Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Detection and partial phylogenetic characterization of adenoviruses in reptiles from the Lisbon Zoo

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE EM BIOLOGIA MOLECULAR EM SAÚDE TROPICAL E INTERNACIONAL

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ABSTRACT

Adenoviruses (AdVs) are double-stranded DNA viruses that infect a wide range of vertebrate hosts, including humans. Viruses of the *Adenoviridae* family are classified into genera *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus* and *Siadenovirus*. A sixth genus (*Testadenovirus*) has been proposed for a new AdV lineage found in Testudinoidea turtles. AdVs, considered one of the most effective vaccine vectors, have also been used as gene transfer vectors (e.g. gene therapy protocols) and as experimental oncolytic agents in cancer therapy.

In this study, 120 faecal samples from 43 species of reptiles from the Lisbon Zoo were screened for AdV DNA, using a consensus nested PCR targeting a conserved region of the DNA polymerase gene, revealing a prevalence of 28.3% (34/120). The PCR amplicons were sequenced and used for phylogenetic analysis, together with 52 homologous reference sequences. Phylogenetic reconstructions, based on nucleotide and amino acid multiple alignments, led to the recognition of several sequences closely related, but distinct from previously described AdVs. The sequences were distributed all over the phylogenetic trees, showing the high genetic diversity of the AdVs circulating in the Lisbon Zoo. An AdV sequence from a red-footed tortoise (the third described for this species) clustered with previously obtained sequences from other Testudinoidea. Altogether, these three sequences may represent a novel testadenovirus species. Phylogenetic inference positioned a sequence detected from a red-eared slider close to frog AdV-1, within the genus *Siadenovirus*, which may be due to environmental contamination. Other sequences obtained from turtles and tortoises clustered with different species of fowl AdV sequences, in *Aviadenovirus* genus, along with sequences from three species of boas. The latter sequences presented very low genetic diversity, which is in accordance with a common origin. The chicks and quails the snakes are fed could be infected with a fowl AdV, which, given tree topology, may represent a new aviadenovirus species. Sequences from three species of lizards and a snake formed a monophyletic group with agamid lizard AdV-1 sequences, within the *Atadenovirus* genus. Agamid AdVs are widespread in inland bearded dragon populations all over the world and our results are consistent with this finding. Finally, several mastadenovirus sequences were found, forming two very distinctive clusters, respectively with murine AdV-2 (nine sequences, from eight species of snakes) and a rat AdV (seven sequences, from two snakes and two lizards). Each of these clusters presented low genetic diversity and this is consistent with common viral sources, the rodents used as food. Irrespective of its origin, it is conceivable that the rat AdV cluster represent a new mastadenovirus species.

It cannot be stated that the DNA detected in these reptiles indicates they are AdV natural hosts. Our findings could be due to ingestion of infected food, or environmental contamination, but also previous AdV host switches from other hosts (birds and rodents, particularly). Also, these reptiles are in zoo facilities close to other animals and the possibility of cross infection with viruses from distant hosts is high. For further characterization of the AdVs detected, complete gene or, preferably, full genome sequences are mandatory.

Keywords: adenovirus, reptiles, faecal samples, DNA polymerase, phylogenetic analysis

RESUMO

Os adenovírus (AdVs) são vírus de genoma de ADN de cadeia dupla que infetam diversos hospedeiros vertebrados, incluindo humanos. A família *Adenoviridae* compreende cinco géneros (*Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus* e *Siadenovirus*) e um sexto género (*Testadenovirus*) foi proposto para acomodar novas espécies de AdVs encontradas em tartarugas da superfamília Testudinoidea. Os AdVs, considerados dos vetores vacinais mais eficazes, têm sido também utilizados como vetores de transferência génica e agentes oncolíticos em protocolos terapêuticos experimentais.

Neste estudo, 120 amostras fecais de 43 espécies de répteis do Jardim Zoológico de Lisboa foram testadas para a presença de ADN de AdVs, utilizando uma *nested* PCR para amplificação de uma região conservada do gene da polimerase de ADN, revelando uma prevalência de 28,3% (34/120). Os fragmentos amplificados foram sequenciados e a sequência utilizada para análise filogenética com 52 sequências homólogas de referência. Esta análise, baseada em alinhamentos múltiplos de nucleótidos e aminoácidos, levou à deteção de sequências geneticamente aparentadas, algumas distintas de AdVs descritos anteriormente, distribuindo-se por vários agrupamentos, demonstrando uma elevada diversidade genética. Uma sequência de AdV de jabuti-de-patas-vermelhas agrupou com sequências obtidas anteriormente de outras tartarugas da superfamília Testudinoidea, podendo, juntamente com outras duas já descritas para esta espécie, representar uma nova espécie de testadenovírus. A inferência filogenética posicionou também uma sequência obtida de uma tartaruga-de-água-doce-americana com o AdV-1 de rãs (género *Siadenovirus*), o que pode ser devido a contaminação ambiental. Outras sequências obtidas de tartarugas agruparam com sequências de diferentes espécies de AdVs aviários (género *Aviadenovirus*), juntamente com sequências de três espécies de boas. Estas últimas apresentaram uma diversidade genética muito baixa, o que está de acordo com uma origem comum. As aves que constituem alimento destas boas podem estar infetadas com AdVs aviários, os quais, dada a topologia da árvore, podem mesmo representar uma nova espécie de aviadenovírus. Sequências de três espécies de lagartos e uma serpente formaram um grupo monofilético com sequências de AdV-1 dos agamídeos (género *Atadenovirus*). Estes AdVs são endémicos em lagartos-dragão em todo o mundo, sendo os resultados descritos consistentes com este facto. Finalmente, foram encontradas várias sequências de mastadenovírus, formando dois agrupamentos distintos, respetivamente com AdV-2 murino (nove sequências, de oito espécies de serpentes) e um AdV de ratazana (sete sequências, de duas serpentes e dois lagartos). Cada um destes agrupamentos apresenta baixa diversidade genética, sendo isso consistente com origens virais comuns, os roedores usados como alimento. Independentemente da sua origem, é concebível que o agrupamento de AdVs de ratazana represente uma nova espécie de mastadenovírus.

Não se pode afirmar que o ADN detetado nestes répteis indique necessariamente que estes sejam hospedeiros naturais de AdVs. Os resultados obtidos podem ser devidos à ingestão de alimentos infetados, ou a contaminação ambiental, mas também a prévia transmissão cruzada entre hospedeiros (de aves e roedores, em particular). Estes répteis são mantidos em instalações próximas de outros animais e a possibilidade de infeção cruzada com vírus de hospedeiros distantes é elevada. Para uma caracterização mais robusta dos AdVs detetados, seria importante a obtenção de sequências génicas completas ou, preferencialmente, do próprio genoma viral.

Palavras-chave: adenovírus, répteis, amostras fecais, polimerase de ADN, análise filogenética

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ABBREVIATIONS AND ACRONYMS

A, C, G, T - Adenine, cytosine, guanine, thymine (organic bases involved in the formation of DNA)

ADB - Agarose Dissolving Buffer

AdV - Adenovirus

BLAST - Bioinformatic program, from "Basic Local Alignment Search Tool"

bp - Base pairs

DNA - Deoxyribonucleic acid

dNTP - Deoxyribose nucleoside triphosphates

dsDNA - Double-stranded DNA

EcoRI - Restriction endonuclease enzyme isolated from *E. coli*

EDS'76 - Egg drop syndrome'76

EDTA - Ethylenediaminetetracetic acid

g (x g) - Times gravity.

HAdV - Human adenovirus

HE - Haemorrhagic enteritis

ICTV - International Committee on Taxonomy of Viruses

IPTG - Isopropyl β-D-1-thiogalactopyranoside

ITR - Inverted terminal repeat

kpb - Kilo base pairs (1000 base pairs)

LB - Lysogeny broth, a nutritionally rich medium primarily used for the growth of bacteria

MAFFT - Bioinformatic program, from "Multiple Alignment using Fast Fourier Transform"

MEGA - Bioinformatic program, from "Molecular Evolutionary Genetics Analysis"

ORF - Open reading frame

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

PEG - Polyethylene glycol

pH - (Potential of hydrogen) is a scale used to specify the acidity or basicity of an aqueous solution

RNA - Ribonucleic acid

rpm - Revolutions per minute

TAdV - Turkey adenovirus

TAE - Tris-acetate-EDTA

TE - Tris-EDTA

TEG - Tris-EDTA-glucose

TP - Terminal protein

Tris - Tris(hydroxymethyl)aminomethane

TSS - Transformation and Storage Solution

USA - United States of America

UV - Ultraviolet radiation

WSAdV - White sturgeon adenovirus

X-Gal -5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

1. Introduction

1.1 Reptiles

Reptiles belong to a class of animals named Reptilia and there are more than 10,000 species recorded on the Reptile Database website (accessed February 2019). Reptiles are tetrapods (as they have four limbs or are descended from ancestors with four limbs, e.g., snakes) and ectotherms - they control their body temperature according to the temperature of their surroundings. Being ectotherms allows them to synchronize growth and reproduction rates with available resources (Shine, 2005).

Reptiles show great diversity in reproduction strategies such as size and number of offspring. They are all amniotes - they use an amniotic egg as a mean of reproduction. Some squamates are viviparous but most reptiles are oviparous (Modesto and Anderson, 2004; Shine, 2005). Reptiles have scales instead of hair or feathers, found in mammals and birds. These scales are oriented asymmetrically, presenting different overlapping patterns for different species and body parts (Di-Poi and Milinkovitch, 2016). They have three-chambered hearts (two atria and one ventricle) except crocodilians, which have two atria and two ventricles. Reptiles are not monophyletic as they do not form a clade where they all have one common ancestor. Some reptiles are more closely related to birds than they are to other reptiles (Modesto and Anderson, 2004).

The definition for Reptilia proposed by Gauthier *et al*. (1988) has been challenged mainly due to the position of turtles (order Testudines). This order has been placed in different phylogenetic positions over the years which has led to controversies. Systematists have not reached a consensus on the position of turtles. Many articles have been published but the results turned out to be ambiguous. Different evolutionary models provide different results and positioning (Chiari *et al*., 2012; Modesto and Anderson, 2004). Due to this lack of consensus, a new phylogenetic definition for Reptilia that considers but is not dependent on the position of turtles is needed. Reptiles are classified into four distinct taxonomic orders: (i) Testudines, (ii) Rhynchocephalia, (iii) Squamata and (iv) Crocodylia.

Testudines (or Chelonia) - Chelonians have lived on Earth for over 200 million years and have survived many environmental changes (Nicholson *et al.*, 2015). This order

includes around 350 living species of turtles, tortoises and terrapins that live in mainland and oceans, between the Arctic and Antarctic circles (Joyce *et al.*, 2016).

Rhynchocephalia - This order includes only one living species of tuatara (*Sphenodon punctatus*) which is often referred to as a ´living fossil´. They can only be found in 30 islands off the coast of New Zealand.

Squamata - Squamata (the scaled reptiles) is the largest and most morphologically and ecologically diverse order of Reptilia, with more than 10,000 members. Squamata is divided into three suborders: (i) Serpentes, which includes the snakes; (ii) Lacertilia, comprising the lizards and (iii) Amphisbaenia, including the worm lizards.

Crocodylia - Crocodylia is a group of reptiles with 25 species divided into three families: (i) Gavialidae, with two species, the gharial and the false gharial; (ii) Alligatoridae, with eight species of alligators and caimans and (iii) Crocodylidae, with 15 species of crocodiles (Wang *et al*., 2016).

1.2 Adenovirus

Adenoviruses (AdVs) are double-stranded DNA (dsDNA) viruses that infect a wide range of vertebrate hosts, including humans. Human adenovirus infections affect patients of all ages and are significant contributors to human morbidity and mortality across the world. These viruses are easily transmissible and can be very contagious (Robinson *et al*., 2011). Most people show evidence of infection by the age of 10 (Arnold and MacMahon, 2017).

The first human adenovirus (HAdV) was isolated from human adenoids, also known as nasopharyngeal tonsils (Rowe *et al*., 1953). This agent caused pathological transformations and degeneration in cell culture. Almost simultaneously, Hilleman and Werner (1954) recovered a new microbial agent from throat washings from a patient with primary atypical pneumonia (in a winter epidemic of acute respiratory illness). Both groups found that this agent multiplied in human cell cultures causing cytopathogenic changes but not in bacteriological media or other laboratory hosts. It was believed that this new viral agent had a possible role in upper respiratory infections, but further investigation was necessary to confirm it.

1.2.1 Taxonomy and classification

Viruses of the *Adenoviridae* family are classified into five genera: *Atadenovirus*, *Aviadenovirus*, *Ichtadenovirus*, *Mastadenovirus* and *Siadenovirus* (Harrach *et al*., 2011). A sixth genus with the name of *Testadenovirus* was proposed for a new AdV phylogenetic lineage found in turtles of the superfamily Testudinoidea (Doszpoly *et al*., 2013).

There is a widely agreed hypothesis that suggests that the different AdV genera co-evolved with their respective vertebrate hosts. A paper published almost two decades ago (Harrach, 2000) shows how phylogenetic trees were used to compare the genetic distance of some host animals and the genetic distance of the different AdV genera. The similarity between the two distance trees supports the theory of a co-evolution between most AdVs and their host animals. Certain AdV genera found in different hosts (other than that it co-evolved with) are hypothesized to have originated from host switches and subsequent adaptation to the new hosts (Szirovicza *et al*., 2016). So, interspecies infections and co-infections might have resulted in AdVs that infect a wide range of vertebrates. The six genera are as follows:

Atadenovirus

The atadenoviruses infect a wide range of hosts including birds, ruminants, opossums and scaled reptiles. According to the International Committee on Taxonomy of Viruses - ICTV (http://www.ictvonline.org), atadenovirus is presently classified into eight different species. The name *Atadenovirus* was given due to the high A+T genomic DNA content in the first virus isolated from this genus (Benkő and Harrach, 1998). The genome size of known strains ranges from 29-33 kbp. Some atadenoviruses are associated with egg drop syndrome'76 (EDS'76) which causes hens and other domestic and wild birds to decrease their egg production and quality (Harrach *et al*., 2011).

Aviadenovirus

Aviadenoviruses have the longest genomes, after that of the white sturgeon adenovirus (WSAdV), the sole representative of the genus *Ichtadenovirus* (see below). As the name implies, aviadenoviruses only infect birds. On the other hand, the majority of AdVs from birds are aviadenoviruses, including species from duck, falcon, goose, pigeon, turkey and several fowl adenoviruses. Interesting to note that some host species can

harbour AdV from very different genera (e.g. turkey, which hosts at least four different aviadenovirus species, but also turkey adenovirus 3 (TAdV-3), which belongs to the genus *Siadenovirus*). The G+C content of aviadenovirus genomes varies between 54%-67% (Harrach *et al*., 2011).

Although aviadenovirus infections are mainly subclinical, there is a wide range of symptoms associated. These symptoms are related to the infected organs and include inclusion body hepatitis, necrotising pancreatitis, haemorrhagic enteritis (HE) and EDS'76 (Hess, 2000; McFerran and Smyth, 2000).

Siadenovirus

This genus consists of six accepted viral species (http://www.ictvonline.org). The name *Siadenovirus* was given because these viruses contain an open reading frame (ORF) that encodes for a protein very similar to bacterial sialidase proteins. Sialidases are enzymes that remove sialic acid residues from oligosaccharide chains (Giacopuzzi *et al*., 2012; Singh *et al*., 2013). Siadenoviruses have a low genomic G+C content and a wide host range. The original host is unknown but the majority of siadenoviruses infect birds (e.g. passerines, raptors, seabirds, penguins), but also amphibians (frogs), and these infections are very pathogenic (Ballmann and Harrach, 2016).

Ichtadenovirus

As previously mentioned, there is only a species in this genus, and it corresponds to the only confirmed fish adenovirus - WSAdV-1. This virus genome is longer than all other adenoviruses, with a genome G+C content of 43%. The virus seems to not cause disease in fish (Harrach *et al.*, 2011).

Mastadenovirus

Mastadenoviruses infect mammals, including humans and other non-human primates, presently comprising the greatest diversity of viral species (45 different species to date) (http://www.ictvonline.org). Besides primates, a significant number of viral species is found in bats (but also in other mammal host species). The G+C content of mastadenovirus genomic DNA varies between 43-63% (Harrach *et al*., 2011). All primate AdVs known to date, including humans (HAdVs), belong to this genus. According to the ICTV, HAdVs are classified into seven species (A to G) with around 60 serotypes, with HAdV-D containing most members. HAdVs present a great level of

genetic diversity and the maintenance of this diversity is partially due to genomic homologous recombination between different species following mixed infections (Robinson *et al*., 2013; Yatsyshina *et al*., 2016).

Testadenovirus

New AdVs have been detected in turtles from the superfamily Testudinoidea. The first from this distinct lineage was detected in an ornate box turtle (*Terrapene ornata ornata*) in Hungary and its genomic G+C content was 49% (Farkas and Gál, 2009). Comparisons of this new AdV genome sequence with corresponding sequences of other AdVs showed that its genetic motifs were different. Genome organization patterns found on other AdVs were less conserved in this new found AdV. Later, similar AdVs were also detected in a captive red-eared slider (*Trachemys scripta elegans*) from the USA and a dead yellow-bellied slider (*Trachemys scripta scripta*) from Hungary. Genetically similar AdVs were subsequently detected on eastern box turtles (*Terrapene carolina carolina*) and a pancake tortoise (*Malacochersus tornieri*) in the USA (Doszpoly *et al*., 2013).

Phylogenetic analysis based on partial genomic sequences clustered these new AdVs separately from the five accepted genera. So, a new genus has been proposed for these new testudinid viruses - *Testadenovirus* (Doszpoly *et al*., 2013). Further analysis using more genes and, ideally, the complete genome sequences is needed to draw final conclusions and to firmly establish its taxonomical position.

1.2.2 Particle structure

Adenoviruses are non-enveloped, non-segmented, DNA viruses with an icosahedral shaped capsid around 90 nm in size (Arnold and MacMahon, 2017). The capsid consists of three major proteins (hexon, penton base and fibre) and minor capsid proteins. The capsid is made of 240 hexons and 12 penton bases at the vertices of the icosahedron, each supporting an extending fibre protein (Figure 1). The number and length of these fibres can vary depending on the type/genera of AdV (Robinson *et al*., 2011). The minor capsid proteins, also called cement proteins, provide stability for the capsid. For example, the outside surface of the capsid is stabilized by the minor proteins VI and IIIa (Benevento *et al*., 2014). As shown in Figure 1, the core of the capsid consists of the viral genomic DNA complexed with four proteins (V, VII e X, also known as mu or μ,

and the terminal protein TP). Protein V is only found in mastadenoviruses. Several copies of the adenoviral protease are also present in the core (Harrach *et al*., 2011; Robinson *et al*., 2011). The protein IVa2 regulates multiple viral processes such as gene transcription and viral assembly (Yang and Maluf, 2014).

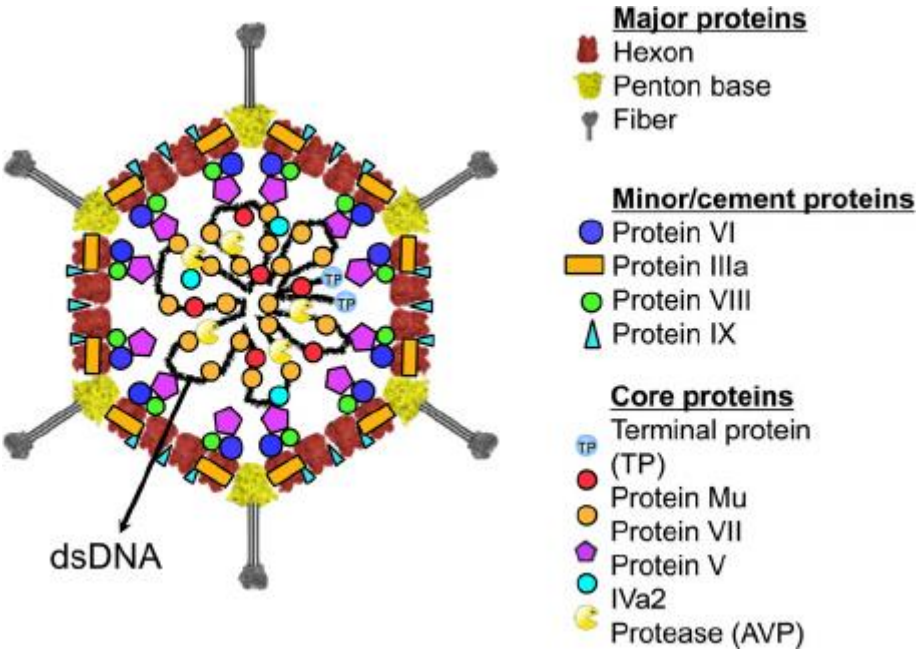


Figure 1 - Structural model of HAdV (from Benevento *et al*., 2014).

1.2.3 Genome

Adenoviral genome is a linear, non-segmented molecule of dsDNA with inverted terminal repeats (ITR). The 5´ends of the DNA strands are covalently attached to a terminal protein (TP), as seen in Figure 1, and the G+C content of AdV genome varies from 33% to 66% (Harrach *et al*., 2011). The middle part of the adenoviral genome is well conserved within the family, whereas the terminal parts show large variation and are characteristic of genera (Benkö *et al*., 2005). The size of the genome varies from 25-48 kbp (Ahi and Mittal, 2016).

1.2.4 Replication cycle

Adenovirus replication takes place in the nucleus of the host cell. This process is highly efficient with one million copies of viral DNA being synthesized in 40 hours (Hoeben

and Uil, 2013). Entry into the host cell is achieved using the fibres glycoproteins to attach to receptors on the surface of susceptible cells. Most strains of AdVs use the Coxsackie-adenovirus receptor (CAR) and CD46 as their primary receptors. AdVs also target cellular receptors such as CD80, major histocompatibility complex I, sialic acid, among others (Arnold and MacMahon, 2017; Sobhy, 2017). A second interaction between the virus and co-receptors (e.g. integrins) must occur to stimulate host cellular entry. The cellular entry can be achieved via clathrin-mediated endocytosis, actin-dependent micropinocytosis and/or dynamin-dependent micropinocytosis (Sobhy, 2017). Inside the cell, the virion goes through structural changes that cause uncoating and it escapes the endosome to get into the cytosol. This process is controlled/affected by pH concentration and endosomal ions. The virus travels to the nuclear membrane by means of dynein/dynactin and microtubules. It is then transported through the nuclear pore to inside the nucleus (Hendrickx *et al*., 2014).

The replication step consists of two phases: an early and a late phase. The early phase (E) is controlled by the early genes and it consists in the expression of regulatory proteins. These proteins main functions are as follows: to control host proteins/enzymes required to synthesize DNA, to switch on other viral genes, needed to subsequent stages of the cycle, and to evade infected host cell death by apoptosis. DNA replication separates the two phases. The late phase (L) is controlled by the late genes and it consists in the production of the structural proteins and the assembly and maturation of the new virions in the nucleus (Fougeroux and Holst, 2017). Then the virus induces cell lysis and it is released.

1.2.5 Epidemiology, infection and clinical symptoms in humans

Adenoviruses are very stable and resistant to disinfectants, physical agents and adverse environmental and pH conditions, which results in extended viability outside the body. Transmission can occur via close personal contact (such as hand shaking and touching), fomites, droplets inhalation (caused by coughing or sneezing, for example) and the faecal-oral route (Arnold and MacMahon, 2017). In some occasions, AdVs can be spread through the water. The incubation period ranges from 2 days to 2 weeks.

Adenoviruses cause respiratory infections in many vertebrates, but the infection can present a wide range of symptoms, mainly due to the tissue tropism in each species

(Cook and Radke, 2017; Yatsyshina *et al*., 2016). Adenoviral infections cause an array of symptoms in humans which vary with the type of AdV, age of the patient and immunological condition. The most common clinical conditions are lower and upper respiratory tract infections, headache, pneumonia, gastroenteritis and conjunctivitis (Arnold and MacMahon, 2017). Different epidemiologic characteristics, clinical conditions and response to treatment are associated with different serotypes of HAdV (Esposito *et al*., 2016). Despite the clinical course of AdV infections in immunocompetent patients is usually asymptomatic and self-limiting, children, immunocompromised patients and people suffering from previous respiratory or cardiac disease are at a greater risk of developing severe or disseminated AdV disease, leading to a fatal outcome.

Very recently, an outbreak of AdV infection at the University of Maryland (USA) has caused medical experts some alarm. The first case was reported on November 1st, 2018 and one month later 22 cases of AdV infection had been reported (University of Maryland 2019). One freshman student died of AdV related complications (pneumonia) and, according to the parents, the student was taking medication for Crohn's disease, which weakened her immune system.

A similar event took place in October 2018 at the Wanaque Center for Nursing and Rehabilitation (USA). A severe viral outbreak took the lives of 11 children from the centre´s paediatric unit. The children at this clinic were seriously ill before the outbreak. The Centers for Disease Control and Prevention (CDC) confirmed that both outbreaks were caused by human adenovirus type 7 (HAdV7). HAdV7 infections are associated with pneumonia, fever and acute respiratory disease syndrome, among others. Transmission can occur through close contact or respiratory droplets and these outbreaks occur mostly in closed living environments, such as hospitals and schools. Human AdVs, especially HAdV7, are often responsible for nosocomial outbreaks in hospitals and chronic care facilities and, in some cases, are fatal. These infections may start with mild respiratory symptoms and, in some cases, the viral load may increase in the lungs which can develop into severe pneumonia (Cui *et al*., 2014). Since there is no treatment for AdV infections it is very important to prevent its transmission by washing hands often, avoiding touching eyes, nose or mouth with hands and avoiding contact with an ill person.

1.2.6 Epidemiology, infection and clinical symptoms in reptiles

In 1985, a group of scientists conducted an experimental transmission study on snakes to demonstrate the pathogenicity of AdVs (Jacobson *et al*., 1985a). A boa constrictor specimen submitted to a veterinarian hospital for post-mortem evaluation presented with head tilt, enlarged liver, lung congestion and excessive amount of mucus in the lumen of the small intestine. Light microscopy revealed severe hepatic necrosis with an infiltrate of heterophils and small mononuclear cells. These symptoms were compatible with an AdV infection. The infection agent was isolated from the dead boa and inoculated into a neonatal boa constrictor and this one died 14 days later with hepatic necrosis and features that were compatible with an AdV infection. This was a pioneer experiment as it fulfilled Koch´s postulates.

Some reptiles with AdV infections are asymptomatic. However, reptiles can present lethargy, hepatitis and some neurological disorders. AdVs are commonly found in lizard species, but they have also been detected in snakes, crocodiles and chelonians. Squamates can present with anorexia associated with lethargy and wasting and central nervous system symptoms (Ariel, 2011; Marschang, 2011). Crocodiles have been reported with hepatic necrosis with intranuclear inclusion bodies, congestion of lungs, kidneys and intestines (Jacobson *et al*., 1985b). Clinical signs described in infected turtles and tortoises include lethargy, weakness, biliverdinuria (early liver disease), wasting and severe systemic disease with high mortality (Farkas and Gál, 2009).

1.2.7 Detection and diagnosis of adenovirus infection in reptiles

Detection of AdV infections in reptiles is carried out by different techniques, including viral isolation in culture, detection of viral antigens, amplification of genomic DNA by polymerase chain reaction (PCR) or serology for the detection of antibodies. AdV infection is inferred by the detection of genomic DNA from cloacal and nasal swabs, kidney, liver, intestine and lung samples by PCR. Nucleotide sequencing and further analysis of the PCR products allow investigators a better understanding of viral phylogenetic classification (Ariel, 2011). One should be aware that AdVs can establish persistent infections but may not always cause illness. So, viruses detected in the respiratory tract and stool specimens are not always sign of disease (Arnold and MacMahon, 2017).

A study conducted in South Korea between 2007 and 2011 tested samples from diseased reptiles for viral infections (Bak, Jho and Woo, 2018). These samples were obtained from local governments, zoos and pet shops. Intestine, liver, lung, kidney and heart samples were collected. Histopathology was performed on all tissues and organs and nucleic acids were extracted. Molecular techniques were used (various models of PCR or RT-PCR, depending on the virus) for viral search. A fragment of the adenoviral polymerase gene was detected in a tropical girdled lizard (*Cordylus tropidosternum*) and, interestingly, this lizard was co-infected with a paramyxovirus. AdV DNA was also detected in a panther chameleon (*Furcifer pardalis*). The fragment detected in the lizard was sequenced and showed similarity (74%) with a previously known AdV (eublepharid AdV-1), so sufficiently different to be considered a part of a novel AdV gene.

For the purpose of this study, it has been decided to screen for AdVs from reptile faeces. Using faecal samples can be done with greater ease when compared to cloacal swabs, for instance. Also, we did not have to collect organ samples post-mortem. As mentioned previously, infected animals could be asymptomatic and shed viruses in high numbers for months or years, which can be detected in their faeces. However, there are challenges when extracting DNA from faecal samples. These samples contain many substances derived from food, cell residues, gastrointestinal tract flora, including several different DNA sources. Also, they may suffer from soil or water contamination, considering where the faecal collection took place. In theory, molecular biology techniques - i.e., PCR - could allow the identification of the majority of the viruses present but there are drawbacks. Some of the previously mentioned molecules are PCR inhibitors, which can have some influence over the reactions results (Hart *et al*., 2015; Smith *et al*., 2011). PCR inhibitors may interact directly with matrix DNA or polymerase co-factors, such as Mg^{2+}, preventing amplification. Bile salts and complex polyssacharides present in faeces are some of these PCR inhibitors and may interfere with the procedure, resulting in altered product yield or no product at all. This can have an important impact in the results and conclusions (Besseti, 2007), especially if a prevalence study is being performed, which is not the case in this work.

1.2.8 Adenoviruses as gene transfer vectors, vaccines and oncolytic agents

For many years now, AdVs have been used as gene transfer vectors, recombinant vaccines and therapeutic tools for experimental cancer therapy. AdVs are one of the most effective vaccine vectors and are considered the vector of choice for gene therapy protocols (Robinson *et al*., 2011).

AdVs have a broad tissue tropism and the ability to infect dividing and non-dividing cells (Bangari and Mittal, 2006). For instance, they are capable of infecting ocular and enteric tissues, cells of the respiratory tract, tonsils, adenoids and myocardial cells, as well as genitourinary cells and meningocytes (Arnold and MacMahon, 2017). AdVs can be used as oncolytic agents in experimental cancer therapy. AdVs genomes can be engineered and used as therapeutic gene vectors specific for cancer cells in a way to reduce cancer spread and potential toxicity. Replacing viral gene promotors with tumour-specific promotors can be done in order to create oncolytic AdVs. The adenoviral genome is well studied and characterized and can be genetically manipulated easily. Non-essential genes can be deleted and replaced with therapeutic genes without affecting their ability to infect cells. Foreign genes can be inserted in AdVs by deleting early genes, thus disabling their expression (Doloff and Waxman, 2014; Fougeroux and Holst, 2017). The most used oncolytic AdVs are based on HAdV type 5, because of its well-studied biology. Unfortunately, it has one very important drawback. Most of the population gets infected with HAdV5 at an early age and develop an anti-HAdV5 immunity response. This widespread preexisting immunity is the reason why vectors based on AdVs of non-human origin are being developed (Cheng *et al*., 2017). As recombinant vaccines, an AdV vectorised vaccine, manufactured by Artemis Technologies Inc. (Guelph, Canada), is currently being used against rabies. This is a HAdV5 based vaccine that uses the rabies virus glycoprotein gene inserted in the AdV genome. This vaccine is administered to animals in baits delivered by aerial distribution and it is safe and effective for numerous animal species, including wild life (e.g. squirrels, rabbits, cats, dogs, horses) (Fougeroux and Holst, 2017). Adenoviral vaccines from human AdV have also been developed to target AdVs and related diseases. A formalin-inactivated AdV vaccine was developed in the USA and it presented with more than 90% efficiency in field trials (Hilleman, 1958). This vaccine was licensed in 1958, two years after its development. Unfortunately, the license to fabricate this

vaccine was revoked due to loss of antigenicity and contamination by an oncogenic virus - simian virus 40 (SV40) - during its production. Also, AdV vaccines were developed in the 1960s based on types 4 and 7. These vaccines were studied at military facilities and proved to be safe, efficient and highly protective, providing a long-lasting immunity (Artenstein *et al*., 2005). These vaccines are only used for specific cases (e.g. in army recruits and rabies in wildlife) and are not available for general public or national vaccination programmes.

Objectives

The purpose of this study was to investigate the presence and diversity of adenoviruses in reptiles from the Lisbon Zoo. The study was designed to: (i) screen for adenoviruses from reptile faeces through nested PCR, targeting a portion of the DNA-dependent DNA polymerase gene; (ii) sequence the PCR products obtained and (iii) characterize the AdVs found using molecular phylogenetic analysis.

2. MATERIAL AND METHODS

2 Material and Methods

2.1 Sample collection

The faecal samples were collected from reptiles housed at the Lisbon Zoo by care takers and veterinarians and sent in clean labelled screw-top containers to the Virology laboratory facilities at the Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa. Once received, the samples were stored in a fridge, until use. The total number of samples was 120 (67 from Squamata, 52 from Testudines and one from Crocodylia). Table 1 outlines the reptile species in this study and the number of samples obtained per species.

Table 1 - List of the reptile faecal samples in this study.

Reptilia	**Common name**	**Scientific name**	**Number of samples**	
Testudines	Adanson´s mud turtle	*Pelusios adansonii*	**1**	**52**
	Alligator snapping turtle	*Macrochelys temminckii*	**1**	
	Common snapping turtle	*Chelydra serpentina*	**2**	
	Egyptian tortoise	*Testudo kleinmanni*	**3**	
	Elongated tortoise	*Indotestudo elongata*	**1**	
	Florida softshell turtle	*Apalone ferox*	**1**	
	Leopard tortoise	*Stigmochelys pardalis*	**1**	
	Marginated tortoise	*Testudo marginata*	**3**	
	McCord´s snake-necked turtle	*Chelodina mccordi*	**10**	
	Red-eared slider	*Trachemys scripta elegans*	**10**	
	Red-footed tortoise	*Chelonoidis carbonaria*	**5**	
	Spiny hill turtle	*Heosemys spinosa*	**4**	
	Spur-thighed tortoise	*Testudo graeca*	**2**	
	Star tortoise	*Geochelone elegans*	**5**	
	Common snapping turtle or Alligator snapping turtle	*Chelydra serpentine or Macrochelys temminckii*	**3**	

Reptilia	Common name	Scientific name	Number of samples	
Squamata	Blood python	*Python curtus*	**1**	**67**
	Blue-tongued skink	*Tiliqua scincoides*	**2**	
	Brazilian rainbow boa	*Epicrates cenchria cenchria*	**1**	
	Burmese python	*Python bivittatus*	**4**	
	California kingsnake	*Lampropeltis getula californiae*	**2**	
	Chuckwalla	*Sauromalus ater*	**1**	
	Common leopard gecko	*Eublepharis macularius*	**2**	
	Cornsnake/Red ratsnake	*Pantherophis guttatus*	**2**	
	Cuban boa	*Epicrates angulifer*	**2**	
	Cuban/Caymans iguana	*Cyclura nubila*	**1**	
	Cuvier´s Madagascar swift	*Oplurus cuvieri*	**1**	
	Dumeril´s boa	*Acrantophis dumerili*	**2**	
	Eastern kingsnake	*Lampropeltis getula getula*	**1**	
	Frilled lizard	*Chlamydosaurus kingii*	**2**	
	Green basilisk	*Basiliscus plumifrons*	**1**	
	Green iguana	*Iguana iguana*	**4**	
	Inland bearded dragon	*Pogona vitticeps*	**3**	
	Jamaican boa	*Epicrates subflavus*	**6**	
	Jungle carpet python	*Morelia spilota cheynei*	**2**	
	Komodo dragon	*Varanus komodoensis*	**2**	
	Madagascar tree boa	*Sanzinia madagascariensis*	**6**	
	Philippine sailfin lizard	*Hydrosaurus pustulatus*	**4**	
	Reticulate Gila monster	*Heloderma suspectum suspectum*	**3**	
	Reticulated python	*Broghammerus reticulatus*	**1**	
	Rhinoceros iguana	*Cyclura cornuta cornuta*	**3**	
	Royal/Ball python	*Python regius*	**5**	

Reptilia	**Common name**	**Scientific name**	**Number of samples**
	Sinaloan milk snake	*Lampropeltis triangulum sinaloae*	**1**
	Veiled chameleon	*Chamaeleo calyptratus*	**2**
Crocodylia	American alligator	*Alligator mississippiensis*	**1**
Total			**120**

As seen in Table 1, samples from three of the four orders of reptiles have been received - 55.8% (67/120) from Squamata, 43.3% (52/120) from Testudines, and 0.8% (1/120) from Crocodylia.

2.2 Faecal sample processing

This procedure was carried out in a laminar flow chamber (Gelaire BSB 4A, Flow Laboratories, Australia) in aseptic conditions. Falcon tubes (15 mL) were labelled and prepared with 5 mL of PBS buffer. Glass microbeads (5-7) were added to the tubes for a rapid sample breakdown. Using an inoculation loop, a loop of the faecal sample (~ 0.5 mL) was transferred to the Falcon tubes, the tubes were vortexed in full speed for one minute and placed on ice before centrifuging at 2,500 x *g*, for 15 minutes, at 4 °C. After centrifugation, the cleared suspension was filtered through sterile 25 mm Acrodisc® PF Syringe Filters with Supor® Membrane, 0.8/0.2 µm (Pall Co., USA), into two sterile 2 mL microtubes (approximately 0.5 mL and 1.5 mL each). These microtubes were labelled and stored at -70 °C. Using disposable Pasteur pipettes, the rest of the cleared suspension was transferred into two non-sterile 2 mL microtubes and stored at -70 °C, as well as the remaining non-processed faecal sample.

2.3 DNA extraction and purification

DNA was extracted from the faecal filtered suspensions using High Pure PCR Template Preparation Kit (Roche, Germany), following the manufacturer's instructions (using the protocol for mammalian whole blood, buffy coat or cultured cells).

200 µL of binding buffer was added to labelled Eppendorf tubes and then 200 µL of each filtered sample to the respective tube. Then, 40 µL of reconstituted proteinase K

(in 4.5 mL double distilled water) was added and the mixtures were vortexed and incubated for 10 minutes at 70 °C. After the incubation, 100 µL of isopropanol was added to each sample and mixed. The samples were then transferred to the upper reservoir of the filter tubes (which were previously assembled into collection tubes). The samples were centrifuged at 8,000 x *g* for one minute. After centrifugation, the flow-through was discarded and the filter tube was assembled into a new collection tube. 500 µL of the inhibitor removal buffer was added and the samples were centrifuged under the same conditions. The flow-through was discarded, the filter tubes were assembled into new collection tubes and 500 µL of wash buffer was added, followed by a centrifugation step again at 8,000 x g for one minute (this washing step was repeated once). As previously, the flow-through was discarded and the filter tubes were assembled into new collection tubes, which have been centrifuged at 13,000 x *g* for 15 seconds. The collection tube was discarded and 120 µL of elution buffer, preheated at 70 °C, was added. The DNA was finally eluted by centrifugation at 8,000 x *g* for one minute. The eluted DNA was stored in three aliquots of 40 µL, at -20 °C, until use.

2.4 DNA amplification by PCR

A nested PCR protocol designed by Wellehan *et al*., (2004) has been proved to be an efficient tool for detecting AdV infections in a wide range of animals, including reptiles (Ballmann and Harrach, 2016; Pénzes *et al*., 2014; Rivera *et al*., 2009). This protocol was selected for this study and resulted in the amplification of a 318-324 bp DNA fragment from a conserved partial sequence of the adenoviral DNA polymerase gene. The primers sequences are shown in Table 2 and, as observed, are all heavily degenerated (B = G, T or C; D = G, A or T; H = A, C or T; M = A or C; N = any base; R = G or A; S = G or C; W = A or T; Y = C or T). The primers were synthesized by STAB Vida (Portugal) and used at a concentration of 10 pmol/µl, after hydration with ultrapure, nuclease-free deionized water, Milli-Q® (Reagent Grade Ultrapure Systems, Merck Millipore, USA) [this type of water was used all throughout the laboratory work].

Table 2 - PCR primers used to amplify a portion of the DNA polymerase gene of AdVs (Wellehan *et al.*, 2004).

PCR round	Primer	Sequence
1	AdVR_polFout	5'-TNMGNGGNGGNMGNTGYTAYCC - 3'
	AdVR_polRout	5'- GTDGCRAANSHNCCRTABARNGMRTT - 3'
2	AdVR_polFin	5'- GTNTWYGAYATHTGYGGHATGTAYGC - 3'
	AdVR_polRin	5'- CCANCCBCDRTTRTGNARNGTRA - 3'

The PCR reaction was setup with the NZYTaq 2x Green Master Mix system (NZYTech, Portugal), whose reaction mix, already supplied, contain a buffer, dNTPs and a modified recombinant Taq DNA polymerase enzyme. The first round of PCR amplification was performed in 25 μL of reaction volume, which contained 12.5 μL of NZYTaq 2× Green Master Mix solution, 1 μL of each first round primer primer (final concentration 0.4 μM), 5.5 μL of deionized water and 5 μL of purified DNA solution. The second round of amplification was performed in the same reaction volume, containing the exact same volume of NZYTaq 2× Green Master Mix solution and second round primers, but with 3 μL of the first PCR product and 7.5 μL of deionized water. A negative control (matrix DNA replaced by sterile deionized water) was performed with each amplification using the same experimental conditions. Amplification was conducted in a PCR Express thermal cycler (Thermo Hybaid, United Kingdom) with the following conditions: an initial denaturation step at 95 °C for 120 seconds, followed by 40 amplification cycles of 95 °C for 30 seconds, 46 °C for 60 seconds and 72 °C for 60 seconds, and a final extension at 72 °C for 7 minutes. Both PCR rounds used the same amplification conditions.

2.5 Gel electrophoresis

DNA fragments separation was carried out by gel electrophoresis on a 1.8-2% (w/v) agarose gel in 0.5x TAE buffer (see Appendix 1), containing 0.5 μg/mL of ethidium bromide. Samples of both PCR rounds (5 μL from the first PCR and 3 μL from the second PCR) were directly placed into the wells of the gel, with 2.5 μL of the molecular

marker NZYDNA Ladder VI (NZYTech, Portugal) (see Appendix 2) in between for size estimation of the DNA bands. The electrophoresis was run for about 60 minutes at 100 V. Afterwards, the gel was visualized under 302 nm UV light, using the Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). Prior to DNA sequencing, some PCR samples required purification by band excision from the gel. Therefore, a second electrophoresis was carried out on a 2% (w/v) agarose gel in 0.5x TAE buffer to separate the fragments further. 15 to 20 μL of the amplification products was used and the gel was run at 40 V for 2-3 hours.

2.6 DNA extraction and purification

After separation by electrophoresis, the samples with bands with the expected size (~ 320 bp) were identified. These samples were presumptively considered positive for the presence of AdVs. Therefore, DNA purification was carried out either directly from PCR products (for specific amplicons with the expected size and acceptable amount of DNA) or from gel fragments after electrophoresis separation (for PCR products presenting several DNA fragments in result of non-specific amplification but containing the DNA fragment of the expected size).

Directly from PCR products

DNA purification was performed using the DNA Clean & Concentrator™-5 kit (Zymo Research, USA), following manufacturer´s instructions. Five volumes of DNA binding buffer were added to each volume of DNA sample, in a 2 mL microtube. The samples were vortexed and transferred to a Zymo-Spin™ column in a collection tube. The samples were centrifuged at 16,000 x *g* for 30 seconds and the flow-through was discarded. After that, 200 μL of wash buffer was added to the columns and the samples were centrifuged at 16,000 x *g* for 30 seconds. This wash step was repeated once. The column was transferred to a 1.5 mL microtube and 19 μL of DNA elution buffer was added directly to the column matrix and left at room temperature for 1-2 minutes. The samples were then centrifuged at 16,000 x *g* for 30 seconds to elute the DNA. To confirm if the purification was successful, 1 μL of each elute was analysed by gel electrophoresis.

From gel fragments

DNA extraction and purification from agarose gel slices were performed using Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), following manufacturer´s instructions. After separation by electrophoresis (See 2.5), selected DNA fragments were excised from the agarose gel under UV light with sterile scalpels and transferred into 2 mL microtubes, previously weighed. Three volumes of ADB (Agarose Dissolving Buffer) were added to each volume of agarose excised from the gel and the microtubes (containing the fragments and ADB) were incubated at 55 °C for 5-10 minutes, until the gel was completely dissolved. After complete homogenization, the melted agarose solution containing the DNA was transferred to a Zymo-Spin™ column, in a collection tube, proceeding the protocol as described in the previous section. To confirm the successful purification of the DNA, 1 µl of each eluate was analysed by gel electrophoresis.

2.7 Plasmid cloning of DNA fragments

Since faecal samples, collected from the environment, and highly degenerate primers were used for PCR, it was expected to find some degree of non-specific amplifications, which proved to be the case. Furthermore, some of the PCR products were present in small amounts when observed on agarose gel, not enough for a direct DNA sequencing reaction. Therefore, plasmid cloning was necessary. After purification, these DNA fragments were cloned using the pGEM®-T Easy Vector system protocol (Promega, USA). (Figure 2).

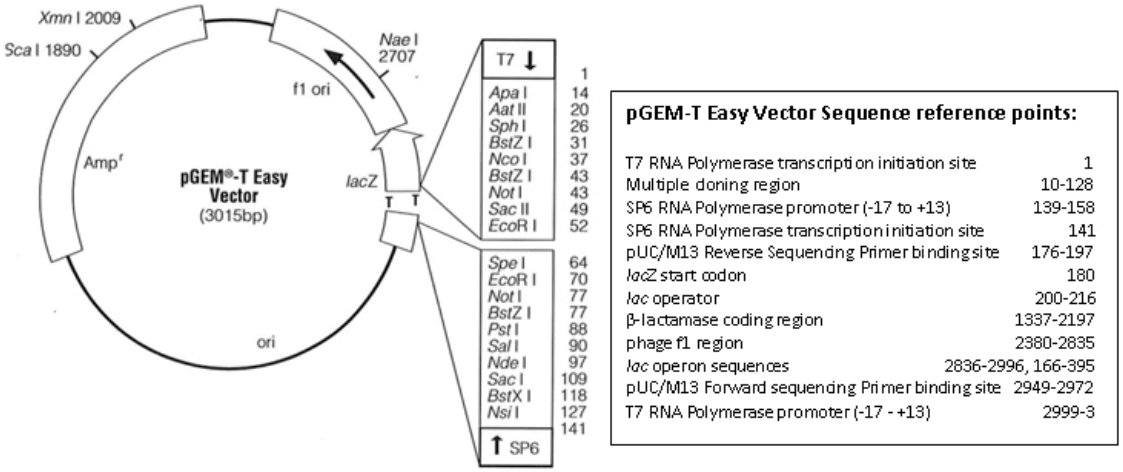


Figure 2 - pGEM®-T Easy Vector map and sequence major reference points (adapted from pGEM®-T and pGEM®-T Easy Vector systems technical manual, Promega).

The pGEM®-T Easy Vector is a linearized plasmid vector with a single 3´-terminal thymidine residue at both ends. This prevents the recircularization of the vector and provides a priming site for the PCR products, when amplified by certain types of polymerases. The PCR fragments generated by the Taq DNA polymerase used have 3´-terminal adenine overhangs which will pair with the complementary 3´ thymidine at the ends of the vector. As seen in Figure 2, the cloning region in the vector is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI. Digestion with any of these enzymes will release the insert, allowing the easy confirmation of the cloning procedure. Moreover, the inserts can be sequenced using the SP6 RNA polymerase promotor or the T7 RNA polymerase promotor primers, which flank the multiple cloning site. The vector has the *bla* gene that encodes the enzyme β-lactamase. β-lactamase (also known as penicillinase) hydrolyses the β-lactam ring in many antibiotics, including ampicillin, which can then be used as a selective marker. The vector also contains the *lacZ* gene that encodes the α peptide of the enzyme β-galactosidase, which hydrolyses X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), an analog of lactose, producing a blue coloured substract. The multiple cloning site is within the coding region of *lacZ* gene. Identification of recombinant clones could therefore be carried out by colour screening on indicator LB plates (See Appendix 1), containing ampicillin, IPTG (isopropyl-β-D-1-

thiogalactopyranoside) and X-Gal (IPTG is a molecular mimic of allolactose and triggers transcription of the lac operon, being used to induce expression of genes under the control of the *lac* operator, as *lacZ*). Since the vector has the *bla* gene, it allows the selection of the bacterial clones transformed with the plasmid as they will be resistant to ampicillin. Inactivation of the β-galactosidase gene can occur if a foreign DNA fragment is inserted, disrupting the open reading frame. As consequence of that, β-galactosidase is not expressed and therefore it will not hydrolyse X-Gal. These recombinant bacterial clones appear as white colonies on the supplemented LB agar plates.

2.7.1 Ligation protocol

Ligation reactions were setup in a 10 μL reaction volume, which contained 5 μL of 2x Rapid ligation buffer, 0.75 μL of pGEM®-T Easy Vector (50 ng /μL), 1 μL of T4 DNA ligase (3 Weiss units/μl) and 3.25 μL of the purified PCR product. The reactions were mixed by pipetting and incubated at 4 °C overnight, with the aim of increasing the efficiency of ligation producing a greater number of transformants.

2.7.2 Preparation of competent *Escherichia coli* cells and transformation by heat shock

After the ligation procedure, *E. coli* JM109 cells were prepared for the transformation step. These cells were inoculated in 2 mL LB medium (see Appendix 1) and incubated overnight at 37 °C at 220 rpm (orbital incubator Infors HT, Switzerland). The next morning, 200 μL of the cellular suspension was transferred to a nephelo culture flask with 20 mL of LB fresh medium and incubated again under the same conditions. Bacterial growth was assessed by measuring optical density at 600 nm until a value of 0.4-0.5 was reached (exponential growth phase). The suspension was transferred to a 50 mL Falcon tube and centrifuged at 4 °C and 4,000 rpm (Centrifuge 5810R, Eppendorf, Germany) for 20 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of cold TSS medium (see Appendix 1). 5 μL of the ligation mixture previously prepared (see previous section) was added to 100 μL of cellular suspension in TSS. At the same time, two transformation controls were also prepared: in the positive control, 1 μL of a concentrated solution of ultrapure DNA of a reference

plasmid (pBluescript II KS+, Stratagene, USA) was added instead of the ligation mixture and in the negative control the DNA was replaced by 5 μL sterile deionized water. The cells were gently mixed to homogenize and left on ice for 30 minutes. After that, the cells were heat-shocked by placing the mixture in a water bath at 42 °C for 90 seconds, immediately returning to ice for 5 minutes. Then, 900 μL of LB medium (preheated at 37 °C) was added and the mixtures were placed in an ornital shaker (Unimax 1010, Heidolph, Germany) at 37 °C and 80 rpm, for 1 hour, so that cells could recover from the heat shock (the recovery period allows time for the plasmid to be incorporated and the antibiotic resistance to be expressed in the cells). After the incubation, the cell suspensions were centrifuged at 6,000 rpm (Centrifuge 5415D, Eppendorf, Germany) for 10 minutes. 100 μL of each transformation culture was added (in triplicates) to LB agar plates containing ampicillin (100 μg/mL), X-Gal (40 μg/mL) and IPTG (0.2 mM) and spread evenly over the surface with sterile glass spheres. The plates were incubated upside down, to prevent condensation, at 37 °C overnight.

2.7.3 Screening of transformants for inserts and DNA extraction by alkaline lysis

As stated before, successful cloning of the PCR amplicons into the plasmid can be recognized by blue/white colour screening of the bacterial colonies. After selecting the presumed recombinant clones (forming white colonies on the indicator plates), the plasmid DNA must be extracted to check for the presence of the correct insert. Individual colonies were scraped up with a sterile toothpick (6 colonies per plate) and immersed in a 15 mL sterile tube containing 2.5 mL of LB medium supplemented with ampicillin (final concentration of 100 μg/mL). The tubes were incubated overnight on an orbital shaker at 37 °C and 220 rpm (orbital incubator Infors HT, Switzerland) for bacterial growth. The following day, 2 mL of each cell culture was transferred to a microtube and centrifuged at 13,000 rpm (Centrifuge 5417C, Eppendorf, Germany) for one minute. The supernatant was discarded and the cell pellet was resuspended in 250 μL of TEG buffer (see Appendix 1) and mixed by vortex. Then, 250 μL of lysis solution (see Appendix 1) was added to promote bacterial cell lysis and the microtubes were gently inverted a few times. 250 μL of potassium acetate solution (3M, pH 4.8) was added and the mixtures were mixed gently by inversion. This allows the neutralization of the highly alkaline medium and, consequently, the renaturing of plasmid DNA, but

not chromosomal DNA. The plasmid DNA is, therefore, separated from the bacterial DNA, which is precipitated out of solution. Potassium acetate also removes the detergent (SDS) from the solution. The mixtures were centrifuged at 13,000 rpm for 15 minutes at 4º C (Centrifuge 3K18, Sigma, Germany) and the supernatant (containing the plasmid DNA) was transferred to a sterile microtube. 700 μL of isopropanol was added to each microtube to precipitate the nucleic acids and the solutions were gently inverted a few times to homogenize before being centrifuged at 13,000 rpm (Centrifuge 5415D Eppendorf, Germany) for 30 minutes to collect the DNA. The supernatant was gently discarded and the pellet washed with 250 μL of 70% (v/v) ethanol. The solution was centrifuged again at 13,000 rpm for 5 minutes and the supernatant was carefully discarded. The pellet, containing the plasmid DNA, was vacuum-dried for 5 minutes (Concentrator 5301, Eppendorf, Germany). The dried sediment was finally resuspended in 50 μL of TE (see Appendix 1) supplemented with RNase A (final concentration of 50 μg/mL) and incubated at 37 °C for 30 minutes for RNA degradation. The samples were stored at -20 °C, until use.

2.7.4 Plasmid DNA observation in agarose gel

Electrophoresis was carried out on a 1.2% (w/v) agarose gel in 0.5x TAE buffer, containing 0.5 μg/mL of ethidium bromide. 6 μL of each sample (in loading buffer 10x, (see Appendix 1) was loaded into the wells of the gel, with 1 μL of a known size unlinearized plasmid vector (internal control) for easier size estimation of the bands. The electrophoresis was run for 90 minutes at 80 V and the gel was visualized under 302 nm UV light. Selection of potentially recombinant plasmids was based on the comparison of the migration patterns presented by the different purified plasmids. Molecules with delayed migration were selected for hydrolysis with the restriction enzyme EcoRI.

2.7.5 Enzymatic hydrolysis of plasmid DNA

As stated before, the pGEM®-T Easy Vector (Promega, USA) cloning region is flanked by recognition sites for the restriction enzymes EcoRI, BstZI and NotI (Figure 2), allowing the excision of the inserts. The enzyme selected in this work was EcoRI. The digestions were performed in 15 μL reaction volumes, containing 4 μL of pDNA

solution, 1.5 µL of EcoRI buffer 10x (see Appendix 1), 1 µL of EcoRI (Thermo Fisher, USA) (10 U/µl) and 8.5 µL of nuclease-free deionized water. The microtubes were incubated at 37 ℃ for three hours. After incubation, electrophoresis was carried out on a 1.2% (w/v) agarose gel in 0.5x TAE buffer, containing 0.5 µg/mL of ethidium bromide. 10 µL of each sample plus loading buffer 10x (10:1) was placed into the wells of the gel, with 2 µL of NZYDNA Ladder VI (NZYTech, Portugal) for size determination. The electrophoresis was run for 60-75 minutes at 100 V and the gel was visualized under 302 nm UV light. If the expected bands, i.e. the insert (~ 320 bp) and the linearized vector (~ 3000 bp), were observed on the agarose gel, further extraction and purification of the plasmid DNA from the corresponding bacterial clones were carried out, using a commercial kit (see next section).

2.7.6 Plasmid DNA extraction and purification for sequencing

This preparation of the plasmid DNA for nucleotide sequencing was carried out using the DNA Clean & Concentrator™ 5 kit (Zymo Research, USA). Two volumes of DNA binding buffer were added to each volume of plasmid DNA sample, extracted in 2.7.3, in a 0.5 mL microtube. From this point on, the purification protocol proceeded fundamentally as previously described for PCR products in section 2.6 (DNA purification. The plasmid DNA was eluted in 19 µL of DNA elution buffer and 15 µL of the samples was stored at -20 ºC in a 0.5 mL microtube to send for DNA sequencing. To confirm if the purification step was successful, 1 µL of each eluate was run on a 1.2% (w/v) agarose gel in 0.5x TAE buffer, under standard conditions previously described.

2.8 DNA sequencing

15 µL of each purified sample, containing a minimum of 400 ng or 1 µg of DNA per sequencing reaction, respectively, for PCR products and recombinant plasmids, was placed into 1.5 mL microtubes and sent for nucleotide by STAB VIDA (Portugal), with chain-terminating inhibitors, as described by Sanger *et al*. (1977). The primers used for sequencing the fragments obtained from the amplification of the DNA polymerase gene were AdVR_polFin (Table 2), for PCR products, at 50 pmol/sequencing reaction, or the universal primer T7 FwdpGEM (Figure 2), for cloned amplicons.

2.9 Bioinformatic analysis of DNA sequences

2.9.1 Chromatogram analysis and editing of sequences

The nucleotide sequences were edited before phylogenetic analysis. The corresponding chromatograms were carefully analysed and sequences edited in the BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999). Regions with overlapping peaks were analysed closely and sequences were corrected when needed. The regions of the sequences corresponding to primer binding - at the 5´and 3´ends - were removed. The plasmid sequences were also removed, when editing cloned sequences. The edited sequences were then compared to previously known sequences deposited in the GenBank from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990), to confirm their identity.

2.9.2 Alignment of sequences and phylogenetic analysis

The classification of the AdV DNA sequences obtained in this study was performed by phylogenetic analysis. The sequences of the DNA polymerase gene obtained from the reptile's faecal samples were used, with homologous and reference sequences, for the construction of phylogenetic trees. Reference sequences from AdVs representing five genera (including from the proposed genus *Testadenovirus*) were retrieved from the GenBank database. Most similar sequences obtained from the BLAST search were also used. A total of 86 sequences were used for the construction of the phylogenetic trees. The GenBank access numbers of the AdV reference sequences used in the analysis are listed in Appendix 3. Alignment of the sequences was performed using the Multiple Alignment using Fast Fourier Transform (MAFFT) program version 7 (Katoh *et al.*, 2002; Katoh & Standley, 2013). Phylogenetic trees were constructed in the Molecular Evolutionary Genetics Analysis (MEGA) version 5 program (Tamura *et al*., 2011). Three phylogenetic trees based on nucleotide sequences were constructed using different combinations of methods and substitution models for the determination of genetic distance matrices. The first tree was based on the neighbor-joining method (Saitou & Nei, 1987), using the Kimura 2-parameter substitution model (Kimura, 1980). Two additional trees were based on the maximum likelihood method - one with application of the 2-parameter model of Kimura and the other with Tamura-Nei

algorithm (Tamura & Nei, 1993). The nucleotide sequences were then translated into amino acid sequences with the European Bioinformatics Institute - EMBL-EBI (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/). Two phylogenetic trees of the amino acidic sequences were constructed; one based on the neighbor-joining method and the other on the maximum likelihood method, both using the Dayhoff algorithm as amino acid substitution model (Dayhoff *et al*., 1978). The reliability of the inferred tree topologies was evaluated by bootstrap analysis (Felsenstein, 1985) of 1,000 replicates of the original sequence data. Bootstrap values equal or greater than 70% were considered to be significant.

2.10 Confirmation of host species of faecal specimens by cytochrome b gene sequence analysis

A PCR for the partial amplification of the mitochondrial cytochrome b gene was carried out on all the positive samples for the presence of AdV DNA, in order to confirm the host species, using a protocol previously described (Kocher *et al*., 1989; Irwin *et al*., 1991). The primer pair L14724_for/H15149_rev (Table 3) was used to amplify a fragment of 424 bp from DNA isolated from the reptile samples. PCR amplification was performed in 25 μL of reaction volume, which contained 12.5 μL of NZYTaq 2× Green Master Mix solution (NZYTech, Portugal), 1 μL of each primer (final concentration 0.4 μM), 5.5 μL of deionized water and 5 μL of purified DNA solution from the faecal samples. Amplification was conducted in a PCR Express thermal cycler (Thermo Hybaid, United Kingdom) with the following conditions: 95 °C for 6 minutes, 40 cycles of 95 °C for 40 seconds, 50 °C for 60 seconds and 72 °C for 45 seconds, and further extension at 72 °C for 5 minutes. A negative control was performed in each amplification experiment.

Table 2 - PCR primers (Irwin *et al*., 1991) used for the amplification of cytochrome b gene.

Primer name	**Sequence**
L14724_for	5’-CGAAGCTTGATATGAAAAACCATCGTTG-3’
H15149_rev	5’-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3’

Analysis of the amplification products was performed by electrophoresis carried out on a 2% (w/v) agarose gel in 0.5x TAE buffer, containing 0.5 μg/mL of ethidium bromide. 5 μL of the PCR samples were analysed along with 2 μL of molecular marker NZYDNA Ladder VI (NZYTech, Portugal) for size determination. The electrophoresis was run for about one hour at 100 V and the gel was visualized under 302 nm UV light, with the Gel Doc™ XR+ Gel Documentation System (Bio-Rad, USA). The amplicons of interest (~ 424 bp) were purified with the commercial kits DNA Clean & Concentrator™-5 or Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA), depending on the band pattern on the gel (specific vs. unspecific amplification). Purification protocols were performed according to the manufacturer's instructions, previously described in section 2.6 (DNA purification). To confirm if the purification was successful, 1 μl of each purified sample was analysed by electrophoresis. The samples were finally sent for nucleotide sequencing under the conditions described in section 2.8. (DNA sequencing) with primer L14724_for (50 pmol/sequencing reaction).

3. RESULTS AND DISCUSSION

3. Results and discussion

3.1 Adenovirus screening using nested PCR

For this study, 120 faecal samples from 43 reptile species were screened for presence of AdV genome. After DNA extraction and purification, a nested PCR was carried out targeting a conserved region of the DNA-dependent DNA polymerase gene to produce amplicons ranging from 318-324 bp (Wellehan *et al*., 2004). Analysis of PCR products on agarose gel revealed complex migration patterns and some non-specific bands due to the use of highly degenerated primers (Table 2) and also the nature of the samples (faecal samples, collected from the ground/water). Nonetheless, 59 samples presented bands (with sizes around 320 bp) compatible with the expected amplified coding sequence of the DNA polymerase gene. Therefore, the preliminary adenoviral detection rate was 49.2% (59/120).

We cannot be sure that failure to generate PCR amplicons from the other samples was due to poor quality or very low amounts of matrix DNA and/or the presence of PCR inhibitors, very frequently found in faecal samples. Similarly, we cannot affirm that all the samples that presented bands in the expected region are positive for AdV, without further investigation. So, all the amplicons that were putatively considered positive were selected for confirmation by DNA sequencing, either directly from PCR reaction mix or after purification from the gel, depending on the migration pattern observed by electrophoresis.

As seen in the Figure 3, as an example of the obtained results, the samples ZLX227, ZLX228, ZLX231, ZLX233, ZLX234, ZLX235 and ZLX236 (highlighted with a red circle) presented compatible bands with the amplicon from coding sequence of the DNA polymerase gene. The samples ZLX227 and ZLX228 belong to Jamaican boas; the samples ZLX231, ZLX233 and ZLX234 belong to Madagascar tree boas; the sample ZLX235 belong to an eastern kingsnake and the sample ZLX236 belong to a jungle carpet python. These samples, at first, were considered positive. Later on, the analysis of the nucleotide sequence confirmed the specific presence of AdV DNA only for ZLX227, ZLX233 and ZLX234.

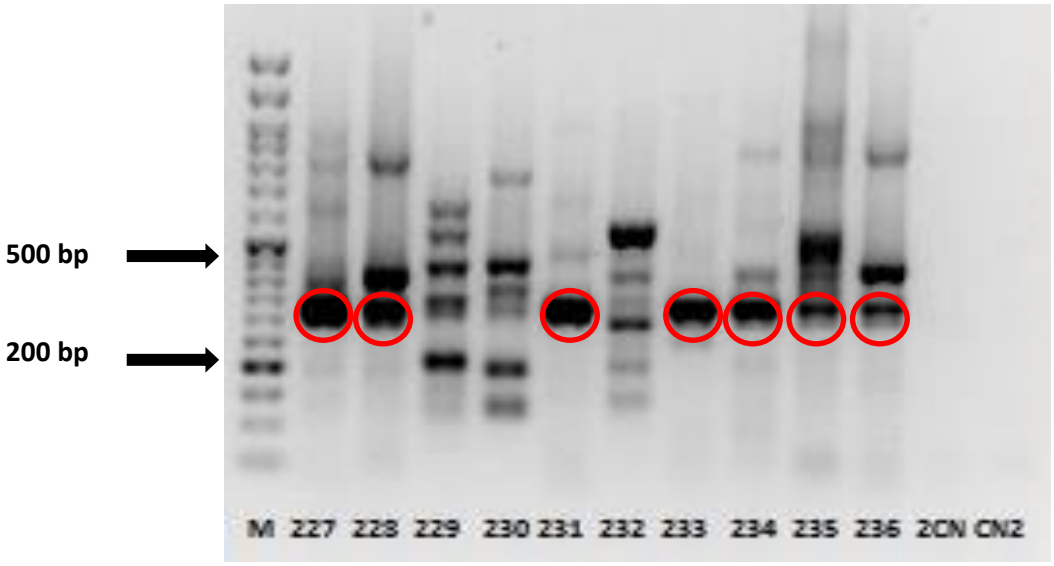


Figure 3 - Results of the nested PCR amplification of a portion of the AdV DNA polymerase gene after separation by electrophoresis in agarose gel. The samples (labelled at the bottom of the photo) were obtained from DNA extracted from reptile faeces. The molecular marker used was the NZYDNA Ladder VI (NZYTech, Portugal)- labelled as M in the pictures- which produces a pattern of bands ranging from 50 to 1500 bp. The 200 and 500 bp bands stain brighter/darker to help identification and are identified by arrows. 2CN and CN2 are negative controls.

All the samples that were considered positive were sent for sequencing.

3.2 Molecular cloning of amplicons on plasmid vector

Some of the DNA sequences from PCR products presented low quality and difficult to interpret chromatograms. This could be due to several reasons such as contamination with other DNAs, small quantities of the desired DNA, the presence of inhibitors in the sequencing reaction or other technical reasons. It is noteworthy that for some DNA samples it was not possible to obtain any nucleotide sequence of acceptable quality, despite the various attempts done. For such problematic samples (n=5), trying to obtain larger quantities of the DNA in pure form, plasmid cloning was performed. This way we could expect to obtain more copies of the DNA fragments of interest and better-quality chromatograms. DNA cloning is performed using recombinant DNA techniques. The DNA fragment of interest is linked to a vector that replicates within a host cell, in this work, a plasmid (see section 2.7, in the previous chapter) and a bacterial cell, respectively. The cloned fragment is replicated with the vector, resulting in large copies of identical DNA molecules that can be purified and sequenced.

After the cloning procedure, the putative recombinant plasmid DNA, purified from bacterial clones on selective agarose plates, was digested with a restriction enzyme

(EcoRI) and run on agarose gel. Two bands were expected per sample, one for the linearized vector plasmid (3015 bp) (see Figure 2) and other for the insert, which was expected to be approximately 320 bp.

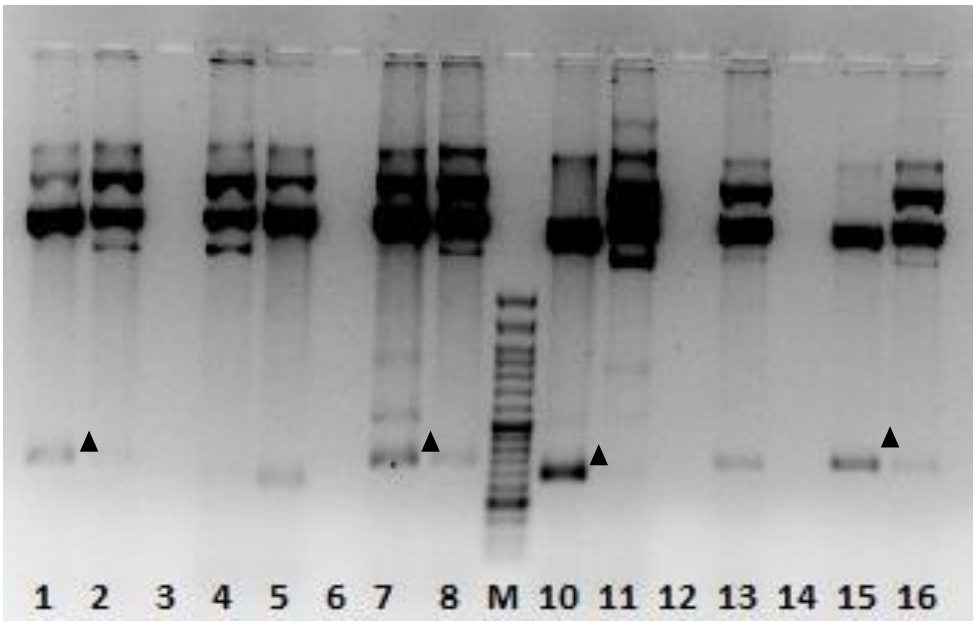


Figure 4 - Migration patterns, af6ter separation by agarose gel electrophoresis, of the plasmid DNA from putative recombinant clones digested with EcoRI. Lane 1- 187.3 (=sample/clone identification); lane 2- 187.6; lane 4- 202.3; lane 5- 202.5; lane 7- 203A.3; lane 8- 203A.4; lane M- molecular marker (NZYDNA Ladder VI, NZYTech, Portugal); lane 10- 203B.3; lane 11- 203B.4; lane 13- 226.3; lane 15- 269.3 and lane 16- 269.4. The molecular marker produces a pattern of bands ranging from 50 to 1500 bp. The 200 and 500 bp bands stain brighter/darker to help identification.

As an example, Figure 4 shows the results of the EcoRI digestion of the putative recombinant plasmids from reptile faecal samples. As it can be seen, the inserts do not all have the same size, ranging from around 250-450 bp (arrow heads), which shows the heterogeneity of the fragments cloned. Although some inserts can be seen (e.g. 1, 7, 8, 10, 13, 15), the digestion was not complete for all the clones. This partial or incomplete digestion can be due to many reasons: not enough units of enzyme used, enzyme not working at its maximum efficiency, digestion time too short and/or salt inhibition, among others. Moreover, under non-optimal conditions, the enzyme may present non-specific cutting, which results in fragments different than expected. When the procedure results in both digested and non-digested plasmids, as in Figure 4, each with a different size and mobility, it shows on the gel as multiple bands, instead of only one for the linearized vector. In this experiment, despite the complex digestion pattern observed, it

was possible to select some clones presenting bands with the predicted insert size that were sent for DNA sequencing, after new round of plasmid DNA extraction and purification. On the whole, seven recombinant plasmids corresponding to five faecal samples were sent for sequencing.

3.3 Bioinformatic analysis of DNA sequences

3.3.1 Chromatogram analysis, editing and identity search of sequences

As stated before, the samples that showed PCR amplicons with compatible size after migration by agarose gel electrophoresis were considered presumably positive and the DNA was sent for sequencing. The DNA sequencing was carried out directly from the purified PCR products, from products excised from agarose gels or after cloning in a vector plasmid. A total of 88 sequencing reactions from 59 samples (including seven recombinant plasmids corresponding to five faecal samples) were set up.

The chromatograms and sequences received from STAB VIDA (Caparica, Portugal) were analysed. Sometimes, a clear reading was complicated because some chromatograms presented 5' ends with very low resolution and/or background low-intensity signals ("background noises") due respectively to technical reasons and, most probably, to the presence of contaminating DNA. The 5' and 3'-end regions, corresponding to the PCR primers, were removed before further analysis and, whenever necessary, the sequences were manually edited with the help of BioEdit software. After editing, the BLAST application from NCBI was used to presumptively identify the sequences, since it would perform a search for regions of local similarity against sequences in the GenBank. BLAST makes a comparison between the submitted sequence (query) and the sequences deposited in the database, showing the percentage of similarity between them. Furthermore, it also estimates the probability of the similarity found being merely due to chance (E value, between 0-1). The sequences in the GenBank with the highest similarity (%) were retrieved (presented in Appendix 4).

Not all the PCR products were of adenoviral origin. Sequencing of the PCR products led to the detection of 34 samples with AdV DNA, leading to a detection rate of 57.6% (34/59), in the putatively positive samples, and 28.3% (34/120), when considering the whole set of samples.

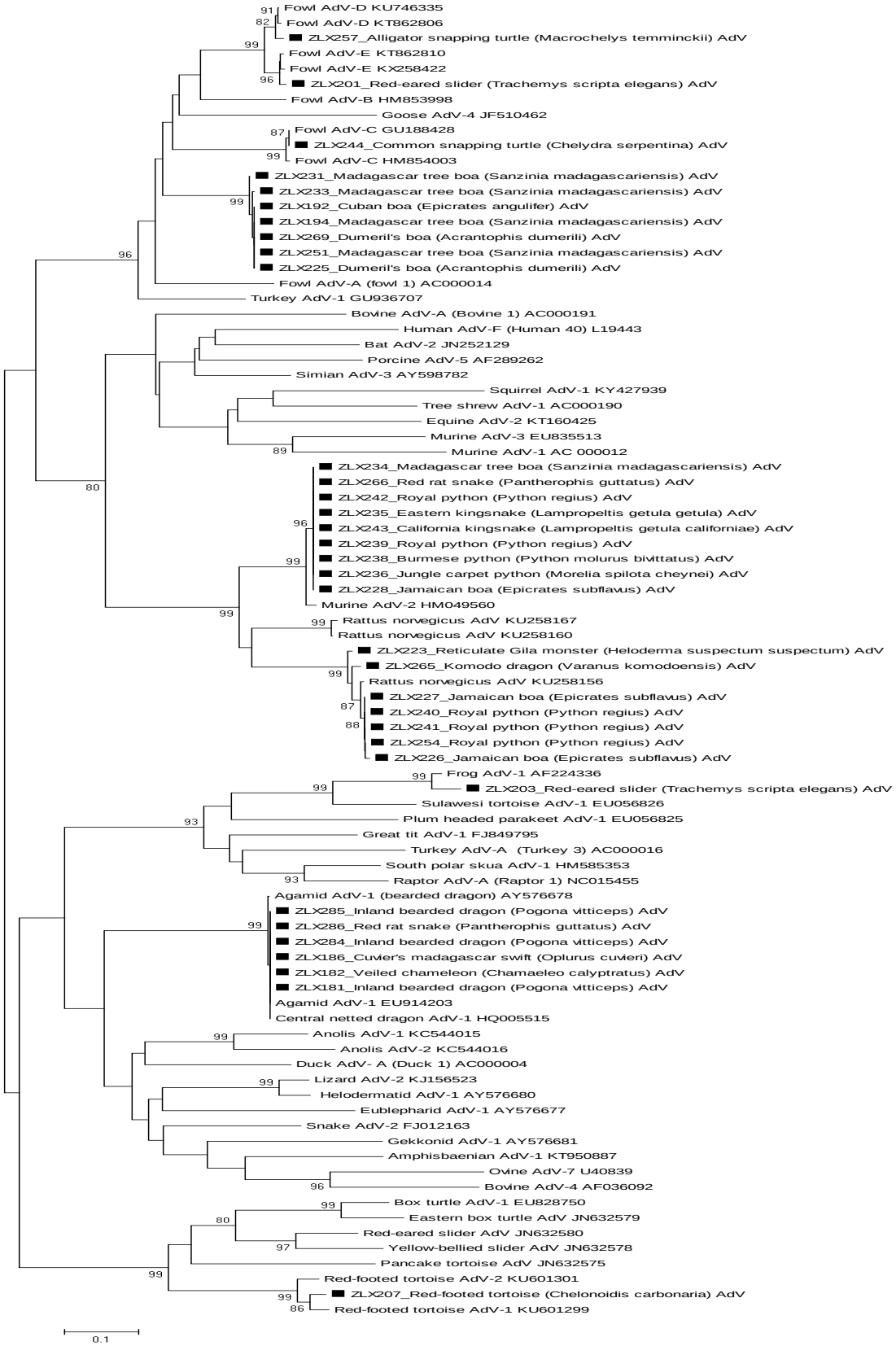
Comparing the partial DNA polymerase sequences of AdVs found in reptiles from the Lisbon Zoo with those deposited in the GenBank, the number of samples with a sequence similarity to known sequences greater than 90% is considerable (27/34), with some presenting 100% similarity. Below are some of the results from these comparisons. The AdV detected in a Jamaican boa (ZLX227) had the highest identity value (99%) of the partial DNA polymerase sequence with an analogous sequence from *Rattus norvegicus*, the common rat, what was considered, at first, a rather unexpected result. The AdV detected in samples from the inland bearded dragon (ZLX181, ZLX284 and ZLX285), the veiled chameleon (ZLX182), the Cuvier´s Madagascar swift (ZLX186), and red rat snake (ZLX286) all demonstrated 100% sequence similarity with agamid lizards AdVs. Four of the five positive Madagascar tree boa samples - ZLX194, ZLX231, ZLX233 and ZLX251 - demonstrated highest identity with fowl AdVs. The remaining Madagascar tree boa sequence - ZLX234 - showed 97% sequence similarity with murine AdV-2. Two turtle samples - common snapping turtle (ZLX244) and alligator snapping turtle (ZLX257) - showed the highest identity with fowl AdVs (99%). The AdV detected in the red-footed tortoise (ZLX207) demonstrated a 94% similarity of the partial DNA polymerase sequence with a previously described AdV-1 isolate from the same species.

There was one result that appear to be out of the ordinary. The sample ZLX270, from a Brazilian rainbow boa, originated a sequence showing 100% similarity with human adenovirus C. One possible explanation for this is contamination of equipment and/or the laboratory station where the procedures were carried out. This sequence was not considered for this study. For the remaining 24 samples, the BLAST identity search mainly revealed amplification of DNA of bacterial origin, from commensal bacteria of animal gut microbiota, or from the reptilian species itself.

3.3.2 Phylogeny of reptilian adenoviruses

Phylogenetic trees were constructed to analyse the evolutionary relationships between the AdVs sequences obtained in this study and homologous sequences retrieved from the GenBank. A terminology was established to present the new sequences in the phylogenetic trees that included: ZLX (from the “Lisbon Zoo“), sample number, common name and scientific name of the host. A total of 86 partial sequences of the

DNA polymerase gene were considered for the construction of the phylogenetic trees shown in Figure 5 (52 corresponding sequences taken from the databases were added to the 34 DNA polymerase sequences from this study). As previously stated, five phylogenetic trees were calculated, based on nucleotide or amino acid alignments, and also on different combinations of methods and substitution models for the determination of genetic distance matrices, and two of them are presented.


Fowl AdV-D KU746335
Fowl AdV-D KT862806
ZLX257_Alligator snapping turtle (Macrochelys temminckii) AdV
Fowl AdV-E KT862810
Fowl AdV-E KX258422
ZLX201_Red-eared slider (Trachemys scripta elegans) AdV
Fowl AdV-B HM853998
Goose AdV-4 JF510462
Fowl AdV-C GU188428
ZLX244_Common snapping turtle (Chelydra serpentina) AdV
Fowl AdV-C HM854003
ZLX231_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX233_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX192_Cuban boa (Epicrates angulifer) AdV
ZLX194_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX269_Dumeril's boa (Acrantophis dumerili) AdV
ZLX251_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX225_Dumeril's boa (Acrantophis dumerili) AdV
Fowl AdV-A (fowl 1) AC000014
Turkey AdV-1 GU936707
Bovine AdV-A (Bovine 1) AC000191
Human AdV-F (Human 40) L19443
Bat AdV-2 JN252129
Porcine AdV-5 AF289262
Simian AdV-3 AY598782
Squirrel AdV-1 KY427939
Tree shrew AdV-1 AC000190
Equine AdV-2 KT160425
Murine AdV-3 EU835513
Murine AdV-1 AC 000012
ZLX234_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX266_Red rat snake (Pantherophis guttatus) AdV
ZLX242_Royal python (Python regius) AdV
ZLX235_Eastern kingsnake (Lampropeltis getula getula) AdV
ZLX243_California kingsnake (Lampropeltis getula californiae) AdV
ZLX239_Royal python (Python regius) AdV
ZLX238_Burmese python (Python molurus bivittatus) AdV
ZLX236_Jungle carpet python (Morelia spilota cheynei) AdV
ZLX228_Jamaican boa (Epicrates subflavus) AdV
Murine AdV-2 HM049560
Rattus norvegicus AdV KU258167
Rattus norvegicus AdV KU258160
ZLX223_Reticulate Gila monster (Heloderma suspectum suspectum) AdV
ZLX265_Komodo dragon (Varanus komodoensis) AdV
Rattus norvegicus AdV KU258156
ZLX227_Jamaican boa (Epicrates subflavus) AdV
ZLX240_Royal python (Python regius) AdV
ZLX241_Royal python (Python regius) AdV
ZLX254_Royal python (Python regius) AdV
ZLX226_Jamaican boa (Epicrates subflavus) AdV
Frog AdV-1 AF224336
ZLX203_Red-eared slider (Trachemys scripta elegans) AdV
Sulawesi tortoise AdV-1 EU056826
Plum headed parakeet AdV-1 EU056825
Great tit AdV-1 FJ849795
Turkey AdV-A (Turkey 3) AC000016
South polar skua AdV-1 HM585353
Raptor AdV-A (Raptor 1) NC015455
Agamid AdV-1 (bearded dragon) AY576678
ZLX285_Inland bearded dragon (Pogona vitticeps) AdV
ZLX286_Red rat snake (Pantherophis guttatus) AdV
ZLX284_Inland bearded dragon (Pogona vitticeps) AdV
ZLX186_Cuvier's madagascar swift (Oplurus cuvieri) AdV
ZLX182_Veiled chameleon (Chamaeleo calyptratus) AdV
ZLX181_Inland bearded dragon (Pogona vitticeps) AdV
Agamid AdV-1 EU914203
Central netted dragon AdV-1 HQ005515
Anolis AdV-1 KC544015
Anolis AdV-2 KC544016
Duck AdV- A (Duck 1) AC000004
Lizard AdV-2 KJ156523
Helodermatid AdV-1 AY576680
Eublepharid AdV-1 AY576677
Snake AdV-2 FJ012163
Gekkonid AdV-1 AY576681
Amphisbaenian AdV-1 KT950887
Ovine AdV-7 U40839
Bovine AdV-4 AF036092
Box turtle AdV-1 EU828750
Eastern box turtle AdV JN632579
Red-eared slider AdV JN632580
Yellow-bellied slider AdV JN632578
Pancake tortoise AdV JN632575
Red-footed tortoise AdV-2 KU601301
ZLX207_Red-footed tortoise (Chelonoidis carbonaria) AdV
Red-footed tortoise AdV-1 KU601299
0.1

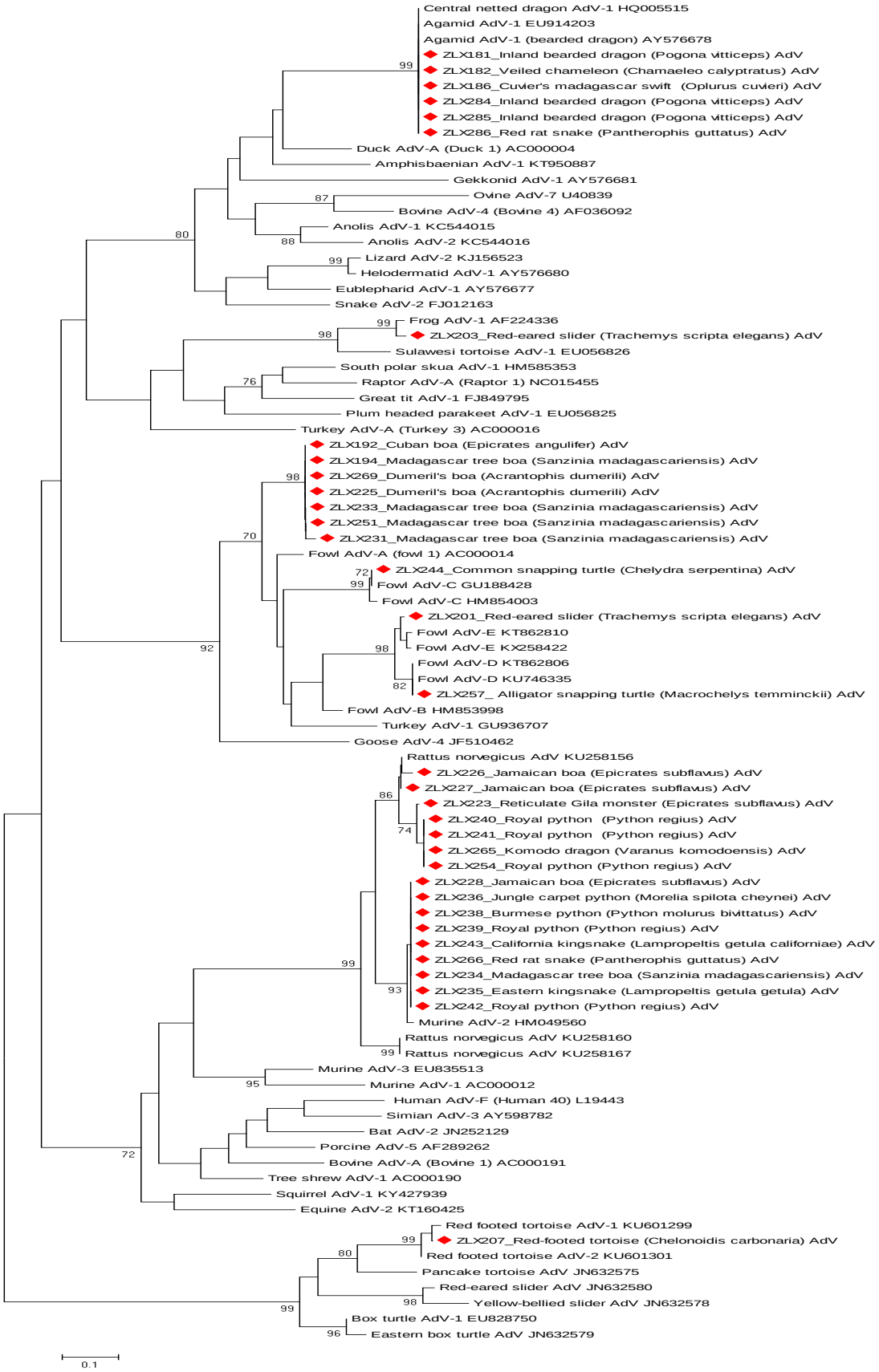

Central netted dragon AdV-1 HQ005515
Agamid AdV-1 EU914203
Agamid AdV-1 (bearded dragon) AY576678
ZLX181_Inland bearded dragon (Pogona vitticeps) AdV
ZLX182_Veiled chameleon (Chamaeleo calyptratus) AdV
ZLX186_Cuvier's madagascar swift (Oplurus cuvieri) AdV
ZLX284_Inland bearded dragon (Pogona vitticeps) AdV
ZLX285_Inland bearded dragon (Pogona vitticeps) AdV
ZLX286_Red rat snake (Pantherophis guttatus) AdV
Duck AdV-A (Duck 1) AC000004
Amphisbaenian AdV-1 KT950887
Gekkonid AdV-1 AY576681
Ovine AdV-7 U40839
Bovine AdV-4 (Bovine 4) AF036092
Anolis AdV-1 KC544015
Anolis AdV-2 KC544016
Lizard AdV-2 KJ156523
Helodermatid AdV-1 AY576680
Eublepharid AdV-1 AY576677
Snake AdV-2 FJ012163
Frog AdV-1 AF224336
ZLX203_Red-eared slider (Trachemys scripta elegans) AdV
Sulawesi tortoise AdV-1 EU056826
South polar skua AdV-1 HM585353
Raptor AdV-A (Raptor 1) NC015455
Great tit AdV-1 FJ849795
Plum headed parakeet AdV-1 EU056825
Turkey AdV-A (Turkey 3) AC000016
ZLX192_Cuban boa (Epicrates angulifer) AdV
ZLX194_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX269_Dumeril's boa (Acrantophis dumerili) AdV
ZLX225_Dumeril's boa (Acrantophis dumerili) AdV
ZLX233_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX251_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX231_Madagascar tree boa (Sanzinia madagascariensis) AdV
Fowl AdV-A (fowl 1) AC000014
ZLX244_Common snapping turtle (Chelydra serpentina) AdV
Fowl AdV-C GU188428
Fowl AdV-C HM854003
ZLX201_Red-eared slider (Trachemys scripta elegans) AdV
Fowl AdV-E KT862810
Fowl AdV-E KX258422
Fowl AdV-D KT862806
Fowl AdV-D KU746335
ZLX257_ Alligator snapping turtle (Macrochelys temminckii) AdV
Fowl AdV-B HM853998
Turkey AdV-1 GU936707
Goose AdV-4 JF510462
Rattus norvegicus AdV KU258156
ZLX226_Jamaican boa (Epicrates subflavus) AdV
ZLX227_Jamaican boa (Epicrates subflavus) AdV
ZLX223_Reticulate Gila monster (Epicrates subflavus) AdV
ZLX240_Royal python (Python regius) AdV
ZLX241_Royal python (Python regius) AdV
ZLX265_Komodo dragon (Varanus komodoensis) AdV
ZLX254_Royal python (Python regius) AdV
ZLX228_Jamaican boa (Epicrates subflavus) AdV
ZLX236_Jungle carpet python (Morelia spilota cheynei) AdV
ZLX238_Burmese python (Python molurus bivittatus) AdV
ZLX239_Royal python (Python regius) AdV
ZLX243_California kingsnake (Lampropeltis getula californiae) AdV
ZLX266_Red rat snake (Pantherophis guttatus) AdV
ZLX234_Madagascar tree boa (Sanzinia madagascariensis) AdV
ZLX235_Eastern kingsnake (Lampropeltis getula getula) AdV
ZLX242_Royal python (Python regius) AdV
Murine AdV-2 HM049560
Rattus norvegicus AdV KU258160
Rattus norvegicus AdV KU258167
Murine AdV-3 EU835513
Murine AdV-1 AC000012
Human AdV-F (Human 40) L19443
Simian AdV-3 AY598782
Bat AdV-2 JN252129
Porcine AdV-5 AF289262
Bovine AdV-A (Bovine 1) AC000191
Tree shrew AdV-1 AC000190
Squirrel AdV-1 KY427939
Equine AdV-2 KT160425
Red footed tortoise AdV-1 KU601299
ZLX207_Red-footed tortoise (Chelonoidis carbonaria) AdV
Red footed tortoise AdV-2 KU601301
Pancake tortoise AdV JN632575
Red-eared slider AdV JN632580
Yellow-bellied slider AdV JN632578
Box turtle AdV-1 EU828750
Eastern box turtle AdV JN632579
0.1

Figure 5 - Phylogenetic trees of partial AdV DNA polymerase nucleotide (A) or amino acid (B) sequences, based on alignments of 220 nucleotide or 62 amino acid positions, respectively, including a total of 86 sequences. All the alignment position gaps were deleted (complete deletion). The percentage of replicate trees in which the associated taxa were grouped together in the bootstrap test (1,000 replicates) is shown next to the branches and only values considered significant (>70%) are presented. The trees are drawn to scale, with branch length proportional to the calculated evolutionary distances (the scale indicates 10% of genetic distance). The sequences in study are highlighted with visual markers: black squares, for nucleotide sequences, and red lozenges, for amino acid sequences. The trees were constructed by (A) the neighbor-joining method, based on the Kimura 2-parameter model. (B) the neighbor-joining method, based on the Dayhoff matrix based model.

As seen in the Figure 5 (A and B), the 34 new sequences from this study are distributed all over the trees in distinct clusters of sequences, proving the existence of a high degree of genetic diversity on the AdVs circulating in the premises of the Lisbon Zoo. The trees display five significant and well defined monophyletic groups that correspond to four AdV genera - *Atadenovirus*, *Siadenovirus*, *Mastadenovirus*, *Aviadenovirus* - and the proposed genus *Testadenovirus* (Doszpoly *et al*., 2013), all of which containing at least one ZLX sequence.

Reference sequences of AdVs detected from turtles and tortoises belonging to the superfamily Testudinoidea (box turtles, sliders and tortoises) cluster closely together, with high significance, separately from the currently accepted AdV genera. These AdVs represent the newly proposed genus - *Testadenovirus* - and it can be seen clearly in these trees. Five samples (ZLX201, ZLX203, ZLX207, ZLX244 and ZLX257) from four different species of the order Testudines were positive for AdV DNA. However, only the sequence ZLX207 (red-footed tortoise) was classified as originating from a testadenovirus. This is only the third time an AdV is detected in this tortoise species and, along with the other two previously described sequences (red-footed tortoise adenovirus 1, KU601299, and red-footed tortoise adenovirus 2, KU601301) they may represent a novel testadenovirus species (candidate species 4). Three candidate species (1-3) have already been proposed for this genus (Harrach, 2014), all represented in the phylogenetic trees (Figure 5). The description of this new sequence from a red-footed tortoise and its relative position in the phylogenetic trees seem to corroborate the distinctness of AdVs from testudinoid turtles and further support the claim for a new *Testadenovirus* genus in the family *Adenoviridae*. The great phylogenetic distance of this lineage from the other genera is probably a consequence of a long period of

coevolution between virus and host. In contrast, it is highly conceivable that the other known AdV from testudinoid turtles, the Sulawesi tortoise adenovirus 1, which is phylogenetically very distinct and classified in the genus *Siadenovirus* (see Figure 5), has been acquired by host switching from a so far unknown animal host (Harrach, 2014).

Still with regard to the sequences obtained from turtles and tortoises, three of these - ZLX201 (red-eared slider), ZLX244 (common snapping turtle) and ZLX257 (alligator snapping turtle) - did not cluster together with similar sequences. Instead, they were allocated to genus *Aviadenovirus* with greater phylogenetic proximity to fowl AdVs. Curiously, these three new sequences presented highly heterogeneous, each one closely clustering with a distinct species of fowl AdVs, namely, *fowl aviadenovirus E, fowl aviadenovirus C* and *fowl aviadenovirus D*, respectively. Among other food items, these reptiles are fed chicks and clean chicken (according to the Lisbon Zoo staff, personal communication), so it is highly likely that the birds they are fed are infected with fowl AdVs and these have been detected by nested PCR from the faecal material. Although less likely, one cannot also discard the hypothesis of these findings correspond to real productive infections of the reptiles as a result of a previous AdV host switch from birds. Host switching is a well-known phenomenon in the evolution of AdVs. For instance, squamate AdVs may have switched to phylogenetically very distant hosts (ruminants and birds) and a siadenovirus lineage, with unknown origin, may have switched to birds, a reptile (Sulawesi tortoise) and an amphibian species (Harrach, 2014). Host switches can also be presumed between more closely related hosts, e.g. among mammals (Kohl *et al*., 2012).

Phylogenetic analysis demonstrated that the sequence detected in the fifth positive faecal sample from testudinoid hosts - ZLX203 (red-eared slider) - is positioned next to the sequence frog AdV-1, type sequence of the species *frog siadenovirus A*, within the genus *Siadenovirus.* This genus includes AdVs from a diverse array of animal hosts, including an amphibian, the above mentioned Sulawesi tortoise and several bird species, which can be a sign of numerous host switches during evolution. So, the evolutionary history of this genus awaits better elucidation (Harrach, 2014). According to the Lisbon Zoo staff (personal communication), some red-eared sliders came from private homes and other locations and what they were fed previously is unknown. Also, some have as

habitat at the Lisbon Zoo an indoor pond and it is possible that frogs had previously inhabited the pond. Therefore, one cannot rule out that the AdV detected in this sample could actually be originated from a frog, resulting from environmental contamination. However, the ZLX203 sequence presents some genetic divergence from frog AdV-1 (see Figure 5).

Regarding AdV sequences identified in faecal samples of animals from the order Squamata (comprising, in this work, lizards and snakes), the sequences ZLX192 (Cuban boa), ZLX194, ZLX231, ZLX233, ZLX251 (all from Madagascar tree boas), ZLX225 and ZLX269 (both from Dumeril's boas) formed a very significant phylogenetic cluster (bootstrap value of 99%, in Figure 5A) within genus *Aviadenovirus*, with no interspersion with any of the reference sequences used from birds (fowl, goose, turkey). At first, this was a very unexpected result and an investigation was set up to know details about the feeding preferences of these species at the Lisbon Zoo. Among other types of meat, all these boas are fed quails and that may be the reason for the phylogenetic positioning of the AdV amplified sequences (the quails could be infected with AdVs). The very low genetic diversity presented by these seven AdV sequences (see Figure 5) is suggestive of a common source of infection, the quails supplied to the Lisbon Zoo by a specific poultry production company. Irrespective of their biological origin, the topology of the cluster is highly suggestive of a new aviadenovirus species, not yet described. This hypothesis could be proven by the sequencing of a larger genomic DNA fragment or, ideally, the complete genome of one of these viral strains.

The genus *Mastadenovirus* is also clearly represented in Figure 5, with corresponding sequences forming a cluster supported by a significant bootstrap value (80%, in Figure 5A). In Figure 5A, the *Mastadenovirus* cluster is further divided in two major subclusters, with the sixteen sequences produced in this work, mainly amplified from snakes, grouping tightly together into two smaller phylogenetically significant sub-subclusters. The sequences obtained from the samples ZLX239 and ZLX242 (royal python); ZLX228 (Jamaican boa); ZLX236 (jungle carpet python); ZLX238 (Burmese python); ZLX235 (eastern kingsnake); ZLX243 (California kingsnake); ZLX266 (red ratsnake); and ZLX234 (Madagascar tree boa) formed a very homogeneous monophyletic group clustering with murine AdV-2, the type species of *murine mastadenovirus B*. It is evident the greater phylogenetic proximity to murine AdV-2

than to other types of murine AdVs in the phylogenetic trees (murine AdV-1 or murine AdV-3). On the other hand, the sequences obtained from the samples ZLX240, ZLX241 and ZLX254 (royal python); ZLX265 (Komodo dragon); ZLX226 and ZLX227 (Jamaican boa); and ZLX223 (reticulate Gila monster) clustered closely together with a sequence from a rat (*Rattus norvegicus*) AdV. The five sequences with origin in snake faecal samples are also genetically remarkable homogeneous, contrasting with the two sequences from lizards (reticulate Gila monster and Komodo dragon) from which some degree of diversity is already perceived (Figure 5A). Considering the topology of this cluster, it is possible that these seven sequences, along with the already known AdV sequences from rats, could establish a new mastadenovirus species in a near future. However, this topology is not exactly maintained when considering amino acid putative sequences (Figure 5B), in what represents the major difference between the two phylogenetic trees presented (the classification of the new sequences remains the same). It is presumed that the AdVs detected in all these samples are not from the host animal, but from the rodents they are fed with. The ZLX viruses from these two sub-subclusters (Figure 5A) present low genetic diversity, suggestive of a common source or infection. According to the Lisbon Zoo staff (personal communication), they keep their own production of rats of vivarium line to feed some of the reptiles (including all the species above mentioned). The mice, on the other hand, are purchased from an external supplier. This might explain the high genetic similarity among these two groups of AdVs as they originate from the same animal lines. This hypothesis could have been put to the test with an additional PCR screening of faecal samples from the rodents used as food for the reptiles at the Lisbon Zoo, but, unfortunately, that was not possible. Besides the ingestion of infected prey, the presence of mammalian AdVs in reptilian faecal samples raises several questions regarding their origin, being possible to advance with a number of alternative hypotheses: the reptiles may have ingested contaminated faecal material from rodents circulating in their enclosures (more probable for murine AdVs); their food have been contaminated by rodent excreta; or this finding is simply due to environmental contamination of the reptilian faecal samples. Finally, the hypothesis that the reptiles are truly productively infected with mammalian AdVs, able to replicate in their digestive tract, cannot be totally excluded, although, until now, mastadenoviruses have been claimed to occur only in mammals.

The sequences obtained from ZLX181, ZLX284 and ZLX285 (inland bearded dragon); ZLX182 (veiled chameleon); ZLX186 (Cuvier's Madagascar swift); and ZLX286 (red ratsnake), clustered closely together in the *Atadenovirus* genus cluster (see Figure 5). The AdVs detected in these six samples are all identical and form a very consistent monophyletic group with agamid AdV-1 and central netted dragon AdV-1, from Australian agamid lizard species *Pogona vitticeps* (inland bearded dragon) and *Ctenophorus nuchalis* (central netted dragon), respectively. Agamid AdV is a type of atadenovirus that infects several reptiles, particularly lizards. This virus is widespread in inland bearded dragon populations in the USA, Europe and Australia having been identified several times (Wellehan *et al.*, 2004; Kübber-Heiss *et al*., 2006; Moormann *et al*., 2009; Parkin *et al*., 2009; Kubiak, 2013; Doneley *et al.*, 2014; Schilliger *et al*., 2016). The results here reported seem to corroborate these findings, since 3 out of 4 faecal samples from Lisbon Zoo inland bearded dragons were found to be positive. In this species, infection with agamid AdV-1 has been related to non-specific clinical symptoms, as lethargy, anorexia, cachexia, diarrhoea and encephalopathy, with the highest morbidity and mortality described in neonates and juveniles (Marschang, 2011). Recent results point for very high prevalence of infection (89%) in inland bearded dragons with signs of disease (Pénzes, 2015). However, no clinical symptoms were reported at the Lisbon Zoo for these animals during the study period. Atadenovirus infections have also been found in other lizards and snakes, as well as in birds and mammals (Harrach, 2014), being presently accepted that genus *Atadenovirus* is of squamate origin (Pénzes, 2015). Several results corroborate this idea and all squamate AdVs to date proved to be atadenoviruses. The fact that in this work several sequences originating from squamata clustered in other AdV genera (*Aviadenovirus* and *Mastadenovirus*) further strengthens the hypothesis of amplification from food (fowl and rodents, respectively). The majority of reptilian atadenoviruses described so far is still unassigned, i.e., not classified in a specific AdV species, including the ones from agamid lizards here described.

No significant differences exist when comparing the topologies of the two phylogenetic trees presented (Figure 5A and B), based, respectively, on nucleotide and amino acid alignments. Minor differences were detected, namely, on the relative position of some reference sequences in major clusters, or in the consistency of the clustering itself (see

bootstrap values), but with no impact on the classification of the sequences under study. As previously mentioned, the major difference can be observed in the topology of the subcluster grouping all the rodent mastadenovirus sequences, but once more without any impact on the classification of the new AdV sequences.

Finally, it is important to consider that the studied reptiles are kept in the zoologic facilities, alongside a variety of other animals of distant origins and carrying different microbiomes. The probability of transmission and co-infection with viruses from taxonomically and evolutionarily very distant hosts is therefore high. On this note, the AdVs detected in reptiles do not necessarily indicate that they are natural hosts, as host switches for AdVs are relatively frequent. In this respect, and as a complement to this study, it would be interesting to evaluate AdV infection in individuals (animal keepers, veterinarians, etc.), and their close relatives, who deal with reptiles at the Lisbon Zoo. Moreover, in this study, only a small part of the DNA polymerase gene was sequenced as our aim was to detect and confirm the presence of AdV in the faecal samples. The primers used were very degenerate, which resulted in the detection of a wide range of sequences. Further studies are needed to fully characterize these AdVs and to better understand their epidemiology. The sequencing of longer genomic DNA fragments is clearly mandatory.

Despite all the possible shortcomes of this study design, the diversity of AdVs in nature is well reflected by our results. Some novel viruses could have been put in evidence, including new species of fowl AdVs (detected in boas) and rodent/mammalian AdVs (detected in lizards and snakes), a new siadenovirus in sliders and a new testadenovirus in tortoises, further strengthening the possibility of a fourth candidate species in this genus. To our knowledge, it is also highly probable that AdVs (of the agamid lizard lineage, genus *Atadenovirus*) were detected for the first time in two reptilian species, the Cuvier's Madagascar swift (*Oplurus cuvieri*) and the veiled chameleon (*Chamaeleo calyptratus*).

3.4 Confirmation of host species of faecal specimens by cytochrome b gene sequence analysis

Our collaborators at the Lisbon Zoo provided an inventory with the faecal samples origin (host species). However, to unequivocally confirm the origin of the samples in

which AdV DNA was successfully identified, a PCR for the partial amplification of the mitochondrial cytochrome b gene was carried out on all these samples (N=34), with a protocol previously described and validated (Kocher *et al*., 1989; Irwin *et al*., 1991). This is one of the most used genes for species identification (Lopez-Oceja *et al*., 2016) and it is often used in the determination of species in cases of illegal trading or trafficking of endangered species and in the food industry to determine the origin of the food/product in question. After analysis of the agarose gels obtained, there were 31/34 samples with DNA bands in the expected region (~ 486 bp). DNA extraction and purification were carried out directly from the PCR products or from the fragments excised from the agarose gel, the amplicons were sequenced, and the sequences compared with those registered in the GenBank database, as previously described.

Species identification using cytochrome b gene partial sequence resulted in 52% (16/31) of samples in accordance with the species origin provided by the Lisbon Zoo (ZLX186, ZLX192, ZLX194, ZLX207, ZLX225, ZLX226, ZLX227, ZLX231, ZLX233, ZLX234, ZLX240, ZLX241, ZLX251, ZLX254, ZLX269 and ZLX286). In one of the remaining samples (ZLX243), it was not possible to unambiguously confirm the identity of the host species, since while the faecal sample was labelled as originating from a California kingsnake (*Lampropeltis getula californiae*), the species with the highest identity in the cytochrome b analysis was the royal python (*Python regius*). This odd result was confirmed once, and it was most probably due to sample mislabelling (during faecal collection at the Lisbon Zoo or laboratorial procedures). In the remaining 14 samples (45%), the confirmation of the host origin also failed due to technical problems related to PCR amplification of the DNA. Briefly, some results pointed for environmental contamination with human or other animal species DNA (ZLX181, ZLX182, ZLX223, ZLX228, ZLX236, ZLX257, ZLX266 and ZLX285), and others for the possible amplification of cytochrome b DNA directly from food items (ZLX201, ZLX203, ZLX235, ZLX244 and ZLX265), as the implicating host species are carnivorous or omnivorous and had been fed with meat (see previous section). In one sample (ZLX242), no significant BLAST similarity was found. The PCR procedure was repeated for all these samples with no improvement in the results. So, the results of species molecular identification of the reptile samples obtained by this method were considered only partially reliable.

4. CONCLUSIONS

4. Conclusions

Here we describe the results of a study intended to screen reptiles for the presence of adenoviruses (AdVs) in the Lisbon Zoo and to characterize their genetic diversity, including phylogenetic relationships. Reptile faecal samples were screened by a nested PCR with consensus primers targeting a conserved region of the DNA-dependent DNA polymerase gene (Wellehan *et al.*, 2004). The PCR protocol used degenerate primers and is very sensitive and efficient for screening AdV infections (of all genera) in a wide range of hosts. Preliminary screening of 120 samples, from 43 different species classified in three out of the four reptilian taxonomic orders, for the presence of AdV DNA revealed that 49.2% (59/120) were putatively positive. After a more detailed study, through DNA sequencing and bioinformatics analysis, 34 samples, from 19 distinct reptilian species, were considered positive, leading to an overall detection rate of 28.3% (34/120) that can be considered relatively high. A set of 52 corresponding reference sequences retrieved from the GenBank database was added to the 34 AdV DNA polymerase sequences from this study, and nucleotide and amino acid phylogenetic trees were constructed from multiple sequence alignments to estimate the evolutionary distances between them. The trees were constructed using different combinations of methods and substitution models and algorithms for the determination of the matrices of genetic distance. In a quick, preliminary analysis, it was clear that the sequences in study were distributed throughout the phylogenetic trees, interspersed between the reference sequences and forming several phylogenetic clusters, showing the high genetic diversity of AdVs circulating in the Lisbon Zoo.

Reference sequences of AdVs detected from turtles and tortoises from the superfamily Testudinoidea clustered closely together, separately from the currently accepted AdV genera, representing the newly proposed genus *Testadenovirus*. The AdV DNA sequence detected in a red-footed tortoise clustered within this group and demonstrated a 94% similarity with a previously described AdV-1 isolate from the same species. To our knowledge, this is only the third time that an AdV is detected in a red-footed tortoise and, along with the other two previously described sequences from the same species, this new sequence may represent a new testadenovirus species. The description of this new sequence from a red-footed tortoise further supports the claim for the

establishment of the candidate *Testadenovirus* genus in the family *Adenoviridae*. AdV sequences obtained from an alligator snapping turtle, a red-eared slider and a common snapping turtle did not cluster with sequences from similar species. These sequences were very heterogeneous and were allocated to the *Aviadenovirus* genus, each one clustering with a different species of fowl AdV. These reptiles are fed chicks and clean chicken at the Lisbon Zoo and it is likely that they are infected with fowl AdVs, being detected by nested PCR. However, these results may also be explained by productive infection of the reptiles as result of previous viral host switches. Moreover, phylogenetic analysis positioned a second sequence detected from a red-eared slider close to a frog AdV-1 sequence, within the genus *Siadenovirus*. This odd result may be due to environmental contamination. Some sliders at the Lisbon Zoo use an indoor pond as habitat and it is possible that frogs had previously inhabited the pond. AdVs sequences detected from a Cuban boa, four Madagascar tree boas and two Dumeril´s boas formed a highly significant monophyletic cluster, within the *Aviadenovirus* genus. These sequences clustered separately from reference sequences from fowl, goose and turkey AdVs and presented very low genetic diversity. All these boas are fed quails (among other types of meat) supplied from a specific poultry company. These quails could be infected with AdVs and that could be the reason for their positioning in the phylogenetic tree. Finally, the topology of this cluster suggests that these sequences may represent a new *Aviadenovirus* species.

Sequences obtained three from inland bearded dragons, a veiled chameleon, a Cuvier´s Madagascar swift and a red rat snake formed a very consistent monophyletic group with agamid AdV-1 and central netted dragon AdV-1. This cluster was allocated to the *Atadenovirus* genus. Agamid AdV infects several reptiles (such as lizards and snakes) and it is widespread in inland bearded dragon populations, having been identified several times, in very distant geographic locations. Three of the four inland bearded dragon samples were positive for AdVs, which attests to these findings. Birds and mammals have also been reported with atadenovirus infections. It is very likely that this is the first time that AdVs (of the agamid lizard lineage, genus *Atadenovirus*) were detected in a Cuvier´s Madagascar swift and a veiled chameleon.

A group of nine sequences, all originating from eight distinct species of snakes, clustered tightly together with murine AdV-2, a representative of *Mastadenovirus* genus

and the type species of *murine mastadenovirus B* species, presenting a very low genetic divergence. Moreover, a second group of five sequences amplified from snake faecal material (two species), along with two more sequences obtained from lizards (the Komodo dragon and the reticulate Gila monster) formed a monophyletic distinctive group with a rat AdV sequence, showing a higher degree of genetic diversity. As discussed before, it is not possible to be sure if the AdVs detected in these reptiles are, in fact, due to productive infection or due to their diet, among other alternative hypotheses. However, it is most likely that these AdVs are from the rodents these reptiles are fed. Finally, considering its topology in the phylogenetic tree, it is conceivable that this rodent cluster may represent a new mastadenovirus species. Only the sequencing of a longer genomic DNA segment may shed some light on this hypothesis.

It must be referred that some of the samples were collected from or in close vicinity of wet locations, such as small lakes and water reservoirs. It is possible that these water reservoirs are contaminated with AdVs from other sources (e.g. frogs, birds), therefore being detected in our PCRs. This study shows the widespread circulation of AdVs in the Lisbon Zoo herpetarium and that AdV infections may be common among the reptiles kept there, although its pathogenic role is not clear, since, as far as it is known, they were all asymptomatic. Also, the likelihood of these reptiles to develop future disease is unknown. Only a limited region of the AdV DNA polymerase gene was sequenced, as the main aim of this work was to detect and confirm the presence of AdV, and so our conclusions have to be taken cautiously. Further investigation is clearly necessary to fully characterize these AdVs and to better understand their phylogenetic relationships and epidemiology at the Lisbon Zoo. Other coding regions such as the hexon gene and/or for other structural proteins of the viral capsid must be analysed. Since interspecific transmissions may have influenced our results, it would be interesting to also screen the Lisbon Zoo workers and animal keepers (and close family members) for AdV infection. In the same logic, other non-reptile animals kept nearby and pest rodents infesting the premises of the Lisbon Zoo could also be screened for AdV.

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5. References

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6. APPENDICES

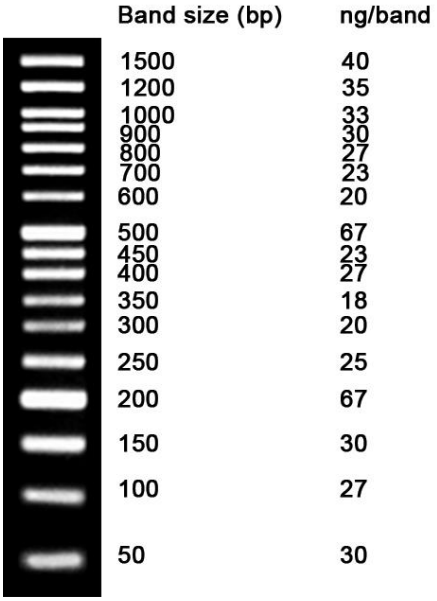
Appendix 1

Composition of media and solutions.

Solution/medium	Contents
IPTG stock solution (0.1M)	1.19 g IPTG Add deionized water to 50 mL final volume. Filter-sterilize and store at 4 °C.
X-Gal stock solution (0.12 M)	100 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside Dissolve in 2 mL N,N´-dimethyl-formamide. Cover with aluminium foil and store at –20 °C.
LB medium (per liter)	10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl. Add deionized water to 1000 mL final volume and heat sterilize.
LB plates with ampicillin	Add 15 g agar to 1 litre of LB medium. Autoclave. Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100 μg/mL Pour 30–35 mL of medium into Petri dishes. Let the agar harden. Store at 4 °C for up to 1 month or at room temperature for up to 1 week.
LB plates with ampicillin/IPTG/X-Gal	Make the LB medium with ampicillin as above; then supplement with 0.2 mM IPTG and 40 μg/mL X-Gal and pour the plates.
2X Rapid Ligation Buffer, T4 DNA Ligase (provided)	60 mM Tris-HCl (pH 7.8), 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% (v/v) polyethylene glycol (MW8000, ACS Grade), Store in single-use aliquots at –20 °C. Avoid multiple freeze-thaw cycles
TAE buffer (Tris-Acetate-EDTA) 50x	242 g Tris-base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0. Add water to 1000 mL final volume.
TE buffer (Tris-EDTA)	10 mM Tris, 1 mM EDTA (pH 8.0)
TSS (transformation and storage solution) medium	LB 1x, 10% (v/v) PEG6000, 5% (v/v) DMSO, 50mM $MgSO_4$, (pH 6.5)
TEG buffer (Tris-EDTA-Glucose)	50 mM glucose, 10 mM EDTA, 25mM Tris-HCl, pH 8.0
Miniprep lysis solution (alkaline SDS)	0.2 M NaOH, 1.5% (w/v) SDS
Gel loading buffer 10x	0.25% (w/v) bromophenol blue, 40% (w/v) sucrose Dilute 1:10 with sample before placing into the wells of the gel.
EcoRI buffer 10x (provided)	50 mM Tris-HCl (pH 7.5, at 37 °C), 10 mM $MgCl_2$, 100 mM NaCl, 0.02% (v/v) Triton X-100, 0.1 mg/mL BSA.

Appendix 2

NZYDNA Ladder VI (NZYTech, Portugal)



NZYDNA Ladder VI is a molecular weight marker specially designed for easy quantification and size determination of small DNA fragments in agarose gels. NZYDNA Ladder VI produces a pattern of 17 regularly spaced bands, ranging from 50 to 1500 bp.

Appendix 3

The GenBank accession numbers of the AdV reference sequences used in the phylogenetic analysis.

Sequence name	Accession number
Agamid adenovirus 1	AY576678
Agamid adenovirus 1 isolate 2	EU914203
Amphisbaenian adenovirus 1	KT950887
Anolis adenovirus 1	KC544015
Anolis adenovirus 2	KC544016
Bat adenovirus 2	JN252129
Bovine adenovirus 4	AF036092
Bovine adenovirus A (bovine 1)	AC000191
Box turtle adenovirus 1	EU828750
Central netted dragon adenovirus 1	HQ005515
Duck adenovirus A (duck 1)	AC000004
Eastern box turtle adenovirus	JN632579
Equine adenovirus 2	KT160425
Eublepharid adenovirus 1	AY576677
Fowl adenovirus 10	HM854003
Fowl adenovirus 2	KT862806
Fowl adenovirus 5	HM853998
Fowl adenovirus 8a	KT862810
Fowl adenovirus A (Fowl 1)	AC000014
Fowl adenovirus C	GU188428
Fowl aviadenovirus D	KU746335
Fowl aviadenovirus E	KX258422
Frog adenovirus 1	AF224336
Gekkonid adenovirus 1	AY576681
Goose adenovirus 4	JF510462
Great tit adenovirus 1	FJ849795
Helodermatid adenovirus 1	AY576680
Human adenovirus F (human 40)	L19443
Lizard adenovirus 2	KJ156523
Murine adenovirus 1	AC000012

Sequence name	**Accession number**
Murine adenovirus 2	HM049560
Murine adenovirus 3	EU835513
Ovine adenovirus 7	U40839
Pancake tortoise adenovirus	JN632575
Plum headed parakeet adenovirus 1	EU056825
Porcine adenovirus 5	AF289262
Raptor adenovirus A (raptor 1)	NC015455
Rattus norvegicus adenovirus isolate RN13GZYX24	KU258156
Rattus norvegicus adenovirus isolate RN13GZYX45	KU258160
Rattus norvegicus adenovirus isolate RN14GZBY56	KU258167
Red-eared slider adenovirus	JN632580
Red-footed tortoise adenovirus 1	KU601299
Red-footed tortoise adenovirus 2	KU601301
Simian adenovirus 3	AY598782
Snake adenovirus 2	FJ012163
South polar skua adenovirus 1	HM585353
Squirrel adenovirus 1	KY427939
Sulawesi tortoise adenovirus 1	EU056826
Tree shrew adenovirus 1	AC000190
Turkey adenovirus 1	GU936707
Turkey adenovirus A (turkey 3)	AC000016
Yellow-bellied slider adenovirus	JN632578

Appendix 4

Preliminary data resulting from the genetic identity search in the BLAST program of the sequences obtained in this study, with information on species of origin (scientific and common name presented). The table shows the sequences in the GenBank with the highest identity (name and accession numbers are presented).

Sample number	Species	Common name	Highest identity	GenBank accession number
ZLX181	*Pogona vitticeps*	Inland bearded dragon	Agamid AdV 100%	EU914203
ZLX182	*Chamaeleo calyptratus*	Veiled chameleon	Agamid AdV 100%	EU914203
ZLX186	*Oplurus cuvieri*	Cuvier´s Madagascar swift	Agamid AdV 100%	EU914203
ZLX192	*Epicrates angulifer*	Cuban boa	Fowl AdV 82%	KX421403
ZLX194	*Sanzinia madagascariensis*	Madagascar tree boa	Fowl AdV 81%	KU991797
ZLX201	*Trachemys scripta elegans*	Red-eared slider	Fowl AdV 98%	KX077988
ZLX203	*Trachemys scripta elegans*	Red-eared slider	Frog AdV 94%	AF224336
ZLX207	*Chelonoidis carbonaria*	Red-footed tortoise	Red-footed tortoise AdV 94%	KU601299
ZLX223	*Heloderma suspectum suspectum*	Reticulate Gila monster	*Rattus norvegicus* AdV 97%	KU258162
ZLX225	*Acrantophis dumerili*	Dumeril´s boa	Fowl AdV 82%	KU991797
ZLX226	*Epicrates subflavus*	Jamaican boa	*Rattus norvegicus* AdV 99%	KU258157

Sample number	Species	Common name	Highest identity	GenBank accession number
ZLX227	*Epicrates subflavus*	Jamaican boa	*Rattus norvegicus* AdV 99%	KU258162
ZLX228	*Epicrates subflavus*	Jamaican boa	Murine AdV 97%	HM049560
ZLX231	*Sanzinia madagascariensis*	Madagascar tree boa	Fowl AdV 81%	KU991797
ZLX233	*Sanzinia madagascariensis*	Madagascar tree boa	Fowl AdV 77%	KT717889
ZLX234	*Sanzinia madagascariensis*	Madagascar tree boa	Murine AdV 97%	HM049560
ZLX235	*Lampropeltis getula getula*	Eastern kingsnake	Murine AdV 97%	HM049560
ZLX236	*Morelia spilota cheynei*	Jungle carpet phyton	Murine AdV 97%	HM049560
ZLX238	*Python bivittatus*	Burmese phyton	Murine AdV 97%	HM049560
ZLX239	*Python regius*	Royal/ball phyton	Murine AdV 95%	HM049560
ZLX240	*Python regius*	Royal/ball phyton	*Rattus norvegicus* 99%	KU258162
ZLX241	*Python regius*	Royal/ball phyton	*Rattus norvegicus* 99%	KU258162
ZLX242	*Python regius*	Royal/ball phyton	Murine AdV 96%	HM049560
ZLX243	*Lampropeltis getula californiae*	California kingsnake	Murine AdV 97%	HM049560
ZLX244	*Chelydra serpentina*	Common snapping turtle	Fowl AdV 99%	KP295475
ZLX251	*Sanzinia madagascariensis*	Madagascar tree boa	Fowl AdV 82%	KU991797

Sample number	Species	Common name	Highest identity	GenBank accession number
ZLX254	*Python regius*	Royal/ball phyton	*Rattus norvegicus* 99%	KU258162
ZLX257	*Macrochelys temminckii*	Aligator snapping turtle	Fowl AdV 99%	KU310942
ZLX265	*Varanus komodoensis*	Komodo dragon	*Rattus norvegicus* 97%	KU258162
ZLX266	*Pantherophis guttatus*	Cornsnake/red ratsnake	Murine AdV 97%	HM049560
ZLX269	*Acrantophis dumerili*	Dumeril´s ground boa	Fowl AdV 77%	HM854003
ZLX284	*Pogona vitticeps*	Inland bearded dragon	Agamid AdV 100%	EU914203
ZLX285	*Pogona vitticeps*	Inland bearded dragon	Agamid AdV 100%	EU914203
ZLX286	*Pantherophis guttatus*	Cornsnake/red ratsnake	Agamid AdV 100%	EU914203