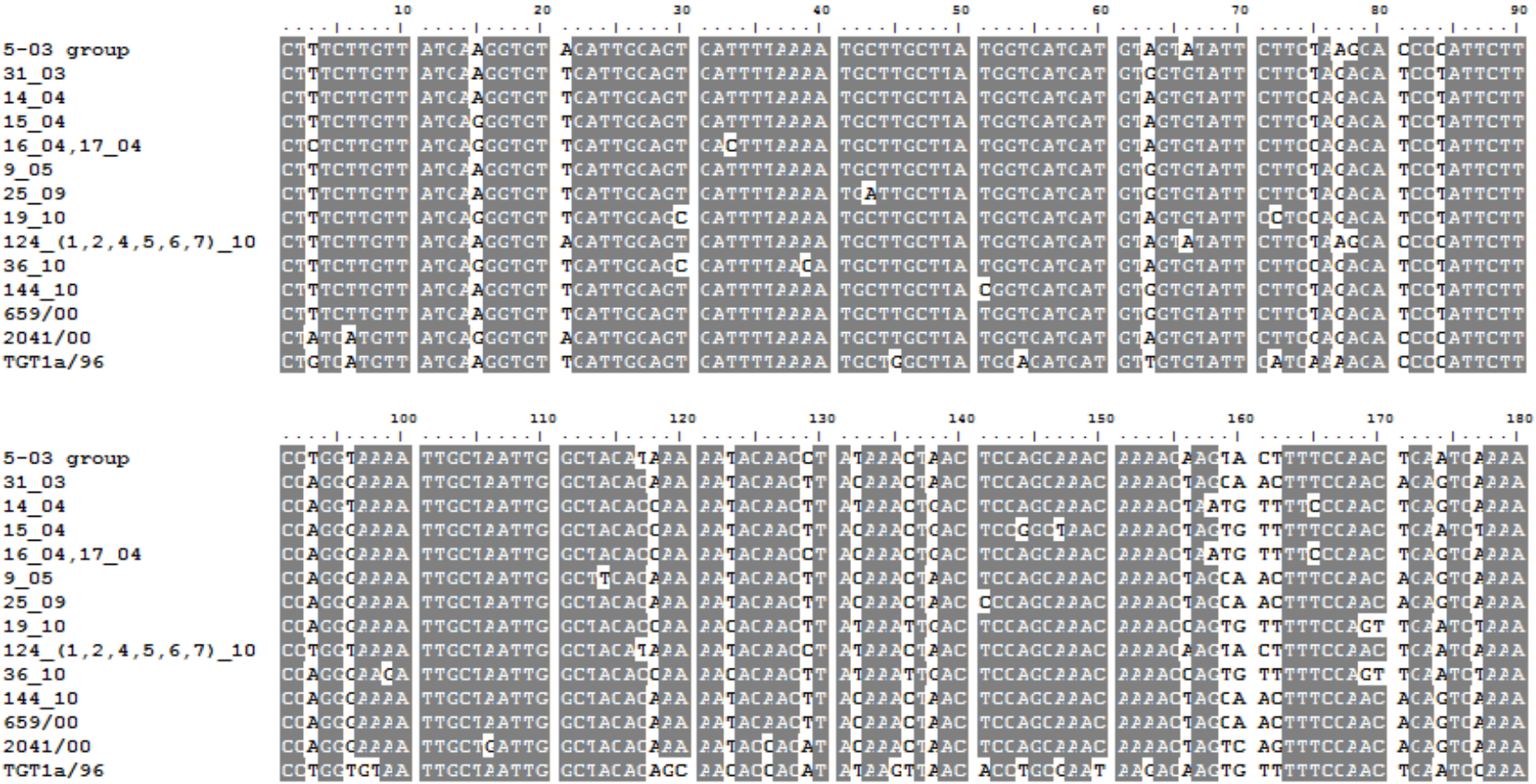
51.	65_2_13	*Testudo hermanni*	kidney	Germany
52.	65_3_13		spleen	Germany
53.	65_4_13		tongue	Germany
54.	65_5_13		lung	Germany
55.	65_6_13+7		stomach+liver	Germany
56.	69_13	Russian tortoise *Testudo horsfieldii*	pharyngeal swab	Flensburg Germany
57.	75_1_13	Hermann's tortoise *Testudo hermanni*	swab	Frankfurt Germany
58.	87_1_13	Tortoises	oral swab	-
59.	87_2_13	Tortoise	swab	-
60.	87_3_13	Tortoise	conjunctiva swab	-
61.	87_4_13	Tortoise	oral swab	-
62.	87_5_13	Tortoise	oral swab	-
63.	87_6_13	Tortoise	cloacal swab	-
64.	87_7_13	Tortoise	cloacal swab	-
65.	87_8_13	Tortoise	conjunctival swab	-
66.	87_9_13	Tortoise	swab from conj. lid	-
67.	89_13	Hermann's tortoise *Testudo hermanni*	swabs from pharynx & eye	Uni Leipzig Germany

68.	90_1_13	Indian star tortoise *Geochelone elegans*	nasal swab	Holzhausen Germany
69.	90_2_13	Indian star tortoise *Geochelone elegans*	nasal swab	Holzhausen Germany
70.	91_13	Hermann's tortoise *Testudo hermanni*	swab	-
71.	97_13	Galapagos giant tortoise *Geochelone nigra*	lung–tongue in paraffin slice	Uni Zürich Switzerland

Table 11.3 Identity values (%) of the detected picornaviruses with four members of the family *Picornaviridae*. 5_03 group includes isolates 5-03, 16-03, 26-03, 2_4_04, 5_04, 19_1_04, 19_2_04, and samples 130_12, 36_13, 62_13. The upper diagonal refers to the values for 308 nucleotides. The lower diagonal shows values for 106 deduced amino acids of the detected viruses.

	5-03, 16-03, 26-03, 2_4_04, 5_04, 19_1_04, 19_2_04,130_12, 36_13, 62_13	31_03	14_04	15_04	16_04 17_04	9_05	25_09	19_10	124_(1,2,4,5,6,7) _10	36_10	144_10	659/00	2041/00	TGT1a/96	HEV	HCV-A13	CanPV	HRV-C
5-03, 16-03, 26-03, 2_4_04, 5_04, 19_1_04, 19_2_04,130_12, 36_13, 62_13	ID	86.3	87.3	87.6	86	86	85.7	86.6	100	86.3	86.3	86.6	87.6	85	40.7	45.9	49.3	38.5
31_03	77.8	ID	91.8	91.2	90.2	99	98.7	89.6	86.3	89.2	99.3	99.6	88.6	79.2	42.2	43.8	49.3	40.7
14_04	78.4	83.1	ID	92.8	96.4	91.5	91.2	93.1	87.3	92.8	91.8	92.2	87	80.8	41.6	43.2	49.3	40.1
15_04	78.6	82.1	83.8	ID	93.1	90.9	90.5	93.8	87.6	93.5	91.2	91.5	87.9	81.4	42.9	44.4	48.4	39.1
16_04 17_04	78.6	82.3	95	86	ID	90.2	89.6	92.2	86	91.8	90.2	90.5	87.9	79.5	40.7	42.5	49.3	40.1
9_05	75.9	98	81.1	81.1	81.3	ID	98.3	89.2	86	88.9	99	99.3	88.3	78.8	42.2	43.8	49	41
25_09	75	98.7	80.1	79.2	79.4	95	ID	88.9	85.7	88.6	98.7	99	88.6	79.5	42.9	44.7	50	40.7
19_10	79.4	79.4	84.8	86.8	83.1	78.4	76.4	ID	86.6	99	89.6	89.9	86	81.8	42.5	43.5	49.3	40.1
124_(1,2,4,5,6,7) _10	100	77.8	78.4	78.6	78.6	75.9	75	79.4	ID	86.3	86.3	86.6	87.6	85	40.7	45.9	49.3	38.5
36_10	78.4	78.4	83.8	85.8	82.1	77.4	75.4	98.4	78.4	ID	89.2	89.6	85.7	81.1	42.2	43.2	50	39.5
144_10	77.8	100	83.1	82.1	82.3	98	97	79.4	77.8	78.4	ID	99.6	88.6	79.2	41.9	43.5	49	40.1
659/00	77.8	100	83.1	82.1	82.3	98	97	79.4	77.8	78.4	100	ID	88.9	79.5	42.2	43.8	49.3	40.4
2041/00	74.7	79.4	74.5	73.5	76.4	78.4	78.4	70.8	74.7	69.9	79.4	79.4	ID	83.1	41	45.6	49.3	38.5
TGT1a/96	71.5	62.8	62.1	65.3	62.5	61.9	62.8	66.9	71.5	66	62.8	62.8	67.3	ID	39.5	42.9	50.9	38.8
HEV	16	19.6	16.9	16	16.9	19.6	20.5	16.9	16	16.9	19.6	19.6	16.8	16.8	ID	73.2	58.5	57.3
HCV-A13	15.8	16.6	14.9	15.8	14.9	17.5	18.5	14.9	15.8	14	16.6	16.6	18.5	15.8	54.8	ID	60.8	64
CanPV	18.8	20.7	21.6	19.8	21.6	20.7	19.8	22.4	18.8	21.4	20.7	20.7	19.8	19.4	32	35.8	ID	58.5
HRV-C	16.8	16.9	17.9	15	17.9	17.9	17.9	15.8	16.8	14.9	16.9	16.9	17.1	17.5	36.1	40.3	40.3	ID

Figure 11.1 Nucleotide alignments (308 nt) of partial polyprotien gene of the detected picornaviruses in tortoises. The 5_03 group includes isolates 5-03, 16-03, 26-03, 2_4_04, 5_04, 19_1_04, 19_2_04, and samples 130_12, 36_13, 62_13.



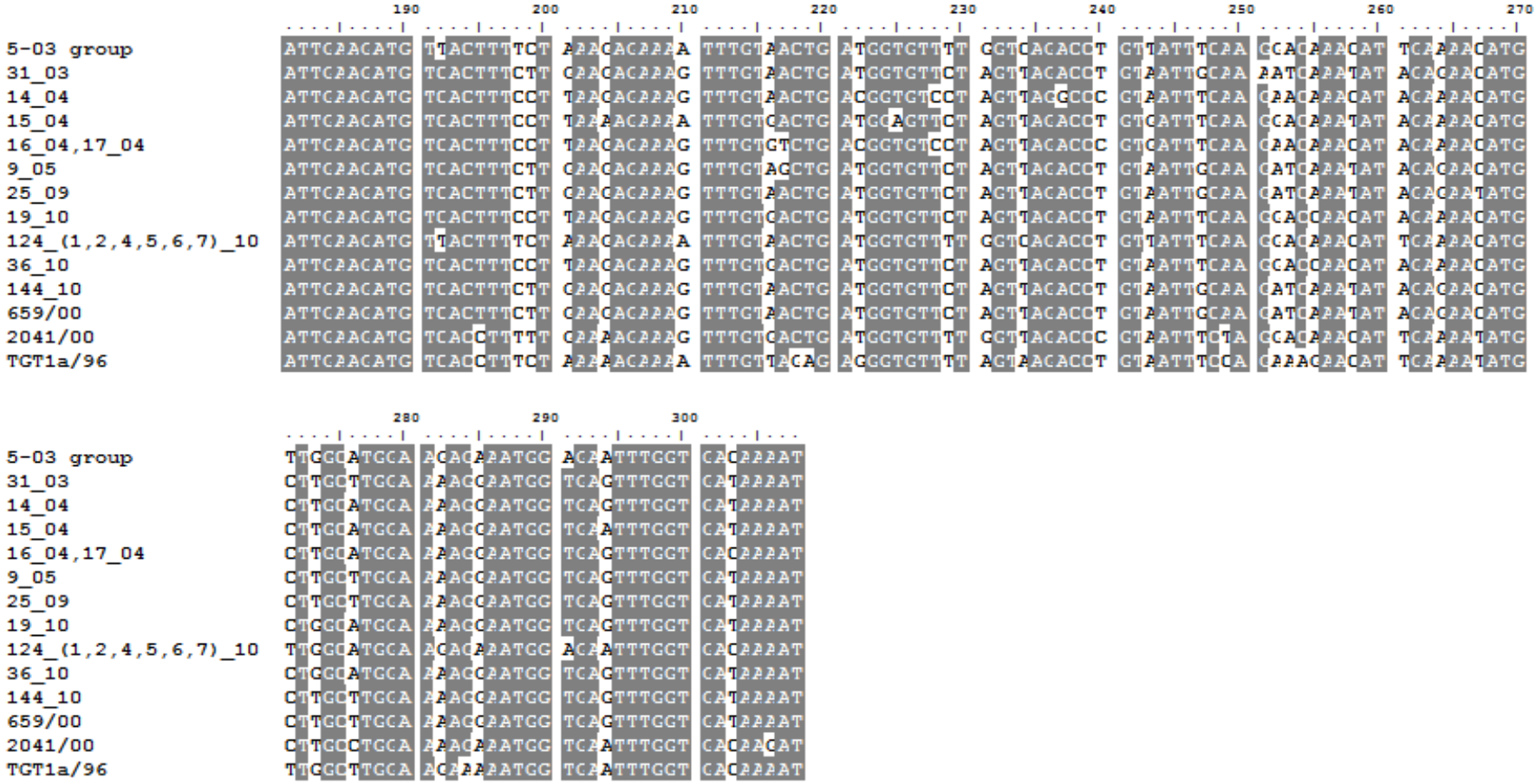

190 200 210 220 230 240 250 260 270
5-03 group
31_03
14_04
15_04
16_04,17_04
9_05
25_09
19_10
124_(1,2,4,5,6,7)_10
36_10
144_10
659/00
2041/00
TGT1a/96
280 290 300
5-03 group
31_03
14_04
15_04
16_04,17_04
9_05
25_09
19_10
124_(1,2,4,5,6,7)_10
36_10
144_10
659/00
2041/00
TGT1a/96

Table 11.4 Arenavirus positive results from diagnostic samples during the study period 2012-2013.
All PCR positives were sequenced. n.a= not available

	Species	**Samples**	**IBD status**	**RT-PCR**	**clinical signs remarks**	**Origin**	**Lab no.**
1.	Reticulated python *Malayopython reticulatus*	kidney brain pancreas intestine lung	n.a.	neg. brain: pos. neg. neg. neg.	-	Leipzig	15_12
2.	Reticulated python *Malayopython reticulatus*	brain	n.a.	pos.	Male	U.K	23_1_12
3.	Ball pythons *Python regius*	brain kidney lung liver feces	IBD: neg.	brain:pos. neg. neg. neg. neg.	Encephalitis	Germany	47_12
4.	Indian python *Python molurus*	kidney liver lung spleen	n.a.	neg. neg. neg. neg.	-	Leipzig	79_12

		brain		brain:pos.			
5.	Boa *Boa constrictor*	lung kidney liver intestine	n.a.	pos. pos. pos. pos.	-	Leipzig	128_12
6.	Boa *Boa constrictor*	blood	IBD neg.	pos.	-	Dr.Michling Germany	134_12: 195A
7.	Boa *Boa constrictor*	blood	IBD neg.	pos.	-	Dr.Michling Germany	134_12: 196A
8.	Boa *Boa constrictor*	blood	IBD neg.	pos.	-	Dr.Michling Germany	134_12: 198A
9.	Boa *Boa constrictor*	blood	IBD neg.	pos.	-	Dr.Michling Germany	134_12: 199A
10.	Boa *Boa constrictor*	blood	IBD ?	pos.	-	Dr.Michling Germany	134_12: 203A
11.	Boa *Boa constrictor*	blood	IBD neg.	pos.	-	Dr.Michling Germany	134_12: 206A
12.	Boa , Albino *Boa constrictor*	pharyngeal swab cloacal swab	n.a.	pos. neg.	-	Germany	1_13
13.	Boa *Boa constrictor*	pharyngeal swab	n.a.	pos.	pneumonia	Germany	3_13
14.	Boa	pharyngeal swab	IBD: neg.	pos.	-	Wilhelma	11_1_13

	Boa constrictor	blood		neg.		Germany	
15.	Boa *Boa constrictor*	pharyngeal swab blood	seven boas were housed together. Six of them were reported IBD pos.	pos. pos.	-	Wilhelma Germany	11_11_13
16.	Boa *Boa constrictor*	pharyngeal swab blood		pos. neg.	-	Wilhelma Germany	11_12_13
17.	Boa *Boa constrictor*	pharyngeal swab blood		pos. pos.	-	Wilhelma Germany	11_13_13
18.	Boa *Boa constrictor*	pharyngeal swab blood		pos. pos.	-	Wilhelma Germany	11_14_13
19.	Boa *Boa constrictor*	pharyngeal swab blood		pos. pos.	-	Wilhelma Germany	11_16_13
20.	Green tree python *Morelia viridis*	pharyngeal swab blood	IBD: neg.	pos. neg.	-	Wilhelma Germany	11_22_13
21.	Boa *Boa constrictor*	pharyngeal swab blood	IBD: neg.	pos. pos.	-	Wilhelma Germany	11_25_13
22.	Boa *Boa constrictor*	pancreas kidney intestine liver lung spleen	IBD: pos	pos. pos. pos. pos. pos. pos.	-	Laboklin Germany	14_1_13
23.	Boa	pancreas	IBD: pos	pos.	-	Laboklin	14_2_13

	Boa constrictor	kidney intestine liver lung		pos. pos. pos. pos.		Germany	
24.	Boa *Boa constrictor*	pancreas kidney intestine liver lung skin	IBD: pos	neg. neg. neg. neg. pos. pos.	-	Laboklin Germany	14_3_13
25.	Reticulated python *Malayopython reticulatus*	kidney liver lung intestine pancreas skin brain	IBD: neg.	neg. neg. neg. neg. neg. neg. brain:pos.	-	Laboklin Germany	14_5_13
26.	Boa *Boa constrictor*	liver trachea kidney spleen lung	IBD: pos.	pos. pos. pos. pos. pos.	-	Laboklin Germany	14_7_13

		intestine		neg.			
27.	Boa *Boa constrictor*	lung heart liver kidney intestine pancreas brain	IBD: neg.	pos. pos. pos. pos. pos. pos. pos.	inappetence	Wilhelma	27_13
28.	Boa *Boa constrictor*	swab	n.a.	pos.	-	Labnoklin Germany	30_13
29.	Boa *Boa constrictor*	pharyngeal swab cloacal swab	n.a.	pos. pos.	-	Germany	32_13
30.	Boa *Boa constrictor*	swab blood	n.a.	neg. pos.		Laboklin Germany	39_13
31.	Boa *Boa constrictor*	pharyngeal swab blood	IBD in collection	pos. neg.	-	Germany	43_2_13
32.	Boa *Boa constrictor*	pharyngeal swab blood		pos. pos.	-		43_6_13
33.	Boa *Boa constrictor*	swab	Individual inclusions in blood smear were	pos.	-		44_13

			observed				
34.	Indian python *Python molurus*	pharyngeal swab cloacal swab skin swab	n.a	pos. pos. pos.	Skin problems	Leipzig Germany	49_13
35.	Python	lung lavage	n.a	pos.	Respiratory symptoms	Germany	55_13
36.	Boa *Boa constrictor*	blood	n.a	pos.		U.K.	94_2_13
37.	Boa *Boa constrictor*	serum swab	n.a	pos. pos.	-	Laboklin U.K.	98_13
38.	Boa *Boa constrictor*	spleen intestine brain pharynx kidney liver ? ? skin	IBD: pos.	neg. neg. neg. neg. neg. neg. pos. neg. pos.	-	Laboklin Germany	116_13
39.	Python	liver pancreas kidney	IBD: neg.	pos. pos. pos.	-	Laboklin Germany	117_13

		skin brain gut		pos. pos. pos.			
40.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin	IBD: neg.	brain:pos. neg. neg. neg. neg. neg.	-	Laboklin Germany	121_13
41.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin lung	IBD: pos.	pos. pos. pos. pos. pos. pos. pos.	-	Laboklin Germany	122_13
42.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin	IBD: pos.	pos. pos. pos. pos. pos. pos.		Laboklin Germany	123_13

		lung liver		pos. pos.			
43.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.	-	Laboklin Germany	124_13
44.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.		Laboklin Germany	125_13
45.	Boa *Boa constrictor*	brain pancreas kidney spleen	IBD: pos.	pos. pos. pos. pos.	-	Laboklin Germany	126_13

		gut skin liver lung		pos. pos. pos. pos.			
46.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: neg.	brain: pos. neg. neg. neg. neg. neg. neg. neg.		Laboklin Germany	127_13
47.	Boa *Boa constrictor*	brain pancreas kidney spleen gut skin	IBD: neg.	brain:pos. neg. neg. neg. neg. neg.	-	Laboklin Germany	128_13
48.	Boa *Boa constrictor*	brain pancreas kidney spleen	IBD: pos.	pos. pos. pos. pos.	-	Laboklin Germany	129_13

		gut skin lung liver		pos. pos. pos. pos.			
49.	Boa *Boa constrictor*	CNS kidney spleen gut skin lung liver pancreas	IBD: neg.	CNS: pos. neg. neg. neg. neg. neg. neg. neg.	-	Laboklin Germany	131_13

Table 11.5 All samples tested for reptarenaviruses during the study period 2012-1013.

	Species	**Tissue samples**	**IBD status**	**RT-PCR Reptarenavirus**	**Origin**	**Clinical signs, Remarks**	**Lab no.**
1.	Reticulated python *Malayopython reticulatus*	trachea	n.a.	neg.	U.K	n.a.	3_12
2.	reticulated python *Malayopython reticulatus*	kidney brain pancreas intestine lung	n.a.	neg. brain: pos. neg. neg. neg.	Leipzig Germany	n.a.	15_12
3.	*Boa* *Boa constictor*	n.a.	n.a.	neg.	n.a.	Male	19_12
4.	*Boa* *Boa constrictor*	n.a.	n.a.	neg.	n.a.	Female	19_5_12
5.	Reticulated python *Malayopython reticulatus*	brain	n.a.	pos.	U.K	Male	23_1_12
6.	*Boa* *Boa contrictor*	brain	n.a.	neg.	U.K	Female	23_2_12
7.	Green tree python	pharyngeal	n.a.	neg.	Leipzig	Inappetence	24_12

	Morelia viridis	swab cloacal swab tracheal flushing water		 neg. neg.	Germany		
8.	Indian python *Python molurus*	pancreas lung kidney intestine	n.a.	neg. neg. neg. neg.	Leipzig Germany	n.a.	25_12
9.	Python	lung liver kidney intestine	n.a.	neg. neg. neg. neg.	n.a.		32_12
10.	Ball python *Python regius*	brain kidney lung liver feces	IBD: neg.	brain: pos. neg. neg. neg. neg.	cuva Germany	Encephalitis	47_12
11.	Ball python *Python regius*	fecal sample	n.a.	neg.	n.a.		50_12
12.	Indian python	pharyngeal	IBD in	neg.	n.a.		51_12

	Python molurus	swab cloacal swab	collection	 neg.			
13.	Indian python *Python molurus*	pharyngeal swab	n.a.	neg.	Leipzig Germany		52_12
14.	Ball python *Python regius*	pharyngeal swab cloacal swab	n.a.	neg. neg.	n.a.		53_12
15.	Indian python *Python molurus*	kidney liver lung spleen brain	n.a.	neg. neg. neg. neg. brain: pos.	Leipzig Germany		79_12
16.	Ball python *Python regius*	lung lavage tracheal pharyngeal flushing water	n.a.	neg. neg.	n.a.		81_12
17.	Ball python *Python regius*	pharyngeal swab	n.a.	neg.	Leipzig Germany		97_12
18.	Ball python	pharyngeal	n.a.	neg.	Leipzig		98_12

	Python regius	swab			Germany		
19.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
20.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
21.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
22.	Reticulated python *Malayopython reticulatus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
23.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
24.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
25.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12

26.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
27.	Indian python *Python molurus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
28.	Reticulated python *Malayopython reticulatus*)	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	n.a.	102_12
29.	Anaconda *Eunectes murinus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München	anorexia	102_12
30.	Anaconda *Eunectes murinus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München		102_12
31.	Anaconda *Eunectes murinus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München		102_12
32.	Anaconda *Eunectes murinus*	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München		102_12
33.	Indian python	oral and	n.a.	neg.	Reptilienauffangst		102_12

	Python molurus) Albino	cloacal swab			München		
34.	Indian python *Python molurus* Albino	oral and cloacal swab	n.a.	neg.	Reptilienauffangst München		102_12
35.	Carpet python *Morelia spilota*	oral swab and tracheal wash	n.a.	neg.	Leipzig Germany		110_12
36.	Indian python *Python molurus*	intestine kidney liver lung	n.a.	neg. neg. neg. neg.	Leipzig Germany		115_12
37.	Ball python *Python regius*	intestine kidney liver pancreas lung brain	n.a.	neg. neg. neg. neg. neg. neg.	Leipzig Germany		115_12
38.	Green tree python *Morelia viridis*	lung kidney intestine	n.a.	neg. neg. neg.	Leipzig Germany		115_12
39.	*Boa*	lung	n.a.	pos.	Leipzig		128_12

	Boa constrictor	kidney liver intestine		pos. pos. pos.	Germany		
40.	Ball python *Python regius*	brain pancreas lung kidney intestine	n.a.	neg. neg. neg. neg. neg.	Leipzig Germany		132_12
41.	*Boa* *Boa constrictor*	blood	IBD: neg.	pos.	Deutschland, Michling		134_12: 195A
42.	*Boa* *Boa constrictor*	blood	IBD: neg.	pos.	Deutschland, Michling		134_12: 196A
43.	*Boa* *Boa constrictor*	blood	IBD: neg.	pos.	Deutschland, Michling		134_12: 198A
44.	*Boa* *Boa constrictor*	blood	IBD: neg.	pos.	Deutschland, Michling		134_12: 199A
45.	*Boa* *Boa constrictor*	blood	IBD ?	neg.	Deutschland, Michling		134_12: 202A
46.	*Boa* *Boa constrictor*	blood	IBD ?	pos.	Deutschland, Michling		134_12: 203A
47.	*Boa* *Boa constrictor*	blood	IBD: neg.	neg.	Deutschland, Michling		134_12: 204A

48.	*Boa* *Boa constrictor*	blood	IBD: neg.	neg.	Deutschland, Michling		134_12: 205A
49.	*Boa* *Boa constrictor*	blood	IBD: neg.	pos.	Deutschland, Michling		134_12: 206A
50.	*Boa* *Boa constrictor*	blood	IBD: neg.	neg.	Deutschland, Michling		134_12: 207A
51.	*Boa* *Boa constrictor*	blood	IBD: neg.	neg.	Deutschland, Michling		134_12: 208A
52.	*Boa* *Boa constrictor*	blood	IBD: neg.	neg.	Deutschland, Michling		134_12: 209A
53.	Ball python *Python regius*	lunge liver brain intestine	n.a.	neg. neg. neg. neg.	Anke	Sunshine +	137_12
54.	Boa *Boa constrictor* Albino	pharyngeal swab cloacal swab	n.a.	pos. neg.	Germany		1_13
55.	Ball python *Python regius*	pharyngeal swab cloacal swab	n.a.	neg. neg.	n.a.	Inappetence, CNS symptoms	2_13

56.	*Boa* *Boa constrictor*	pharyngeal swab	n.a.	pos.	Germany	Pneumonia	3_13
57.	Ball python *Python regius*	kidney liver lung intestine	IBD: neg.	neg. neg. neg. neg.	Germany (via Silvia Blahak)	Suspected to be infected with Sunshine virus	8_13
58.	Ball python *Python regius*	lung leavage	n.a.	neg.	n.a.	Pneumonia	9_13
59.	*Boa* *Boa constrictor*	pharyngeal swab blood	IBD: neg.	pos. neg.	Wilhelma Germany		11_1_13
60.	Indian python *Python molurus*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_2_13
61.	Rainbow boa *Epicrates cenchria*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_3_13
62.	Rainbow boa *Epicrates cenchria*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_4_13
63.	Rainbow boa *Epicrates cenchria*	pharyngeal swab	IBD: neg.	neg.	Wilhelma Germany		11_5_13

		blood		neg.			
64.	Rainbow boa *Epicrates cenchria*	pharyngeal swab blood	IBD:?	neg. neg.	Wilhelma Germany		11_6_13
65.	Green tree python *Morelia viridis*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_7_13
66.	Green tree python *Morelia viridis*	Pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_8_13
67.	*Boa* *Boa constrictor*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_9_13
68.	*Boa* *Boa constrictor*	pharyngeal swab blood	Seven boas were housed together. Six of them were reported IBD pos.	neg. neg.	Wilhelma Germany		11_10_13
69.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. pos.	Wilhelma Germany		11_11_13
70.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. neg.	Wilhelma Germany		11_12_13

71.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. pos.	Wilhelma Germany		11_13_13
72.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. pos.	Wilhelma Germany		11_14_13
73.	*Boa* *Boa constrictor*	pharyngeal swab blood		neg. neg.	Wilhelma Germany		11_15_13
74.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. pos.	Wilhelma Germany		11_16_13
75.	Garden tree boa *Corallus hortulanus*	pharyngeal swab blood	n.a.	neg. neg.	Wilhelma Germany		11_17_13
76.	Garden tree boa *Corallus hortulanus*	pharyngeal swab blood	n.a.	neg. neg.	Wilhelma Germany		11_18_13
77.	Garden tree boa *Corallus hortulanus*	pharyngeal swab blood	n.a.	neg. neg.	Wilhelma Germany		11_19_13
78.	Ball python	pharyngeal	n.a.	neg.	Wilhelma		11_20_13

	Python regius	swab blood		 neg.	Germany		
79.	Ball python *Python regius*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_21_13
80.	Green tree python *Morelia viridis*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_22_13
81.	Green tree python *Morelia viridis*	pharyngeal swab blood	IBD: neg.	pos. neg.	Wilhelma Germany		11_22_13
82.	*Boa* *Boa constrictor*	pharyngeal swab blood	IBD: pos	neg. neg.	Wilhelma Germany		11_24_13
83.	*Boa* *Boa constrictor*	pharyngeal swab blood	IBD: neg.	pos. pos.	Wilhelma Germany		11_25_13
84.	Indian python *Python molurus*	pharyngeal swab blood	IBD: neg.	neg. neg.	Wilhelma Germany		11_26_13
85.	*Boa* *Boa constrictor*	liver kidney	IBD neg.	neg. neg.	n.a		13_13

		lung brain intestine heart		neg. neg. neg. neg.			
86.	*Boa* *Boa constrictor*	pancreas kidney intestine liver lung spleen	IBD: pos	pos. pos. pos. pos. pos. pos.	Laboklin Germany		14_1_13
87.	*Boa* *Boa constrictor*	pancreas kidney intestine liver lung	IBD: pos	pos. pos. pos. pos. pos.	Laboklin Germany		14_2_13
88.	*Boa* *Boa constrictor*	pancreas kidney intestine liver lung skin	IBD: pos	neg. neg. neg. neg. pos. pos.	Laboklin Germany		14_3_13
89.	Ball python	brain	IBD: neg.	neg.	Laboklin		14_4_13

	(*Python regius*)	lung liver pancreas kidney intestine		neg. neg. neg. neg. neg.	Germany		
90.	Reticulated python *Malayopython reticulatus*)	kidney liver lung intestine pancreas skin brain	IBD: neg.	neg. neg. neg. neg. neg. neg. brain: pos.	Laboklin Germany		14_5_13
91.	Ball python *Python regius*	liver kidney pancreas intestine spinal cord trachea blood skin pharyngeal swab	IBD: neg.	neg. neg. neg. neg. neg. neg. neg. neg. neg.	Laboklin Germany		14_6_13

92.	*Boa* *Boa constrictor*	liver trachea kidney spleen lung intestine	IBD: pos	pos. pos. pos. pos. pos. neg.	Laboklin Germany		14_7_13
93.	Ball python *Python regius*	liver kidney pancreas intestine spinal cord trachea brain skin	IBD: neg.	neg. neg. neg. neg. neg. neg. neg. neg.	Laboklin Germany		14_8_13
94.	Indian python *Python molurus*	liver kidney spinal cord	IBD: neg.	neg. neg. neg.	Laboklin Germany		17_13
95.	*Boa* *Boa constrictor*	oral and cloacal swab	n.a.	neg.	Laboklin Germany	Male	21_13
96.	*Boa* *Boa constrictor*	oral and cloacal	n.a.	neg.	Laboklin Germany	Female	21_13

		swab					
97.	Ball python *Python regius*	brain liver lung kidney skin	n.a.	neg. neg. neg. neg. neg.	Laboklin Germany	Rana virus pos.	23_13
98.	*Boa* *Boa constrictor*	lung heart liver kidney intestine pancreas brain	IBD: neg.	pos. pos. pos. pos. pos. pos. pos.	Wilhelma Germany	Inappetence	27_13
99.	*Boa* *Boa constrictor*	swab	n.a.	pos.	Labnoklin Germany		30_13
100.	*Boa* *Boa constrictor*	pharyngeal swab cloacal swab	n.a.	pos. pos.	Germany		32_13
101.	*Boa* *Boa constrictor*	tissue pool	n.a.	neg.	Laboklin Germany	Losing weight	38_1_13
102.	Indian python	kidney	n.a.	neg.	Laboklin	Skin problems	38_2_13

	Python molurus	brain lung heart pancreas		neg. neg. neg. neg.	Germany		
103.	*Boa* *Boa constrictor*	swab blood	n.a.	neg. pos.	Laboklin Germany		39_13
104.	*Boa* *Boa constrictor*	pharyngeal swab cloacal swab skin	n.a.	neg. neg. neg.	Laboklin Germany		40_13
105.	*Boa* *Boa constrictor*	pharyngeal swab blood	IBD in collection	neg. neg.	Germany		43_1_13
106.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. neg.	Germany		43_2_13
107.	African rock python *Python sebae*	pharyngeal swab blood		neg. neg.	Germany		43_3_13
108.	Indian python *Python molurus*	pharyngeal swab		neg.	Germany		43_4_13

		blood		neg.			
109.	Indian python *Python molurus*	pharyngeal swab blood		neg. neg.	Germany		43_5_13
110.	*Boa* *Boa constrictor*	pharyngeal swab blood		pos. pos.	Germany		43_6_13
111.	Indian python *Python molurus*	pharyngeal swab blood		neg. neg.	Germany		43_7_13
112.	Indian python *Python molurus*	pharyngeal swab blood		neg. neg.	Germany		43_8_13
113.	Ball python *Python regius*	pharyngeal swab blood		neg. neg.	Germany		43_9_13
114.	Ball python *Python regius*	pharyngeal swab blood		neg. neg.	Germany		43_10_13
115.	Unknown	pharyngeal swab blood		neg. neg.	Germany		43_11_13

116.	Madagascar boa *Arcantophis* sp.	pharyngeal swab blood		neg. neg.	Germany		43_12_13
117.	*Boa* *Boa constrictor*	swab blood	n.a.	neg. neg.	Germany		44_13
118.	Rainbow boa *Epicrates cenchria*	swab	n.a.	neg.	Germany		44_13
119.	Rainbow boa *Epicrates cenchria*	swab	n.a.	neg.	Germany		44_13
120.	Rainbow boa *Epicrates cenchria*	swab	n.a.	neg.	Germany		44_13
121.	Green tree python *Morelia viridis*	swab	n.a.	neg.	Germany		44_13
122.	Indian python *Python molurus*	swab	n.a.	neg.	Germany		44_13
123.	Garden tree boa *Corallus hortulanus*	swab	n.a.	neg.	Germany		44_13
124.	Garden tree boa *Corallus hortulanus*	swab	n.a.	neg.	Germany		44_13
125.	Ball python *Python regius*	swab	n.a.	neg.	Germany		44_13
126.	Ball python	swab	n.a.	neg.	Germany		44_13

	Python regius	blood		neg.			
127.	Green tree python *Morelia viridis*	swab	n.a.	neg.	Germany		44_13
128.	Green tree python *Morelia viridis*	swab	n.a.	neg.	Germany		44_13
129.	*Boa* *Boa constrictor*	swab	Individual inclusions in blood smear were observed	pos.	Germany		44_13
130.	Indian python *Python molurus*	swab	n.a.	neg.	Germany		44_13
131.	Indian python *Python molurus*	swab blood	n.a.	neg. neg.	Germany		44_13
132.	Indian python *Python molurus* albino	swab blood	n.a.	neg. neg.	Germany		44_13
133.	*Boa* *Boa constrictor*	pharyngeal swab cloacal swab	n.a.	neg. neg.	Germany		46_13
134.	Indian python	pharyngeal	n.a	pos.	Germany	skin problems	49_13

	Python molurus	swab cloacal swab skin swab		 pos. pos.			
135.	Python	lung lavage	n.a	pos.	Germany	respiratory symptoms	55_13
136.	Rosy boa *Lichanura trivirgata*	swab	n.a	neg.	Germany		56_13
137.	*Boa constrictor*	swab blood	n.a.	neg. neg.	Germany		57_13
138.	*Boa constrictor*	swab blood	IBD: pos.	neg. neg.	Germany	Stomatitis, dermatitis	58_13
139.	Green tree python *Morelia viridis*	brain lung intestine kidney swab blood	n.a	neg. neg. neg. neg. neg. neg.	Leipzig Germany		60_13
140.	Rainbow boa *Epicrates cenchria*	swabs	n.a	neg.	Laboklin Germany		63_13
141.	Boa	swab	n.a	neg.	Laboklin, UK		70_2_13

	Boa constrictor						
142.	Boa *Boa constrictor*	swab	n.a.	neg.	Laboklin, UK		70_3_13
143.	Ball python *Python regius*	swab	n.a	neg.	Laboklin, UK		73_13
144.	*Boa* *Boa constrictor*	swab blood	n.a	neg. neg.	Laboklin, UK		80_13
145.	Green tree python *Morelia viridis*	swab blood	n.a	neg. neg.	Laboklin, UK		80_13
146.	Boa *Boa constrictor*	RNA	n.a	neg.	Laboklin, UK		86_13
147.	Boa *Boa constrictor*	swab blood	n.a	neg. neg.	Laboklin, UK		92_13
148.	Carpet python *Morelia spilota*	swab	n.a	neg.	U.K.		94_1_13
149.	*Boa* *Boa constrictor*	blood	n.a	pos.	U.K.		94_2_13
150.	*Boa* *Boa constrictor*	blood	n.a.	neg.	UK		94_3_13
151.	Boa *Boa constrictor*	serum swab	n.a	pos. pos.	Laboklin UK		98_13
152.	Ball python	tissue pool	IBD: neg.	neg.	Laboklin Germany		115_13

	Python regius						
153.	*Boa* *Boa constrictor*	spleen intestine brain pharynx kidney liver skin two undefined organs	IBD: pos.	neg. neg. neg. neg. neg. neg. pos. neg. pos.	Laboklin Germany		116_13
154.	Python	liver pancreas kidney skin brain gut	IBD: neg.	pos. pos. pos. pos. pos. pos.	Laboklin Germany	PCR and RNA extraction repeated	117_13
155.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut	IBD: neg.	brain: pos. neg. neg. neg. neg.	Laboklin Germany		121_13

		skin		neg.			
156.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin lung	IBD: pos.	pos. pos. pos. pos. pos. pos. pos.	Laboklin Germany		122_13
157.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.	Laboklin Germany		123_13
158.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin	IBD: pos.	pos. pos. pos. pos. pos. pos.	Laboklin Germany		124_13

		lung liver		pos. pos.			
159.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.	Laboklin Germany		125_13
160.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin liver lung	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.	Laboklin Germany		126_13
161.	*Boa* *Boa constrictor*	brain pancreas kidney spleen	IBD: neg.	brain: pos. neg. neg. neg.	Laboklin Germany		127_13

		gut skin lung liver		neg. neg. neg. neg.			
162.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin	IBD: neg.	brain: pos. neg. neg. neg. neg. neg.	Laboklin Germany		128_13
163.	*Boa* *Boa constrictor*	brain pancreas kidney spleen gut skin lung liver	IBD: pos.	pos. pos. pos. pos. pos. pos. pos. pos.	Laboklin Germany		129_13
164.	Ball python *Python regius*	brain kidney spleen gut	IBD: neg.	neg. neg. neg. neg.	Laboklin Germany		130_13

		skin lung liver pancreas		neg. neg. neg. neg.			
165.	*Boa* *Boa constrictor*	CNS kidney spleen gut skin lung liver pancreas	IBD: neg.	CNS: pos. neg. neg. neg. neg. neg. neg. neg.	Laboklin Germany		131_13
166.	Ball python *Python regius*	gut CNS pancreas skin liver kidney lung testis	IBD: neg.	neg. neg. neg. neg. neg. neg. neg. neg.	Laboklin Germany		132_13
167.	Ball python *Python regius*	gut CNS	IBD: neg.	neg. neg.	Laboklin Germany		133_13

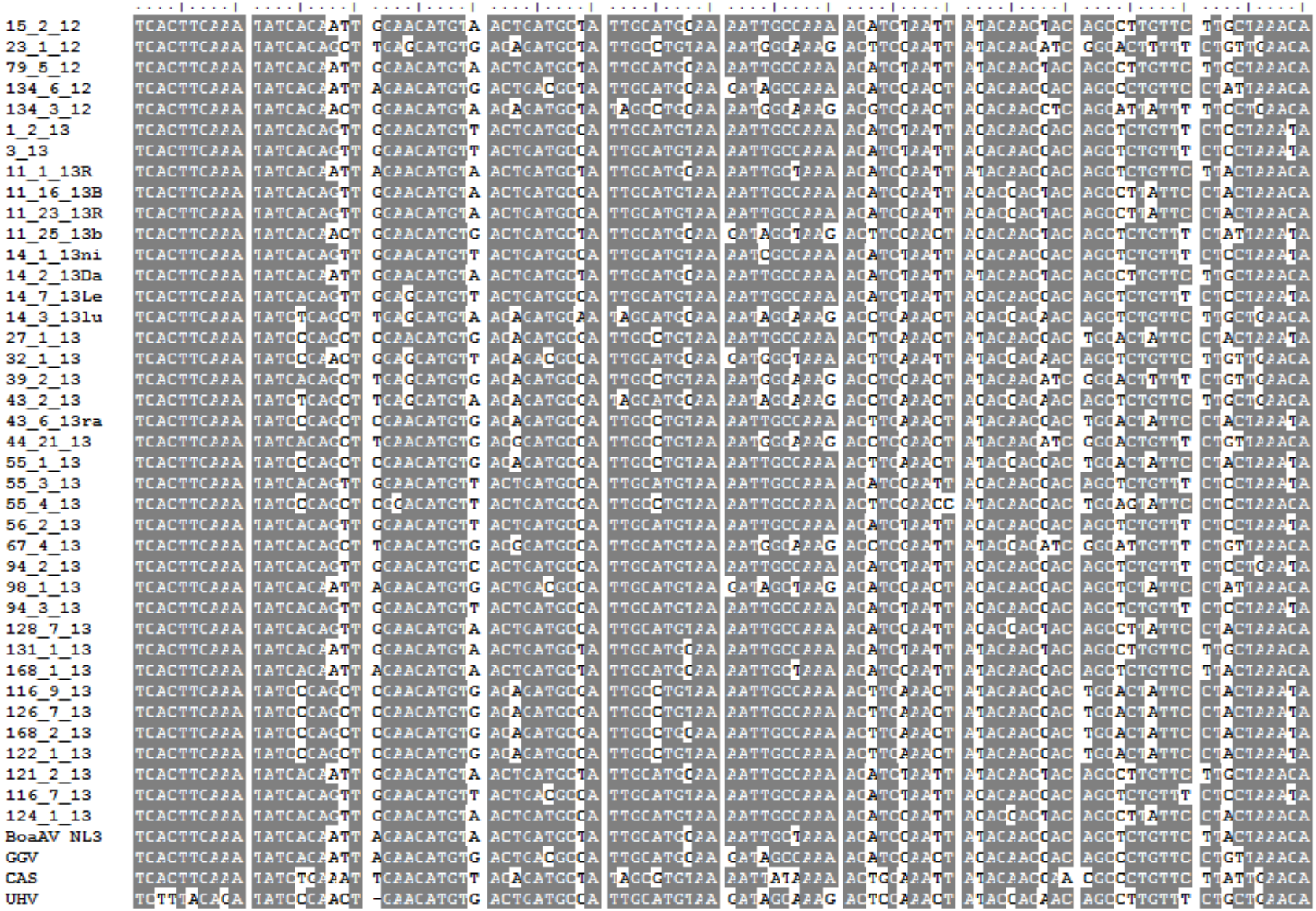
		pancreas skin liver kidney lung testis		neg. neg. neg. neg. neg. neg.			
168.	Ball python *Python regius*	pharyngeal + cloacal swab	n.a.	neg.			145_13
169.	Python albino	blood	n.a.	neg.	U.K.		156_13
170.	Green tree python *Morelia viridis*	swab lung liver heart intestine pancreas brain	IBD: neg.	neg. neg. neg. neg. neg. neg. neg.	Wilhelma Germany		143_13 and 158_13

Table 11.6 Identity values (%) of the detected reptarenviruses and GGV, Boa AV NL B3,UHV and CASV. The upper diagonal refers to the values for 187 nucleotides. The lower diagonal shows values for 62 deduced amino acids of the detected viruses. GenBank accession numbers: Golden Gate Virus (JQ717264), Boa Arenavirus Netherlands (KC508669), California Academy of Sciences (JQ717262) and University of Helsinki Virus (KF297880).

Seq->	15_2_12	23_1_12	79_5_12	134_6_12	134_3_12	1_2_13	3_13	11_1_13R	11_16_13B	11_23_13R	14_1_13ni	14_2_13Da	14_7_13Le	14_3_13lu	27_1_13	32_1_13	39_2_13	43_2_13	43_6_13ra	55_1_13	55_3_13
15_2_12	ID	77.5	100	86.6	77.5	83.9	83.9	94.1	88.7	89.3	83.4	99.4	83.4	79.1	77.5	80.2	76.4	79.1	77.5	78	84.4
23_1_12	93.6	ID	77.5	78	79.6	78	78	77	74.8	74.8	78	77	78.6	78	80.2	80.7	96.2	78	80.2	79.6	79.1
79_5_12	100	93.6	ID	86.6	77.5	83.9	83.9	94.1	88.7	89.3	83.4	99.4	83.4	79.1	77.5	80.2	76.4	79.1	77.5	78	84.4
134_6_12	96.8	93.6	96.8	ID	76.4	83.9	83.9	87.1	84.4	84.4	83.9	86	83.4	75.9	80.2	81.2	78.6	75.9	80.2	80.2	85.5
134_3_12	90.4	92	90.4	90.4	ID	75.9	75.9	78	75.9	75.9	75.9	77.5	75.4	78	75.4	75.9	79.6	78	75.4	74.8	75.9
1_2_13	98.4	93.6	98.4	98.4	88.8	ID	100	83.9	86.6	86.6	99.4	83.4	99.4	76.4	81.2	79.1	78.6	76.4	81.2	81.2	98.3
3_13	98.4	93.6	98.4	98.4	88.8	100	ID	83.9	86.6	86.6	99.4	83.4	99.4	76.4	81.2	79.1	78.6	76.4	81.2	81.2	98.3
11_1_13R	100	93.6	100	96.8	90.4	98.4	98.4	ID	87.1	87.7	83.4	94.6	83.4	78.6	79.1	80.7	75.9	78.6	79.1	79.6	84.4
11_16_13B	98.4	93.6	98.4	98.4	88.8	100	100	98.4	ID	99.4	86	89.3	86	77	79.6	75.9	74.3	77	79.6	80.2	86
11_23_13R	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	ID	86	89.8	86	77	79.1	76.4	74.3	77	79.1	80.7	86
14_1_13ni	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	ID	82.8	98.9	76.4	80.7	79.1	78.6	76.4	80.7	80.7	97.8
14_2_13Da	100	93.6	100	96.8	90.4	98.4	98.4	100	98.4	98.4	98.4	ID	82.8	79.6	77.5	80.7	75.9	79.6	77.5	78	83.9
14_7_13Le	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	ID	77	80.7	79.6	79.1	77	80.7	80.7	97.8
14_3_13lu	95.2	93.6	95.2	95.2	87.3	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2	ID	77	82.8	80.7	99.4	77	77.5	76.4
27_1_13	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	ID	79.6	81.8	77.5	100	98.3	81.2
32_1_13	95.2	96.8	95.2	95.2	90.4	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2	98.4	98.4	ID	81.2	82.8	79.6	80.2	79.6
39_2_13	93.6	98.4	93.6	95.2	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	93.6	95.2	96.8	ID	80.7	81.8	81.2	79.6
43_2_13	95.2	93.6	95.2	95.2	87.3	95.2	95.2	95.2	95.2	95.2	95.2	95.2	95.2	100	98.4	96.8	93.6	ID	77.5	78	76.4
43_6_13ra	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	100	98.4	95.2	98.4	ID	98.3	81.2
55_1_13	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	100	98.4	95.2	98.4	100	ID	81.2
55_3_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	ID
55_4_13	85.7	82.5	85.7	87.3	79.3	87.3	87.3	85.7	87.3	87.3	87.3	85.7	87.3	84.1	85.7	84.1	82.5	84.1	85.7	85.7	87.3
56_2_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
11_25_13b	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
44_21_13	92	96.8	92	93.6	92	92	92	92	92	92	92	92	92	92	93.6	95.2	98.4	92	93.6	93.6	92
67_4_13	92	96.8	92	92	90.4	92	92	92	92	92	92	92	92	92	93.6	95.2	96.8	92	93.6	93.6	92
94_2_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
98_1_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
94_3_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
128_7_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
131_1_13	100	93.6	100	96.8	90.4	98.4	98.4	100	98.4	98.4	98.4	100	98.4	95.2	96.8	95.2	93.6	95.2	96.8	96.8	98.4
168_1_13	96.8	90.4	96.8	93.6	87.3	95.2	95.2	96.8	95.2	95.2	95.2	96.8	95.2	92	93.6	92	90.4	92	93.6	93.6	95.2
116_9_13	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	100	98.4	95.2	98.4	100	100	96.8
126_7_13	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	100	98.4	95.2	98.4	100	100	96.8
168_2_13	96.8	95.2	96.8	98.4	90.4	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	98.4	96.8	96.8	98.4	98.4	96.8
122_1_13	96.8	95.2	96.8	96.8	88.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	98.4	100	98.4	95.2	98.4	100	100	96.8
121_2_13	92	87.3	92	90.4	85.7	90.4	90.4	92	90.4	90.4	90.4	92	90.4	88.8	90.4	88.8	87.3	88.8	90.4	90.4	90.4
116_7_13	98.4	93.6	98.4	98.4	88.8	100	100	98.4	100	100	100	98.4	100	95.2	96.8	95.2	93.6	95.2	96.8	96.8	100
124_1_13	96.8	92	96.8	96.8	87.3	98.4	98.4	96.8	98.4	98.4	98.4	96.8	98.4	93.6	95.2	93.6	92	93.6	95.2	95.2	98.4
BoaAV NL3	100	93.6	100	96.8	90.4	98.4	98.4	100	98.4	98.4	98.4	100	98.4	95.2	96.8	95.2	93.6	95.2	96.8	96.8	98.4
GGV	96.8	93.6	96.8	100	90.4	98.4	98.4	96.8	98.4	98.4	98.4	96.8	98.4	95.2	96.8	95.2	95.2	95.2	96.8	96.8	98.4
CAS	87.3	84.1	87.3	85.7	79.3	87.3	87.3	87.3	87.3	87.3	87.3	87.3	87.3	87.3	85.7	84.1	84.1	87.3	85.7	85.7	87.3
UHV	92	90.4	92	92	84.1	92	92	92	92	92	92	92	92	93.6	95.2	93.6	90.4	93.6	95.2	95.2	92

55_4_13	56_2_13	11_25_13b	44_21_13	67_4_13	94_2_13	98_1_13	94_3_13	128_7_13	131_1_13	168_1_13	116_9_13	126_7_13	168_2_13	122_1_13	121_2_13	116_7_13	124_1_13	BoaAV NL3	GGV	CAS	UHV
79.1	83.4	83.4	79.1	80.2	84.4	82.8	83.9	89.3	99.4	91.9	78	78	78.6	77.5	92.5	83.4	88.7	94.1	86	76.4	78
75.9	77.5	77	90.3	87.7	79.1	77.5	78	74.8	77	76.4	80.2	80.7	80.2	80.2	74.8	77.5	75.4	77	77.5	73.2	78
79.1	83.4	83.4	79.1	80.2	84.4	82.8	83.9	89.3	99.4	91.9	78	78	78.6	77.5	92.5	83.4	88.7	94.1	86	76.4	78
77	83.4	88.2	80.2	78	83.4	88.7	83.9	84.4	86	86	79.6	81.8	80.7	80.2	81.2	84.4	85	87.1	98.3	73.7	77.5
72.7	75.9	75.9	79.6	77.5	75.9	74.3	75.9	75.9	77.5	77.5	75.4	76.4	76.4	75.4	75.9	75.4	75.4	78	75.9	71.1	74.8
79.6	99.4	83.4	80.2	79.6	97.8	82.8	100	86.6	83.4	82.8	80.7	81.8	80.7	81.8	78	99.4	87.1	83.9	83.9	78	75.9
79.6	99.4	83.4	80.2	79.6	97.8	82.8	100	86.6	83.4	82.8	80.7	81.8	80.7	81.8	78	99.4	87.1	83.9	83.9	78	75.9
77.5	84.4	86	78	78	84.4	86.6	83.9	87.7	94.6	97.8	79.6	79.6	80.2	79.1	87.1	83.4	87.1	100	86.6	78.6	76.4
80.2	87.1	82.8	77.5	80.7	86	84.4	86.6	99.4	89.3	85.5	80.2	81.2	79.6	80.2	82.3	86	98.3	87.1	85	76.4	74.8
80.7	87.1	83.4	77.5	80.7	86	85	86.6	100	89.8	85.5	79.6	80.7	79.1	79.6	82.8	86	98.3	87.7	85	75.9	75.4
79.1	98.9	83.4	80.2	79.6	97.3	82.8	99.4	86	82.8	82.3	80.2	81.2	80.2	81.2	77.5	98.9	86.6	83.4	83.9	77.5	75.9
79.1	83.9	83.9	78.6	79.6	83.9	83.4	83.4	89.8	100	92.5	78	78	78.6	77.5	91.9	82.8	89.3	94.6	86.6	75.9	77.5
79.1	98.9	82.8	79.6	79.1	97.3	82.3	99.4	86	82.8	82.3	80.2	81.2	80.2	81.2	77.5	98.9	86.6	83.4	83.4	77.5	75.4
73.7	77	77	78.6	78	77	78.6	76.4	77	79.6	78	76.4	75.9	76.4	77	77	75.9	77	78.6	77	72.7	79.6
86.6	81.2	79.6	80.2	78.6	80.7	80.7	81.2	79.1	77.5	77.5	99.4	98.3	98.3	99.4	75.4	80.7	79.6	79.1	79.6	75.4	75.9
74.3	79.6	80.7	79.6	81.2	78	81.2	79.1	76.4	80.7	79.1	79.1	79.1	79.1	80.2	75.4	79.6	75.9	80.7	82.8	74.3	80.7
77	78	77	93	88.7	79.6	79.1	78.6	74.3	75.9	75.4	81.2	82.3	81.2	82.3	74.3	78	75.4	75.9	79.1	72.1	79.1
74.3	77	77	78.6	78	77	78.6	76.4	77	79.6	78	77	76.4	77	77	77	75.9	77	78.6	77	72.7	79.6
86.6	81.2	79.6	80.2	78.6	80.7	80.7	81.2	79.1	77.5	77.5	99.4	98.3	98.3	99.4	75.4	80.7	79.6	79.1	79.6	75.4	75.9
87.1	81.2	80.2	80.2	79.6	80.7	80.7	81.2	80.7	78	77.5	97.8	97.8	96.7	97.8	75.4	80.7	80.7	79.6	79.6	73.7	77
79.6	97.8	83.9	80.7	80.2	96.2	83.4	98.3	86	83.9	83.4	80.7	81.8	80.7	81.8	78	97.8	86.6	84.4	85.5	77	74.8
ID	79.6	76.4	78	76.4	79.6	77.5	79.6	80.7	79.1	75.4	86	87.1	85.5	86	75.4	79.1	80.7	77.5	77	71.1	71.6
87.3	ID	83.9	79.6	79.1	97.3	83.4	99.4	87.1	83.9	83.4	80.7	81.8	80.7	81.8	77.5	98.9	87.7	84.4	84.4	77.5	75.4
87.3	100	ID	79.1	78	82.8	90.9	83.4	83.4	83.9	83.9	79.1	79.1	80.2	79.6	76.4	82.8	83.4	86	87.7	74.3	79.6
80.9	92	92	ID	94.1	80.2	78.6	80.2	77.5	78.6	76.4	79.6	81.2	79.6	80.7	75.4	79.6	77.5	78	80.7	72.1	78
80.9	92	92	95.2	ID	79.1	77.5	79.6	80.7	79.6	76.4	79.1	80.2	77.5	79.1	75.4	79.1	80.7	78	78.6	71.6	79.1
87.3	100	100	92	92	ID	82.3	97.8	86	83.9	83.4	80.2	81.2	80.2	81.2	78.6	97.3	86.6	84.4	83.4	78.6	75.4
87.3	100	100	92	92	100	ID	82.8	85	83.4	85	80.2	79.6	80.2	81.2	77.5	83.4	85	86.6	89.3	74.3	79.1
87.3	100	100	92	92	100	100	ID	86.6	83.4	82.8	80.7	81.8	80.7	81.8	78	99.4	87.1	83.9	83.9	78	75.9
87.3	100	100	92	92	100	100	100	ID	89.8	85.5	79.6	80.7	79.1	79.6	82.8	86	98.3	87.7	85	75.9	75.4
85.7	98.4	98.4	92	92	98.4	98.4	98.4	98.4	ID	92.5	78	78	78.6	77.5	91.9	82.8	89.3	94.6	86.6	75.9	77.5
82.5	95.2	95.2	88.8	88.8	95.2	95.2	95.2	95.2	96.8	ID	78	78	78.6	77.5	85	82.3	85.5	97.8	85.5	77.5	74.8
85.7	96.8	96.8	93.6	93.6	96.8	96.8	96.8	96.8	96.8	93.6	ID	97.8	98.3	98.9	75.4	80.2	79.6	79.6	79.1	74.8	75.4
85.7	96.8	96.8	93.6	93.6	96.8	96.8	96.8	96.8	96.8	93.6	100	ID	96.7	97.8	75.4	81.2	81.2	79.6	81.2	75.4	75.9
85.7	96.8	96.8	95.2	93.6	96.8	96.8	96.8	96.8	96.8	93.6	98.4	98.4	ID	97.8	76.3	80.2	80.1	80.2	80.2	74.8	74.3
85.7	96.8	96.8	93.6	93.6	96.8	96.8	96.8	96.8	96.8	93.6	100	100	98.4	ID	75.4	81.2	80.2	79.1	80.2	75.4	76.4
82.5	90.4	90.4	87.3	85.7	90.4	90.4	90.4	90.4	92	88.8	90.4	90.4	90.4	90.4	ID	77.5	83.3	87.1	80.7	70.5	75.4
87.3	100	100	92	92	100	100	100	100	98.4	95.2	96.8	96.8	96.8	96.8	90.4	ID	86.6	83.4	84.4	77.5	75.4
85.7	98.4	98.4	90.4	93.6	98.4	98.4	98.4	98.4	96.8	93.6	95.2	95.2	95.2	95.2	88.8	98.4	ID	87.1	85.5	76.4	75.4
85.7	98.4	98.4	92	92	98.4	98.4	98.4	98.4	100	96.8	96.8	96.8	96.8	96.8	92	98.4	96.8	ID	86.6	78.6	76.4
87.3	98.4	98.4	93.6	92	98.4	98.4	98.4	98.4	96.8	93.6	96.8	96.8	98.4	96.8	90.4	98.4	96.8	96.8	ID	72.1	78
74.6	87.3	87.3	82.5	82.5	87.3	87.3	87.3	87.3	87.3	84.1	85.7	85.7	85.7	85.7	79.3	87.3	85.7	87.3	85.7	ID	70
80.9	92	92	88.8	88.8	92	92	92	92	92	88.8	95.2	95.2	93.6	95.2	85.7	92	90.4	92	92	84.1	ID

Figure 11.2 Nucleotide alignments (187 nt) of partial GP gene of the detected reptarenaviruses and GGV, Boa AV NL3, UHV and CAS. Identity shading refers to 80% among arenaviruses. GenBank accession numbers: Golden Gate Virus (GGV JQ717264), Boa Arenavirus Netherlands (Boa AV NLB3 KC508669), California Academy of Sciences (CASV JQ717262) and University of Helsinki Virus (UHV-1 KF297880).



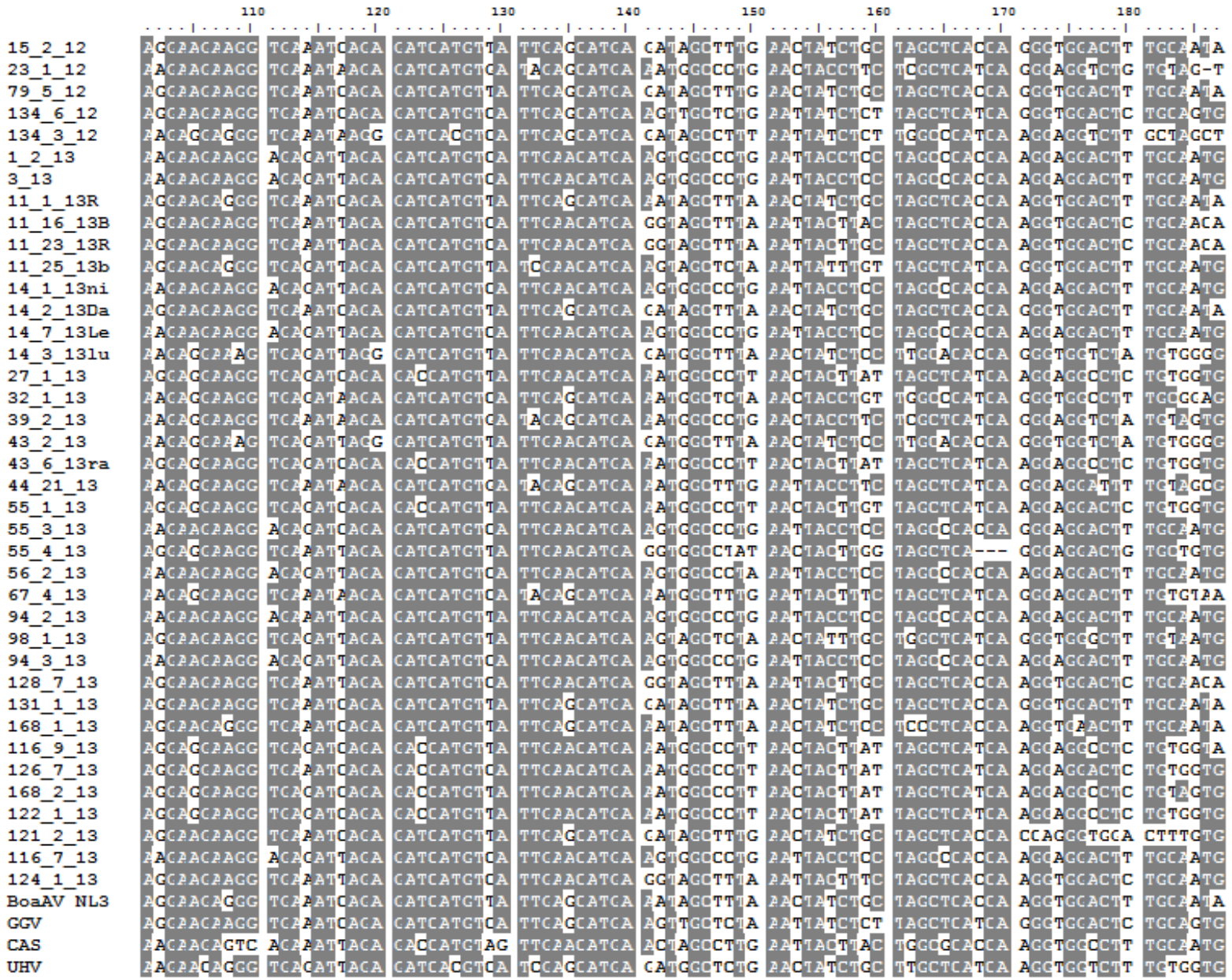
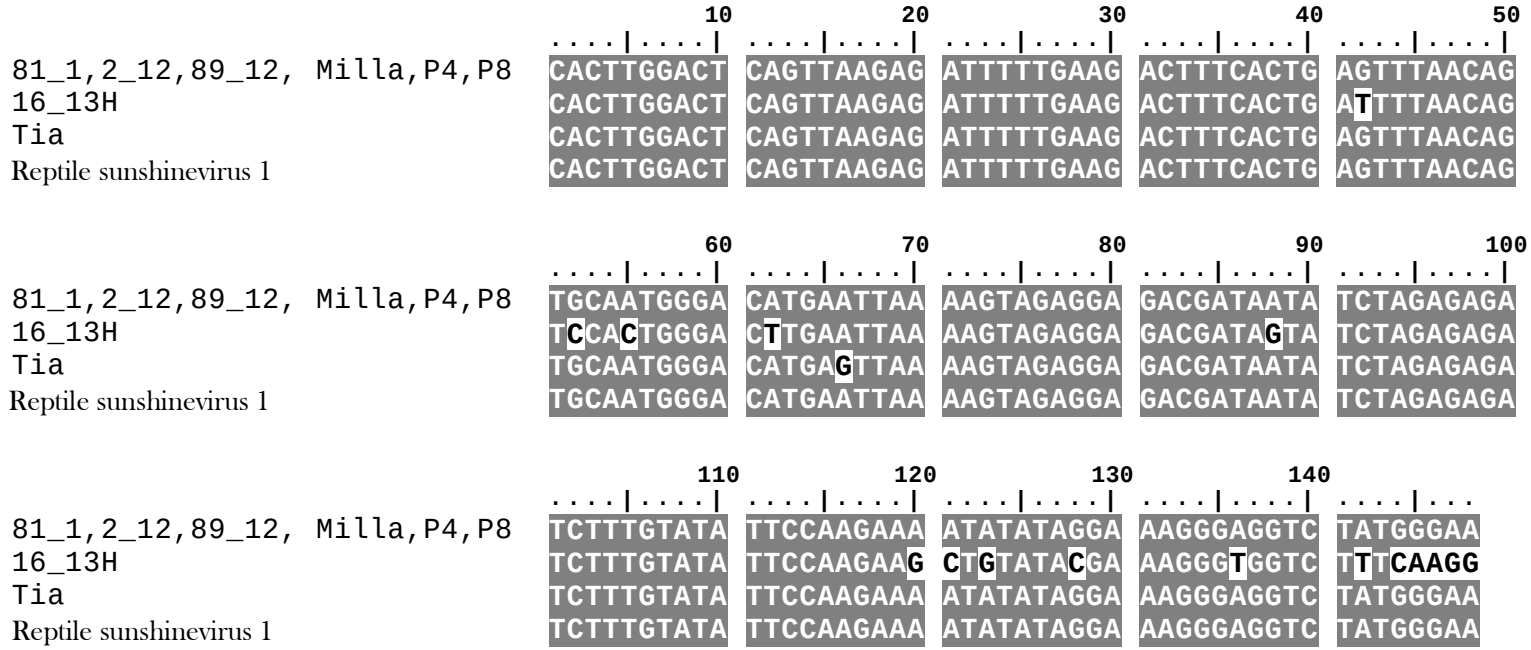

110
120
130
140
150
160
170
180
15_2_12
23_1_12
79_5_12
134_6_12
134_3_12
1_2_13
3_13
11_1_13R
11_16_13B
11_23_13R
11_25_13b
14_1_13ni
14_2_13Da
14_7_13Le
14_3_13lu
27_1_13
32_1_13
39_2_13
43_2_13
43_6_13ra
44_21_13
55_1_13
55_3_13
55_4_13
56_2_13
67_4_13
94_2_13
98_1_13
94_3_13
128_7_13
131_1_13
168_1_13
116_9_13
126_7_13
168_2_13
122_1_13
121_2_13
116_7_13
124_1_13
BoaAV NL3
GGV
CAS
UHV

Figure 11.3 Nucleotide alignments (148 nt) of the detected sunshinevirus in snakes in Germany and *Reptile sunshinevirus 1* Genbank accession number: JN192445



Affirmation

I hereby ensure that this dissertation has been completed by my own effort, has not been developed, described or submitted in any other thesis, paper or test and no other sources, tools, or programs were used other than those cited.

Stuttgart 26/11/2017

M.Sc. Tara Aqrawi

Lebenslauf

M.Sc. Tara Aqrawi. Thomas-Mann-Str. 14, 70469 Stuttgart
Mobil: 017670542282 tara_hikmat@yahoo.com

Persönliche Angaben

Geboren: 07/08/1981 in Bagdad

Staatsangehörigkeit: Deutsch

Familienstand: verheiratet, 2 Kinder

Praktische Erfahrungen

10/2011– 09/2013 **Doktorstudentin**

Umwelt und Tierhygiene Institut, Universität Hohenheim, Stuttgart, Fachbereich Virologie (S2 Diagnostik Labor) im Rahmen der Doktor Arbeit

- Proben einnehmen und vorbereiten mit physische und chemische Methoden
- Virale DNA/ RNA Präparation und Extraktion
- PCR, RT-PCR
- Sequenzen analysieren und Viren Identifizieren
- Zellkultur und mikroskopische Untersuchung
- Ergebnisse dokumentieren

10/2005 – 4/2008 **Masterstudentin**

Food Science and Biotechnology Institut, Universität Hohenheim, Stuttgart, Fachbereich Lebensmittel Mikrobiologie

- Fleisch Konservierung via natürliche Extrakten im Rahmen der Master Arbeit unter der Titel: Antimicrobial activity of natural extracts; Application in meat, gefördert durch Cognis GmbH
- Optimierung der Aufrechterhaltung der Frische von Fleisch und Hemmung von Krankheitserreger
- Brühe-Assay, titrieren
- Hackfleisch mit Bakterien Beimpfen und Zählen von CFU
- Beobachtungen dokumentieren

10/2003 – 01/2005 **Laborlehrerin**

College of Medicin, Dohuk Universität, Irak

- Betreuung von Studenten im 2.Semester
- Unterrichtsvorbereitungen und Vorlesungen

05/2001 – 10/2002 **Praktikantin**

Dohuk Haupt Krankenhaus – Azadi, Irak

- Alle mikrobiologische Labordiagnostik (Hämatologie-Parasitologie- Bakteriologie)
- Blutabnahme, Blutuntersuchung analysieren
- Urin und Kotuntersuchung
- Diagnostische Bakterienkultur
- Gram-Staining von Bakterien

03/2008 – 09/2008 Teilnehmen an HACCP Konzept und der Planung von hygienischen Anforderungen in Zusammenarbeit mit dem

Institut für Lebensmittelwissenschaft und Biotechnologie,
Universität Hohenheim

Weitebildung und Studium

Forschungspublikationen im Rahmen der Doktorabeit:

03/2016 Marschang RE, Ihász K, Kugler R, Lengyel G, Fehér E, Marton S, Bányai K, Aqrawi T, Farkas SL. Development of a consensus reverse transcription PCR assay for the specific detection of tortoise picornaviruses.
In: ***Journal of Veterinary DiagnosticInvestigation***,
Vol. 28(3) 309 –314

05/2015 Aqrawi T, Stöhr AC, Knauf-Witzens T, Krengel A, Heckers KO, Marschang RE,. Identification of snake arenaviruses in live boas and pythons in a zoo in Germany.In:***Tierarztlische Praxis Ausg K, Kleintiere/ Heimtiere*** 43(4):239-47

03/2013 Marschang RE, Stöhr AC, Aqrawi T, Hyndman TH, Plenz B, Blahak S & Pees M. First detection of Sunshine virus in pythons (*Python regius*) in Europe.***In Proceedings of the Association of Reptilian and Amphibian Veterinarians Conference***.p. 15.**Association of Reptilian and Amphibian Veterinarians ARAV.**

09/2005 – 04/2008	Master in Agrarwissenschaften unter der Titel "Environmental protection and Agricultural food production" mit Abschluss, Hohenheim Universität, Stuttgart • Ausflug beim EnBw , Müllentsorgung mit Umwelt freundliche Methoden und Strom gewinnen • CO_2 Emission Messung in Stuttgart Mitte • Schadstoffmessung im Körsch Bach in Plieningen • Ernährungswissenschaften • Lebensmitteltechnologie • Tierhaltung mit Umweltfreundlichen Methoden
04/2005 – 08/2005	Integrationssprachkurs Deutsch mit Abschluss B1 Goethe Institut, Göttingen • gefördert durch das DAAD Stipendium
09/1999 – 06/2003	Bachelor in Biologie Wissenschaften (Mikrobiologie und allgemeine Biologie) mit Abschluss Salah-Al-Din Universität, Erbil, Irak • Grundkenntnisse für Laborarbeit • Abschluss Projekt: Die Umwelt: Noise and Color Pollution
09/1987– 07/1999	Allgemeinbildende Schule mit Abschluss, Irak

Kenntnisse und Fähigkeiten

Sprachen	Arabisch: Muttersprache Kurdisch: Fließend sprechen Deutsch: sehr gute Grundlagen in Wort und Schrift Englisch: sehr gute Grundlagen in Wort und Schrift
EDV	MS-Office: sehr gute Kenntnisse in Word, Power Point, Excel, Adobe Acrobat und Photoshop