SIADENOVIRUSES: A COMPARATIVE MOLECULAR AND PHYLOGENETIC STUDY

Ph.D. Thesis

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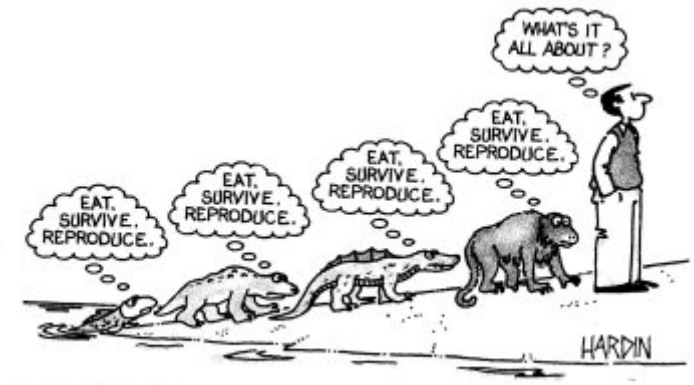
"There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

Charles Darwin



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1 INTRODUCTION

1.1 HISTORY AND SIGNIFICANCE OF ADENOVIRUS RESEARCH

Adenoviruses have been known since the middle of the twentieth century. Rowe *et al.* in 1953 cultured surgically removed human pharyngeal tonsils, which led to the observation of spontaneous cellular degeneration. At about the same time, a virus with similar cell-damaging effects was isolated from the tracheal phlegm of American enlisted men suffering from acute respiratory illness (Hilleman & Werner, 1954). Upon recognition of the close relatedness of the two viruses emerged the appellation *adenovirus*, indicating the adenoid origin (Enders *et al*., 1956). Further isolations and serological experiments confirmed that these viruses were prevalent with numerous serotypes. The first animal adenovirus was isolated from cattle in 1959 (Klein *et al.*, 1959), and further ones were later found in several mammalian and avian hosts. Adenoviruses can be found globally, isolated or detected with various methods (serology, EM, PCR) from representatives of all major classes of vertebrates (Russell & Benkő, 1999).

The family *Adenoviridae*, especially human adenovirus 2 (HAdV-2) and 5 (HAdV-5), is one of the most thoroughly studied groups of viruses. In the course of the adenovirus research as of the 1960's, two major discoveries took place that facilitated a boom in the field. Trentin *et al*. (1962) recognised a transforming effect of adenoviral DNA sparking considerable interest in cancer research. According to the original observation, different human AdV serotypes inoculated into new-born hamsters were able to induce tumours to a variable extent. Adenoviruses used to be temporarily attributed an important causative role in the development of cancer. Although adenoviruses are indeed able to transform cultured cells, a direct role in bringing forth human malignant tumours was later disproved. They nonetheless proved to be beneficial in understanding the process of oncogenesis.

Another discovery of great impact was the recognition of the post-transcriptional modification of mRNA, specifically the phenomenon of splicing (Chow et al., 1977; Weber et al., 1977). As soon as it became obvious that this mechanism is widely distributed in eukaryotes, adenoviruses became the model organisms for the study of eukaryotic gene expression.

It is worth noting that at the time of the upsurge of AIDS and the recognition of the viral origin thereof, adenoviruses fell under suspicion again as prospective triggers of the disease, because numerous new HAdV types were isolated from patients with AIDS.

The pace of research has not seemed to slacken in the last two decades. Adenoviruses turned out to be extraordinarily efficient gene expression systems, and the proteins coded by their genomes are as a rule expressed with great efficacy (Russell, 2000). No wonder adenoviruses were among the first candidates for gene therapy vectors. However, the most commonly used serotype HAdV-5 in the course of the application did not live up to the great expectations. Pre-existing antibody levels in the population for the most part did not allow for the efficient application of these vectors. Mainly due to this finding, attention shifted towards animal-infecting adenoviruses, which seemed a theoretically plausible alternative. Thus, an upshoot in genome sequencing of animal adenoviruses began to take place in the 1990's.

Adenovirus research has a strong tradition in Hungary as well. Important studies were performed as to antigen structure (Ádám *et al*., 1988; Rusvai *et al*., 1988; Nász & Ádám, 2006) and the adenoviral effect of inducing interferon production (Tóth *et al*., 1983 and 1984; Mándi *et al*., 1987; Tóth *et al*., 1987; Berencsi *et al*., 1991). Hungarian veterinarian scientists in cooperation with physicians took part in the analysis of DNA and genomic structure of animal adenoviruses (Belák *et al*., 1983 and 1986; Fejér *et al*., 1992; Rusvai & Belák, 1992). Three new bovine AdV serotypes were isolated in Hungary (Bartha & Áldásy, 1966; Bartha & Csontos, 1969; Bartha *et al.*, 1970). Another one was described with the participation of Hungarian scientists (Horner *et al.*, 1989). The peculiarity of certain bovine AdVs was first recognized by these reports, questioning the traditional classification of the serotypes long before the era of molecular genetics. They subsequently undertook an important role in renewing the taxonomy of the virus family (Bartha, 1969; Benkő *et al*., 2000).

1.2 MORPHOLOGY

Adenoviruses are medium-sized (70–90 nm), non-enveloped (naked), double-stranded DNA viruses with an icosahedral capsid symmetry. If not indicated otherwise, the following overview is taken mainly from Russell (2000 and 2009) and is based on the study of HAdV-2. Principal capsid proteins include hexon (polypeptide II), penton base (III) and the homotrimer fiber (IV). These proteins had been designated by Roman numerals in order of their decreasing molecular weight in SDS-PAGE (Maizel *et al.*, 1968). The hexon is a pseudo-hexagonal trimer of two major kinds. Peripentonal hexons are associated with the penton bases at the 12 apices. Groups of nine hexons (GONs) are positioned on the 20 facets of the icosahedron. The penton capsomere is built as a complex of two proteins — the homopentameric penton base and the homotrimeric fiber that protrudes from the 12 vertices of the virion. In the fiber, three distinct regions can be differentiated: tail, shaft and knob. The

number of fibers on each vertex is one except for members of the *Aviadenovirus* genus, in which two fibers protrude from the vertices. The number of fibers is no function of the number of fiber genes in the aviadenovirus genome.

Additional minor structural components contribute to the stability of the capsid, without which the assembled virion would not be infective (with the exception of polypeptide IX). Polypeptide IIIa is positioned below the penton base and is highly phosphorylated. Polypeptide VI is in contact with the inner cavity of hexons. An 11-amino acid peptide, cleaved from the precursor of protein VI, acts as a cofactor of the viral protease (see section 1.3). The function of polypeptide VIII is little known, it may have a role in stabilizing the GONs. Polypeptide IX is only present in members of the genus *Mastadenovirus*, it stabilizes the capsid on the external facets. It is also a transcriptional activator of viral and cellular promoters (Rosa-Calatrava *et al.*, 2001).

Within the capsid, the linear genome is intimately associated with protein V, a minute core protein called μ (X), and the histone-like protein VII. Protein V seems to be dispensable in replication, since it is present only in mastadenoviruses. The DNA in complex with protein VII (a highly basic polypeptide) serves as a functional template for replication and transcription *in vivo*. Protein VII is also a mediator of virus DNA import into the nucleus (Hindley *et al.*, 2007). The terminal protein (TP) is covalently linked to the 5' end of each strand of the viral genome, present in two copies per virion, and is indispensable for replication (see below).

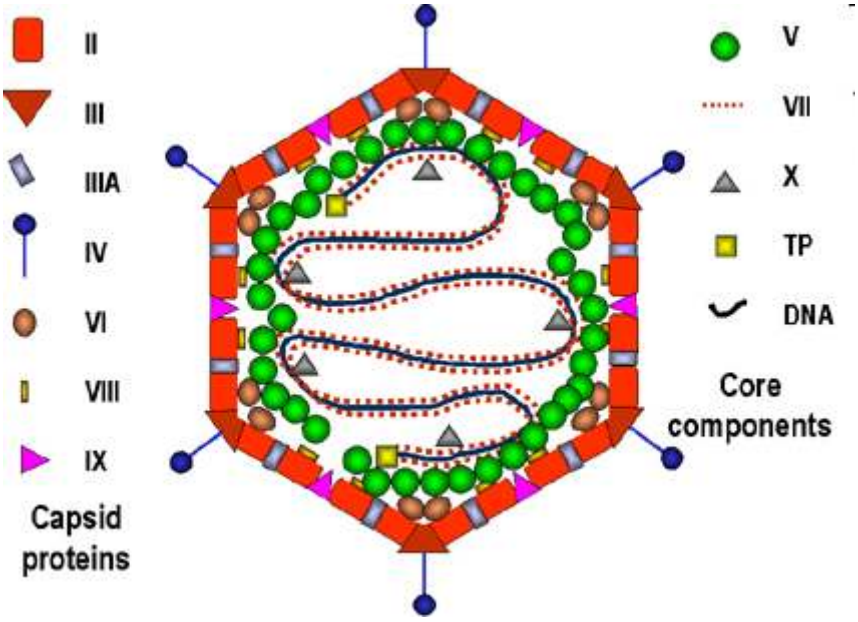


Figure 1. Schematic reconstruction of the adenovirus virion. II, hexon; III, penton base; IV, fiber; TP, terminal protein. Proteins V and IX are only present in mastadenoviruses. (Modified from Stewart & Burnett, 1993)

1.3 TAXONOMY AND GENOME ORGANIZATION

Historically, adenoviruses used to be divided into groups on the basis of serological reactivity. The family *Adenoviridae* had included two easily identifiable taxa: serotypes infecting mammals (*Mastadenovirus*) were sufficiently distinct serologically from those infecting birds (*Aviadenovirus*) to merit the status of separate genera. It appeared that the host provided a good basis for classification, but the isolation of certain bovine serotypes cast doubt on it (Bartha & Áldásy, 1966; Bartha & Csontos, 1969; Bartha *et al.*, 1970). Upon Bartha's (1969) proposal, the bovine serotypes were divided into two subgroups based on their biological properties. Subgroup I included serotypes that exhibited cross-reactivity with other mammalian AdVs in complement-binding assays. Contrarily, subgroup II bovine AdVs (BAdVs) reacted with neither subgroup I serotypes, nor with other mastadenoviruses examined (human, porcine) (Bartha, 1969; Bürki *et al.*, 1979). Since the criterion of difference between mastadenoviruses and aviadenoviruses was the lack of a common complement-binding antigen (Wigand *et al.*, 1982), subgroup II BAdVs were proposed to be recognized as a separate genus. However, this proposal was rejected by the International Committee on Taxonomy of Viruses (ICTV) for lack of further evidence (Bartha, 1974).

A similar problem emerged in case of AdVs coming from birds. The avian AdVs turkey hemorrhagic enteritis virus (THEV) and egg drop syndrome virus (EDS) did not cross-react with chicken AdV strains, therefore three groups were established in order to separate serologically distinct avian AdVs (McFerran & Smyth, 2000): group I included various cross-reacting fowl AdVs related to chicken embryo lethal orphan virus (CELO, fowl AdV-1), group II included THEV (turkey AdV-3), and group III included the EDS virus (duck AdV-1, DAdV-1). Neither of the latter two viruses cross-reacted with each other or with fowl AdVs in group I.

Later, when the use of modern molecular biology techniques started to gain prevalence, however, the former, outdated classification system soon became untenable. By the 1990's and especially in the new millennium, the taxonomy of adenoviruses gradually shifted towards what may be described as molecular taxonomy based on genome sequencing and phylogeny reconstruction. Benkő *et al.* (1988) subjected the bovine AdV serotypes to restriction enzyme analysis and confirmed the significant divergence between subgroup I and II BAdVs. Extended sequence homology was shown between the molecularly cloned and mapped BAdV-4 and other subgroup II BAdVs (Benkő *et al.*, 1990). Subgroup II BAdVs and the group III aviadenovirus (DAdV-1) were identified as similar on the molecular level, and were eventually classified together in a separate genus (*Atadenovirus*) (Harrach *et al.*, 1997;

Benkő & Harrach, 1998). The great phylogenetic distance suggested that atadenoviruses may have codiverged with members of a different vertebrate class (Harrach, 2000). This led to the investigation of AdVs found in less advanced vertebrates. Likewise, the group II aviadenovirus (TAdV-3) along with the frog AdV were put in their newly established own genus (*Siadenovirus*) (Davison *et al.*, 2000; Davison & Harrach, 2002). Currently, the family consists of five, firmly established genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus* (Benkő *et al.*, 2005). The *Ichtadenovirus* genus contains the only AdV found in fish.

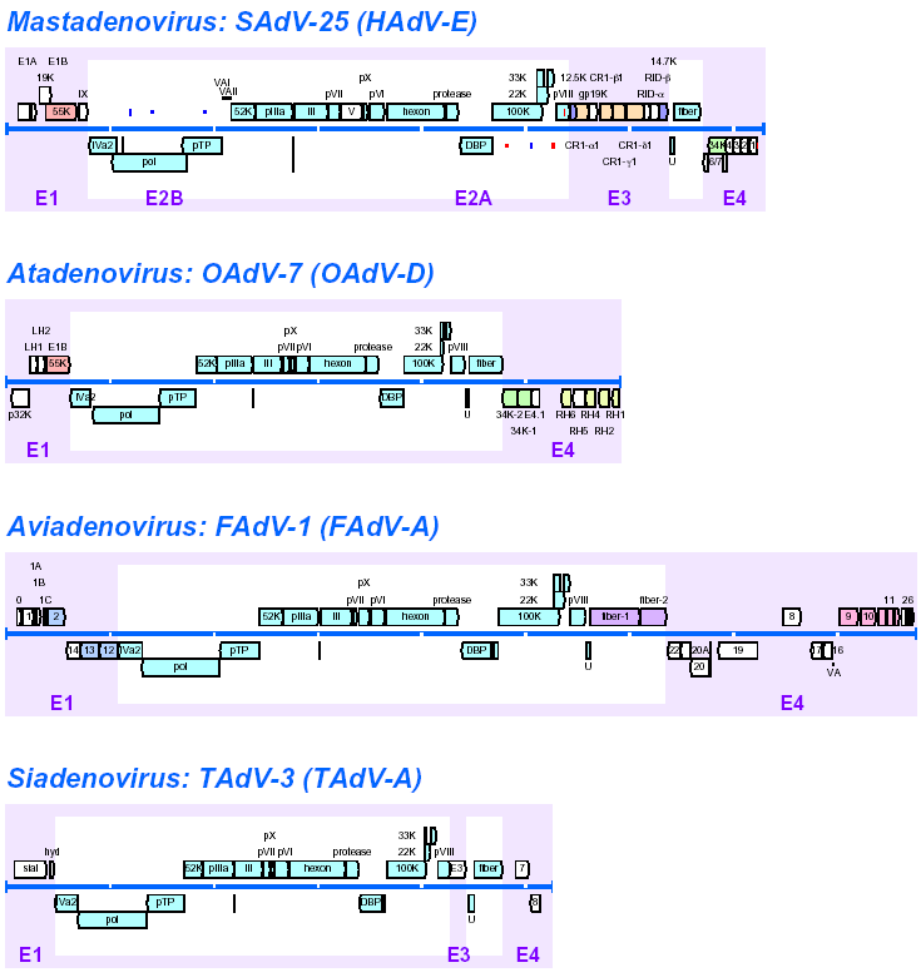


Figure 2. Gene layout in representatives of four adenovirus genera. The genome map of the ichtadenovirus is not yet available. Each genome is represented by a central blue horizontal line marked at 5 kbp intervals. Protein-encoding regions are shown as arrows. Cyan denotes genus-common genes, other colours indicate related genus-specific genes, and genus-specific genes that lack relatives are white. E1 and E4 are marked in all genera and E3 in the *Mastadenovirus* and *Siadenovirus* genera, and these regions are shaded violet. E2A and E2B genes are present in all genera, and these regions are indicated for the *Mastadenovirus* genus. Untranslated leader exons in the *Mastadenovirus* genus are shown for late, E2, E3 and E4 genes, with early transcripts in red, late transcripts in blue and 5'-terminal exons broader than internal exons. The VA RNA genes in the *Mastadenovirus* and *Aviadenovirus* genera are also included. (Davison *et al.*, 2003)

All adenoviruses hold a distinct ORF arrangement, in so far as they conform to a basic "genetic blueprint" (Figure 2). From a bird's eye view, the central part of the genome contains genes whose transcription is driven by the major late promoter (MLP), that are the rather conserved structural proteins (L region), as well as those essential for viral replication (E2A and E2B) (Davison *et al.*, 2003). These "genus-common" genes (except that of polypeptide V) are present in every single adenovirus. The flanking regions consist of the so-called "genus-specific" ORFs conserved within a certain genus, but having no counterparts (*i.e.* identifiable homologs) in other genera (Davison *et al.*, 2003). This basic plan is maintained throughout the family, only differing in one particular case of the fish AdV.

This location pattern of a conserved, central part versus variable parts is not restricted to adenoviruses, it is also characteristic to other large DNA viruses, such as herpesviruses (McGeoch & Davison, 1999) and poxviruses (Upton *et al.*, 2003).

In adenoviruses, the conserved region contains a predominantly fixed number of 17+1 genes, the extra one pertaining to the U exon. It is a spliced ORF present in almost all adenoviruses, U denoting unknown, so named on account of the fact that its 3' exon could not be located in any adenovirus. Lately however, the 3' exons have been found in the leader and coding region of the DNA binding protein (DBP), and its protein product UXP is expressed in all HAdV-C types (Tollefson *et al.*, 2007).

Moreover, extra copies (paralogs) of the fiber gene can be found in certain aviadenoviruses (Chiocca *et al.*, 1996; Kaján *et al.*, 2010), mastadenoviruses (HAdV-40 and 41, certain simian AdVs) (Kidd & Erasmus, 1989; Kidd *et al*., 1990; Davison *et al.*, 1993; Kovács *et al.*, 2005) as well as in the ichtadenovirus genome.

What sets apart each genus is the intergeneric diversity of the flanking regions. Only conserved within the genera, the gene composition of these regions varies greatly across the family. For instance, the E1 transcriptional unit at the left end of the genome contains 2–9 genes depending on which particular genus is in question. Genes in this unit are generally used to subvert cellular metabolism, and its products trigger apoptosis and often interact with the nuclear transactivator NF-κB and the tumour suppressor p53 (Russell, 2000; Russell, 2009). Proteins in the E3 unit, between pVIII and fiber, tackle the host's immune response (Wold & Chinnadurai, 2000; Lichtenstein *et al.*, 2004), but are dispensable for *in vitro* replication (Russell, 2000) UA mint a 35.oldal. E4 gene products are involved mainly with viral messenger RNA metabolism (Leppard, 1997). These findings are based on human AdV (*Mastadenovirus*) studies, and the function of the genes in these early regions are yet to be studied in the other genera.

On the grounds of this specific genome arrangement, in addition to the "traditional" genera *Mast-* and *Aviadenovirus*, three additional clades were recognized in the past decade.

1.3.1 Mastadenovirus

This traditional genus encompasses all human AdVs (currently 54 types in 7 species) and most of the types derived from mammals including simian, canine, bovine, ovine, caprine, equine, murine, and porcine AdVs (Harrach *et al.*, 2011). Understandably, this genus is the most studied of the five. The bulk of the knowledge with regard to the function of genes in the conserved and the genus specific regions stems from the study of types HAdV-2 and HAdV-5 in species HAdV-C. The size of mastadenovirus genomes ranges roughly between 30 and 36 kb. Their inverted terminal repeat (ITR) sequences are longer and more complex than in members of the rest of the genera (Benkő *et al.*, 2005). The structural polypeptides IX and V are unique to this genus. The early transcription unit E3 features numerous (with some notable exceptions, *e.g.* MAdV-1) ORFs involved in the evasion mechanisms against the host's immune response (Russell, 2000). Virus associated (VA) RNAs (transcribed by RNA polymerase III) are discernible in primate AdV genomes (Kidd *et al.*, 1995; Ma & Matthews, 1996), and are involved in translational control and inhibition of the interferon response (Mori *et al.*, 1996). Naturally occurring recombination events have been suggested between certain human AdV types (Pring-Akerblom *et al.*, 1995; Ebner *et al.*, 2005), which may even cross the species boundary.

1.3.2 Aviadenovirus

Twelve AdV types isolated from chicken and classified in five species populate this genus. Two turkey and a goose AdV also belong here, a species from falconids (Schrenzel *et al.*, 2005), as well as a number of candidate avian AdV types. Currently five full genomes are deposited in GenBank, those of FAdV-1 (Chiocca *et al.*, 1996), FAdV-9 (Ojkic *et al.*, 2000), TAdV-1 (Kaján *et al.*, 2010), FAdV-8 (Grgić *et al.*, 2011), and most recently FAdV-4 (Griffin & Nagy, 2011). The latter represents the longest published AdV genome to date with ~46 kb. In all FAdVs, two fibers project from each vertex of the capsid, which can be different in size if two genes are present (in FAdV-1 and 4). The aviadenovirus genome is characterized by a balanced or elevated G+C content, long terminal regions (E1 and E4), two fiber genes (except in FAdV-8 and 9), and the complete lack of the E3 region. ITRs are shorter than those of

mastadenoviruses. A VA RNA is located in the E4 region of FAdV-1 (Larsson *et al.*, 1986). Aviadenoviruses were presumed to be optimal candidates for gene delivery vectors (Johnson *et al.*, 2003; Francois *et al.*, 2004; Corredor & Nagy, 2010), because as much as 2–3 kb is dispensable from the terminal regions for *in vitro* replicaton.

1.3.3 Atadenovirus

Atadenovirus contains types with a diverse array of hosts. These include peculiar types from cattle (Benkő & Harrach, 1998; Dán *et al.*, 1998; Élő *et al.*, 2003; Graham *et al.*, 2005), sheep (Barbezange *et al.*, 2000; Both, 2004), goat (Lehmkuhl *et al.*, 2001), and mule deer (*Odocoileus hemionus*) (Zakhartchouk *et al.*, 2002) with low G+C content; the only known AdV from a marsupial (Thomson *et al.*, 2002); a duck AdV that causes the EDS disease (Harrach *et al.*, 1997; Hess *et al.*, 1997); or a snake AdV with a balanced G+C content (Farkas *et al.*, 2008). Eredetileg hüllőnek gondolták, és célzottan ezért lezdték az alacsonyrendű gerincesek AdV-it vizsgálni (Harrach, 2000). Current knowledge indicates that the original host vertebrates of this genus might be the squamate reptiles (Squamata) *viz*. lizards including snakes (Benkő & Harrach, 2003; Kovács & Benkő, 2011). However diverse the species constituting the genus with respect to pathological or epidemiological implications might be, they all share the same genome plan described above, with genus-specific genes (p32K, LH1–3, RH genes) present only within *Atadenovirus*. The genus, along with *Siadenovirus* (see section 1.6), was acknowledged by the International Committee on Taxonomy of Viruses (ICTV) in 2002. A universal PCR for the detection of any AdV was first used to amplify sequences of various lizard AdVs (Wellehan *et al.*, 2004). The atadenovirus genome is comparable to siadenoviruses in its compactness, but possesses quite a long E4 region (Davison *et al.*, 2003).

1.3.4 Siadenovirus

The subject matter of the present study has been relatively under-represented with only two known members for years. These include TAdV-3 (Pitcovski *et al.*, 1998) and FrAdV-1 (Davison *et al.*, 2000). TAdV-3 (THEV) used to be classified as a group II aviadenovirus until the genus *Siadenovirus* was established in 2002. Serologically indistinguishable strains of TAdV-3 are capable of causing three different pathologies in three different bird species, HE in turkeys, marble speen disease in pheasants, and splenomegaly in chickens (Pierson & Fitzgerald, 2008; Smyth & McNulty, 2008).

1.3.5 Ichtadenovirus

The fifth genus *Ichtadenovirus* has recently been approved, and is considered a distinct, ancestral adenoviral lineage (often used as an outgroup in phylogenetic calculations), whose sole known representative was isolated from a chondrostean fish of great economic value, the white sturgeon (*Acipenser transmontanus*) (Benkő *et al*., 2002; Kovács *et al*., 2003). Its genome is currently subjected to analysis and exceeds all expectations as to size and arrangements of genes, which is most peculiar in comparison with any other AdV genomes (personal communication).

1.4 BASIC BIOLOGY

The following description is based mainly on the studies of human AdVs, especially HAdV-C. Entry takes place by a primary contact through the fiber knob to the coxsackie-adenovirus receptor (CAR), a member of the immunoglobulin superfamily, in species HAdV-A, -C, -E and -F (Philipson & Pettersson, 2004; Coyne & Bergelson, 2005 and 2006). Most of the HAdV-B viruses attach to the membrane complement regulatory molecule CD46 (Segerman *et al.*, 2003; Gaggar *et al.*, 2005; Marttila *et al.*, 2005). In case of certain types associated with epidemic keratoconjunctivitis (such as human AdVs 8, 19, and 37 in species D), surface sialic acid residues were evidenced to mediate endocytosis in lieu of or in concert with CAR (Arnberg *et al.*, 2000 and 2002). In primate AdVs, a secondary connecting of the RGD motif of the penton base to cellular *αvβ*3/*αvβ*5 integrins (Mathias *et al.*, 1994) takes place during internalization via clathrin-coated vesicles (Patterson & Russell, 1983).

The membrane lytic capsid protein VI facilitates entry by fragmenting membranes of the endosomes. The virion is uncoated (eclipse) and the viral DNA is transported to the nucleus by way of the microtubular system in association with the microtubule organizing centre (MTOC) (Lee *et al.*, 2003; Wodrich *et al.*, 2006). Polypeptide μ (X) targets the nucleolus and modulates the expression of E2 proteins (Lee *et al.*, 2004). Genome replication (reviewed in de Jong & van der Vliet, 1999), as deduced from the study of HAdV-2 and 5, is brought about by three gene products encoded by the E2 transcription unit. These are the adenoviral DNA-dependent DNA polymerase (pol), precursor terminal protein (pTP) and DBP. The pTP is subject to viral protease activity whereby two copies of TP are released and covalently linked to the 5' termini of the genome. It, along with DBP and the *cis*-acting ITR sequences, plays a role in the initiation process of replication. The adenoviral pol exhibits 3'–

5' proof-reading exonuclease activity and catalyses strand displacement synthesis (Liu *et al.*, 2003; Webster *et al.*, 1997).

Besides pTP, several other precursor proteins go through post-translational modification before they attain their final, active form. Adenoviruses have their own endopeptidase whose gene is located between those of hexon and DBP. This is one of the most conserved adenoviral genes. Precursors pIIIa, pVII, pX, pVI, and pVIII are all subject to protease cleavage (Russell, 2000; Russell, 2009). An 11 residue peptide (pVIc), the product of the cleavage of pVI by the protease, binds to the same protease and increases its activity by a factor of 300. The viral DNA itself increases the catalytic rate of the protease 6000-fold (Mangel *et al.*, 1996; Baniecki *et al.*, 2001). This makes this enzyme the only known protease that requires DNA for maximum activity.

The structural protein IVa2 plays a major part in both encapsidation and regulation of late transcription. Packaging of the virus DNA involves a multimeric complex of DNA, IVa2, pVII, and 52/55K (Tyler *et al.*, 2007; Zhang & Arcos, 2005). There is evidence of another non-structural protein, 22K, being involved in packaging as well (Ewing *et al.*, 2007; Ostapchuk *et al.*, 2006). The major late promoter (MLP) is regulated by a transcriptional complex made up of IVa2 and the non-structural protein L4 33K (Ali *et al.*, 2007; Pardo-Mateos & Young, 2004).

1.5 ADENOVIRUS INFECTION IN BIRDS OF PREY AND IN SONGBIRDS

Adenoviruses have a history of occurrence in various raptorial birds (*Falconiformes*), in some cases with serious conservational implications. Lesions consistent with adenoviral disease have been found in several species of birds of prey, including American kestrels (*Falco sparverius*) (Sileo *et al.*, 1983; Tomaszewski & Phalen, 2007); a merlin (*F. columbarius*) (Schelling *et al.*, 1989); Mauritius kestrels (*F. punctatus*), once the rarest bird on the planet (Forbes *et al.*, 1997) (it has been brought back from the brink of extinction from a single breeding pair); Taita falcons (*F. fasciinucha*) and hybrid falcons (*F. rusticolus/peregrinus*) (Van Wettere *et al.*, 2005; Dean *et al.*, 2006); orange-breasted falcons (*F. deiroleucus*) and Northern aplomado falcons (*F. femoralis septentrionalis*) (Oaks et al., 2005; Schrenzel *et al.*, 2005). The above occurrances are summarized in Table 1. Antibodies against avian adenoviruses have been detected in common buzzards (Frölich *et al.*, 2002).

No adenovirus has ever been reported from perching birds (*Passeriformes*) to date, however, avian poxvirus has been repeatedly detected in tits (Holt & Krogsrud, 1973; Gruber *et al*., 2007; Palade *et al*., 2008).

Table 1. Adenoviruses reported from raptors (Falconiformes). Asterisk (*) indicates identical strains. Adenovirus nomenclature is inconsistent.

Host	Species/Type	Sequence data	Accession number	Reference
American kestrel (*Falco sparverius*)	Falcon adenovirus A/ Falcon adenovirus 1	partial hexon	DQ460220	Sileo *et al.* (1983), Tomaszewski & Phalen (2007)
Merlin (*F. columbarius*)	-	partial hexon	EF675482	Schelling *et al.* (1989), Schrenzel *et al.* (2005)
Mauritius kestrel (*F. punctatus*)	-	-	-	Forbes *et al.* (1997)
Orange-breasted falcon (*F. deiroleucus*)	Falcon adenovirus A/ Orange-breasted falcon adenovirus*	partial hexon	AY683555	Oaks *et al.* (2005), Schrenzel *et al.* (2005)
Northern aplomado falcon (*F. femoralis septentrionalis*)	Falcon adenovirus A/ Northern aplomado falcon adenovirus*	complete: III, pVII, pX, pVI partial: hexon, pol	EF675479 AY685146 AY683541	Schrenzel *et al.* (2005 & 2007)
Taita falcon (*F. fasciinucha*)	Falcon adenovirus A/ Taita falcon adenovirus*	partial hexon	AY683554 EF675481	van Wettere *et al.* (2005), Schrenzel *et al.* (2005)
Gyrfalcon × Peregrine hybrid (*F. rusticolus/peregrinus*)	*	-	EF675478	Schrenzel *et al.* (2005)

1.6 HISTORY OF *SIADENOVIRUS*

Siadenovirus is a relatively recent taxonomical term proposed by Davison & Harrach as late as 2000, established by the ICTV. Before that, the only known siadenovirus had been referred to as group II avian adenovirus. The first documented occurrence of hemorrhagic enteritis (HE) in turkeys can be traced back to 1937 (Pomeroy *et al.*, 1937). Observations on the disease were made 20 years later, but no direct link could be established between the disease and any viral agents (Gale & Wyne, 1957). Marble spleen disease (MSD) was first reported in ring-necked pheasants (*Phasianus colchicus*) in 1966 (Mandelli *et al.*, 1966).

A new adenovirus was isolated from the granuloma-bearing kidney of the Vermont leopard frog (*Rana pipiens*) (Clark *et al.*, 1973), the first instance of adenovirus in a poikilothermic vertebrate. Named FAV-1 (now FrAdV-1), it appeared to be antigenically distinct from avian and mammalian adenoviruses; and it could not be cultivated in either of two cell lines of homologous genus origin (RP132 from *R. pipiens* and FT from *R.*

catesbeiana). It was propagated eventually on the TH-1 cell culture derived from the heart tissue of Eastern box turtle (*Terrapene carolina*).

Electron microscopy photographs were taken of icosahedral virions swarming in the cell nuclei of turkey inflicted by HE, whereupon adenoviruses were proposed to be implicated in the disease (Tolin & Domermuth, 1975). Serologic examination could not show the presence of HEV/MSDV antibodies in the serum samples of 618 free-ranging wild birds representing 42 species (Domermuth *et al*., 1977). Since the HE/MSD group of viruses had appeared to be serologically unrelated to other avian adenoviruses, the provisional taxon of "group II avian adenoviruses" was established to include them; whereas those related to CELO (Chiocca *et al*., 1996; Benkő *et al.*, 2000) by common precipitating antigens were designated "group I avian adenoviruses" (Domermuth *et al*., 1979).

All avian adenoviruses (FAdVs és TAdVs) infect standard monolayer cultures and propagate easily *in vitro*. However, the first successful *in vitro* propagation of THEV took place in MDTC-RP16 and -RP19 B-type lymphoblastoid cell lines (Nazerian & Fadly, 1982), previously established from spleen and liver tumours of turkeys infected with Marek's disease virus (Nazerian *et al*., 1982). This method did not prove lasting due to poor virus titers; THEV is routinely isolated from the spleen of turkeys, infected orally beforehand.

In the course of a characterization of THEV proteins, eleven structural and two core polypeptides were identified (van den Hurk, 1992). Capsids were shown to have a single fiber at each vertex, in contrast with group I avian adenoviruses, which have two. No significant differences between the protein properties of the avirulent (HEV-A) and virulent (HEV-V) strains of the virus were detected.

In a pathological study, spleno-enteritis of psittacine birds showed similar pathography to turkey HE (Gomez-Villamandos *et al*., 1995). Positive immunoreaction to group II avian adenovirus antigens was also demonstrated.

Variations in immune responses to MSD virus infection were reported in various lines of ring-necked pheasants (*Phasianus colchicus*), tested by lymphoblastogenesis assays (Kunze *et al*., 1996).

In a study involving DNA sequencing, the penton base and certain core proteins of THEV were characterized molecularly for the first time. The distinctiveness of the virus within the genus *Aviadenovirus* was corroborated with sequence data (Jucker *et al*., 1996). The complete genome of THEV was sequenced two years later (Pitcovski *et al*., 1998) and was found to be significantly shorter than that of another avian adenovirus sequenced at the time, CELO (Laver *et al*., 1971, Chiocca *et al*., 1996). Pitcovski *et al*. listed nine ORFs (>300

nt) that had no counterparts in other adenovirus genomes, which number later proved supernumerary. Apparently, the size of the capsid does not correlate with the genome size, since the capsid of both THEV and CELO is 70–80 nm in diameter. The capsid size appears to be more dependent on the size of the major capsid protein hexon, which in turn is very similar in both viruses. G+C content of the THEV genome was found to be surprisingly low with 35%, another striking difference from other avian AdVs. Similar G+C content had been demonstrated only in strange mammalian AdVs such as ovine AdV-7. The first PCR test for specific detection of THEV was developed in 1999 (Hess *et al*., 1999).

The commercial THEV vaccine (vxHEV), effective though it may have been, turned out to be largely immunosuppressive (Sharma, 1994). A recombinant fowl poxvirus (rFPV) expressing the native hexon of THEV was shown to act just as effectively against the virus, but with significantly lower immunodepression rates (~78% vs. 20%) (Cardona *et al*., 1999). A model for THEV pathogenesis and immunosuppression was developed by Rautenschlein & Sharma (2000).

Due to the extensive serological and molecular divergence manifest in THEV and the EDS virus, the traditional classification of avian adenoviruses was no longer tenable. Accordingly, the 7th Report of the ICTV removed THEV from *Aviadenovirus* and listed it as an unassigned species within *Adenoviridae* (Benkő *et al*., 2000). In the same year, the complete sequence of the frog AdV was published and found to be the closest relative of THEV on the basis of phylogenetic calculations (Davison *et al*., 2000). The two genomes were to a great extent similar in size, G+C content and genetic layout. These data strongly supported the establishment of a fourth genus to separate THEV and FrAdV-1 as a distinct lineage, which is duly proposed in the paper. The new taxonomical proposal was in turn approved by the ICTV in 2002 (Mayo, 2002). The genus *Siadenovirus* with the above two distinct species was then described formally in the 8th Report of the ICTV (Benkő *et al*., 2005). A calculated phylogenetic distance of 10% was laid down as species demarcation criterion within the new genus.

1.7 OBJECTIVES

PCR has revolutionized virological techniques in so far as direct detection and determination of genome sequences of non-isolated viruses have become possible. By means of a consensus, nested PCR suitable to detect probably all members of the *Adenoviridae* family, numerous novel adenoviruses were found in samples of various animals in the last couple of years. The highly degenerate primers of the PCR target an adenoviral enzyme with the most conserved

amino acid motifs, namely the gene of the DNA dependent DNA polymerase. Although the length of the fragment that can be aquired from the gene is only 300 nucleotides (nt), it is still appropriate for preliminary phylogenetic calculations and the genus-level classification of newly detected viruses.

In this manner was the existence of a seemingly new siadenovirus proven, which was detected in the internal organs of dead raptorial birds. Since the same virus occurred in several host species, it was officially named raptor adenovirus 1 (RAdV-1). On the basis of the short polymerase sequence it was determined to be a pathogen presumably representing a new virus species that was significantly distinct from TAdV-3 in spite of the similar pathological effects. Tissue culture isolation of the virus was unsuccessful, so we decided to make an attempt at the further characterization of the virus by means of PCR and sequencing done directly on the pathological material. In addition, the examination of further novel siadenoviruses found in the course of screening PCR investigations was also planned, as far as the conditions allowed. The nucleotide sequence of a significant genome fragment was determined from a new siadenovirus found in the sample of a Great tit (*Parus major*). This virus was temporarily named great tit adenovirus 1 (GTAdV-1). In case of the successful execution of the planned work, the genome organization considered characteristic to the members of the *Siadenovirus* genus was expected to be confirmed. Furthermore, the rise of the number of known siadenoviruses would be expected to augment the reliability of the phylogenetic calculations. The ultimate goal of the study was the clarification of the host origin of the lineage represented by siadenoviruses.

2 MATERIALS AND METHODS

2.1 SAMPLE ORIGINS

2.1.1 Raptor adenovirus

The sample material originated from two separate British aviaries where sporadic deaths of various exotic raptorial bird species were experienced. Adenovirus infection was suspected on the basis of necropsy and histological findings such as hepatomegaly, splenomegaly, renomegaly, proventricular and ventricular dilation, ulceration, erythema, hepatic and splenic necrosis, hepatitis, and basophilic inclusion bodies (Zsivanovits *et al.*, 2006). One specimen from each of the following three species were involved (Figure 3):

- Harris hawk (*Parabuteo unicinctus*),
- Bengal eagle owl (*Bubo bengalensis*),
- Verreaux's eagle owl (*B. lacteus*).

None of them are native to Europe. Major necropsy findings of the individual birds are outlined in Table 2. The enteric disease signs are characteristic to those caused by THEV, a siadenovirus.

Table 2. Host species in which RAdV-1 was detected.

Species	Age	Pathological necropsy findings
Harris hawk (*Parabuteo unicinctus*)	20 weeks	Moderate hepato- and splenomegaly, congested lungs, severe renomegaly
Bengal eagle owl (*Bubo bengalensis*)	1 year	Mild hepato- and splenomegaly, severe dilation of the proventriculus, ventriculus and duodenum, moderate airsacculitis, pericarditis, severe renomegaly
Verreaux's eagle owl (*B. lacteus*)	3 years	Moderate hepatomegaly, ulcerated ventriculus, mild airsacculitis, moderate renomegaly

A preliminary PCR study (Zsivanovits *et al.*, 2006) revealed that the infective agent was a siadenovirus, and a new species at that. Isolation was attempted on two separate occasions involving two different laboratories, first in chicken embryo liver cells (Zsivanovits *et al.*, 2006), then on the RP-19 lymphoblastoid cell line (permissive for THEV) (unpublished), but the virus did not grow *in vitro*. Eventually, the organ samples (homogenized spleen, liver and small intestines in PBS buffer) were transported to our laboratory. After extraction, we commenced molecular work on the virus genome.

Figure 3. Host organisms of the raptor adenovirus: 1. Harris hawk (*Parabuteo unicinctus*) 2. Bengal eagle owl (*Bubo bengalensis*) 3. Verreaux's eagle owl (*B. lacteus*). None of them are native to Europe.

2.1.2 Great tit adenovirus

Dead animals, organ samples, as well as various virological samples (mainly if tested negative for any other viruses) are routinely sent to our laboratory to look for the occasional presence of adenoviruses. A nation-wide avian influenza virus (AIV) survey took place in Hungary in 2006. The already extracted nucleic acid sample, found negative for AIV, came from a dead European great tit (*Parus major*) (Figure 4). The organ homogenates had been extracted manually with the aid of TRIzol® Reagent (Invitrogen). The sample was subjected to a nested PCR (Wellehan *et al.*, 2004), and tested positive for a novel siadenovirus species. Additional passerines were also found positive, such as Common blackbirds (*Turdus merula*), a Bohemian waxwing (*Bombycilla garrulus*), and a Reed warbler (*Acrocephalus scirpaceus*), but subsequent PCRs were unsuccessful in these cases.

Figure 4. Host organism of the great tit adenovirus: *Parus major*, a common passerine throughout Europe.

2.2 DNA EXTRACTION

DNA was extracted from the homogenized organ samples according to the following protocol, which is a slightly modified version of the method previously described by Dán *et al.* (2003). Two hundred µl of organ homogenate in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) was mixed with 10 µl of 10% sarcosyl (N-lauroylsarcosine, Sigma-Aldrich) and 4 µl proteinase K (20 mg/ml) solution. The mixture was incubated at 55ºC overnight. Subsequently, 20 µl 7.5 M ammonium acetate and 300 µl 8 M guanidine HCl (Sigma-Aldrich) were added and the mixture was further incubated at room temperature for 1 h. The DNA was then precipitated by adding 1 ml ice-cold absolute ethanol. After short mixing, the tubes were centrifuged at 13,000 rpm (13,226 × *g*) in an Eppendorf centrifuge (5417C) for 15 min. The resulting DNA pellet was washed with 1 ml 70% ice-cold ethanol. After a second centrifugation at 13,000 rpm for 5 min, the ethanol was discarded. The DNA pellet was dried briefly in a vacuum centrifuge, then resolved in 50 µl sterile, distilled water. Without quantifying the amount of DNA (part of which was obviously of chromosomal and bacterial origin), we used 1–5 µl from the sample as target in the PCRs.

2.3 NON-SPECIFIC WHOLE GENOME AMPLIFICATION

Due to the low quantity of the extracted nucleic acid in the great tit sample, an attempt was made to preserve the putative adenovirus therein. To this end, a non-specific DNA amplification method was performed with the REPLI-g® Mini Kit (Qiagen). It is based on the method of isothermal multiple displacement whole genome amplification (IMDA) (Luthra & Medeiros, 2004). This one-cycle amplification reaction requires exonuclease resistant random primers and Φ29 DNA polymerase of great processivity. The REPLI-g kit provides highly uniform amplification across the entire genome, with negligible sequence bias (Hosono *et al*., 2003). Instructions of the manufacturer was strictly observed. Five µl of the sample (with the template DNA) was mixed with 5 µl denaturation buffer and incubated at room temperature for 3 min. Next, 10 µl neutralization buffer was added. Finally, 30 µl master mix (consisting of the REPLI-g Mini DNA Polymerase and Mini Reaction Buffer) was added to the denatured sample DNA. The mixture was incubated at 30ºC overnight (10–16 h). At the end of the incubation, the DNA polymerase was inactivated by heating the sample at 65ºC for 3 min. The amplified DNA was diluted 1:20 and 3 µl of the sample was used in each subsequent PCR. Whenever it was necessary, additional IMDA reactions were performed to re-amplify the depleting DNA in the original sample (Kovács *et al.*, 2010).

2.4 PRIMER DESIGN AND PRIMER TYPES

Three types of PCR primers were used. Initially, two pairs of degenerate consensus primers (type 1) to target conserved parts of the genes penton base (III) an hexon (II) were designed on the basis of multiple aa sequence alignments from various adenoviruses. Additional consensus primers were designed, based on highly conserved aa sequence motifs, preferably with few codon variations, from pTP and DBP. Another approach was to find non-degenerate consensus primer sequences by comparing the complete sequence of TAdV-3 and FrAdV-1 with the use of BLASTn. On grounds of this method eight 19–20-nt-long primers (labelled as type 2) were designed from regions where the nt sequences of the two genomes were identical. Oligonucleotides complementary to the selected regions were synthesized in both senses to allow for dual use as forward and reverse primers. Thirdly, primers specific to RAdV-1 or GTAdV-1 were labelled as type 3. The sequence of such primers was derived from DNA fragments previously obtained with type 1 and/or 2 primers. Type 1 and 2 primers successfully used to acquire parts of the RAdV-1 genome are summarized in Table 3, while the properties of all the primers used to amplify the GTAdV-1 genome are summarized in Table 4. Feasibility and compatibility of the primers in various pair combinations were screened *in silico* by Primer Designer 2.0 (Scientific & Educational Software, State Line, PA, USA). When degenerate primers were used, the PCR conditions (especially the hybridization temperature and the duration of elongation) required optimization.

Table 3. Sequence and origin of PCR primers applicable for the amplification of RAdV-1 DNA. Type 1 primers are highly degenerate consensus primers designed on conserved aa motifs from three proteins. For nt variations the ambiguity code recommended by the IUPAC (Cornish-Bowden, 1985) is used. Type 2 primers were designed on the basis of nt sequence comparison of the two known siadenovirus genomes (TAdV-3 and FrAdV-1). The corresponding sequence of RAdV-1 proved to be identical with the exception of the primer derived from the pTP, in which the two underlined nucleotides were different (C and G instead of Ts).

Primer sequence (5'–3')	**Gene**	**Position (nt)**	
		TAdV-3	**FrAdV-1**
Type 1			
GGNGCNARRTTYTCIARCAT	pTP	8,371–8,391	8,379–8,399
TGGAAYCARGCNGTNGAYGAYTAYGA	hexon	14,630–14,656	14,635–14,661
GRTGRTTRAANGGRTTNACRTTRTCC	hexon	15,052–15,078	15,072–15,098
TGYGAYTTYAARYTNWSNATGATHG	DBP	17,125–17,150	17,187–17,212
Type 2			
CCTCTATTTCTTCTCATTTG	pTP	7,563–7,582	7,533–7,552
GGAGAAGGAAGAATCTAAAG	52K	8,691–8,710	8,679–8,698
GGGCTCTTTAGGTTCTTCC	pVI	12,998–13,016	13,045–13,063
CCTTTTGCTACTGGAACTGC	hexon	16,300–16,319	16,347–16,366

Table 4. Detailed information on all the primers used in amplifying and sequencing the genome fragments of great tit adenovirus type 1. Amplicon numbers correspond to those in Figure 10.

Amplicon	Primer name	Primer type	Target gene	Product size (nt)	Primer sequence (5'–3')	Comment
I.	PolFouter	family-specific degenerate consensus	DNA polymerase	324	TNMGNGGNGGNMGNTGYTAYCC	designed by Wellehan et al. (2004); nested PCR; detection
	PolRouter				GTDGCRAANSHNCCRTABARNGMRTT	
	PolFinner				GTNTWYGAYATHTGYGGHATGTAYGC	
	PolRinner				CCANCCBCDRTTRTGNARNGTRA	
II.	sipVIfo	genus-specific non-degenerate	pVI	3,251	GGGCTCTTTAGGTTCTTCC	primers identical in TAdV-3, FrAdV-1 and RAdV-1
	sihexprotrev		Hexon		CCTTTTGCTACTGGAACTGC	
III.	sipTPdegre	genus-specific degenerate	pTP	273	GGNGCNARRTTYTCNARCAT	semi-degenerate PCR, annealing temp. varies between 46°C and 56°C
	si52Krev	genus-specific non-degenerate	52K		CTTTAGATTCTTCCTTCTCC	
IV.	si52Kfo	genus-specific non-degenerate	52K	4,660	GGAGAAGGAAGAATCTAAAG	spans seven genes; cloned into pKS/*EcoR*V
	5957pVI	specific to GTAdV-1	pVI		TCTCCAGCAAGTCCAGCAAC	
V.	szpolre	specific to GTAdV-1	DNA polymerase	3,619	GGTAGCGGATCTAGCAACTCG	species-specific
	szpTPfo		pTP		AATTCCTACGTTCTGTACCTG	
VI.	Cpoloutward	specific to GTAdV-1	DNA polymerase	2,201	TGTATGCTAGTGCTCTCACACATCC	semi-degenerate PCR
	IVa2deg2	genus-specific degenerate	IVa2		YTTICCISWICCIGTIGGICC	

2.5 PCR

The reactions were generally performed in a volume of 50 µl in a *Tpersonal* thermocycler (Biometra). The reaction conditions including the choice of DNA polymerase had to be tested and optimised for almost every individual target and primer pair. Accordingly, the temperature and duration of the steps in the cycles ranged as follows:

- Initial denaturation: 95 or 98ºC, 5 min,
- Denaturation: 94 or 98ºC, 30 sec,
- Hybridization: 42–60ºC, 30 sec,
- Elongation: 72ºC, 1–4 min,
- Final elongation: 72ºC, 5–10 min.

Depending on amplicon size, 35–40 cycles were carried out. Three types of DNA polymerases were used, as summarized in Table 5.

Table 5. Types of DNA polymerase used in the PCRs

Brand	Application	Manufacturer
RedTaq® DNA Polymerase	<1–2 kb amplicons	Sigma-Aldrich
Phusion™ High-Fidelity DNA Polymerase	longer amplicons	Finnzymes
FailSafe™ PCR System, contains 12 premixes	problematic targets, no or multiple amplicons	Epicentre

If applicable, the DNA of TAdV-3 was used as positive control. Ten µl aliquots of the reactions were checked by electrophoresis in 1% agarose gels (Biorad). Gels were dyed with GelRed (Biotium). GeneRuler™ DNA Ladder Mix (Fermentas) was used for molecular mass standard. Photographs were taken with a Kodak Gel Logic 212 Imaging System (Carestream). DNA fragments of the expected size were excised from the gels and purified with the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. If the reaction resulted in one specific band, then it was purified directly from the PCR tube with the GenElute™ PCR Clean-Up Kit (Sigma).

2.6 MOLECULAR CLONING

Every single PCR product was first sequenced directly with the PCR primers to verify its identity and homogeneity. Subsequently, specific DNA fragments exceeding 0.8 kb in size were cloned into one of the following vectors:

- pBluescript® II KS(+/-) (Stratagene), a phagemid, previously cleaved with *Eco*RV rendering the vector blunt;
- pGEM®-T Easy (Promega), a linearized plasmid with a single thymidine added to each 3'-end.

The choice of vector depended on the DNA polymerase used in the PCR. Both Phusion™ and FailSafe™ polymerases generate blunt ended fragments, whereas RedTaq® polymerase links A-overhangs to the 5'-ends. Ligation was achieved by incubating the mixture of the PCR product, vector and DNA ligase in the appropriate buffer at 16ºC overnight. The ligate was then either chemically transformed into DH5α *E. coli* cells using heat shock, or electronically by an EasyjecT Plus Electroporator (Equibio). Mini-preparations of the plasmid DNA were obtained by the alkaline lysis method (Sambrook et al., 1989). Selected clones were subsequently subjected to sequencing by primer walking.

2.7 POLY-A TAILING

The 5' ends of the adenovirus DNA strands are capped by the terminal protein (TP), which prevents the effective ligation of the end fragments during molecular cloning. The amplification of these terminal sequences can be accomplished by circumventing the 5' ends by means of the 5'/3' RACE Kit (Roche) using a modified protocol. The kit is designed for the rapid amplification of either 5' or 3' cDNA ends. The mechanism of poly-A tailing is depicted in Figure 5, and it was performed under the following conditions: extracted total DNA (19 µl) was mixed with reaction buffer (2.5 µl) and 2 mM dATP (2.5 µl), heated at 94ºC for 8 min, then 1 µl terminal transferase was added. The mixture was incubated at 37ºC for 20 min, then the tailing reaction was terminated by heating to 70ºC. The poly-A-tailed DNA was subjected to a semi-nested PCR using an oligo dT primer and two specific primers annealed to the opposite strand. Cycling conditions: initial denaturation at 94ºC for 1 min, denaturation at 95ºC for 30 sec + hybridization/elongation at 68ºC for 3 min, 25 cycles, terminal elongation at 68ºC for 3 min. Phusion™ High-Fidelity DNA Polymerase (Finnzymes) was used.

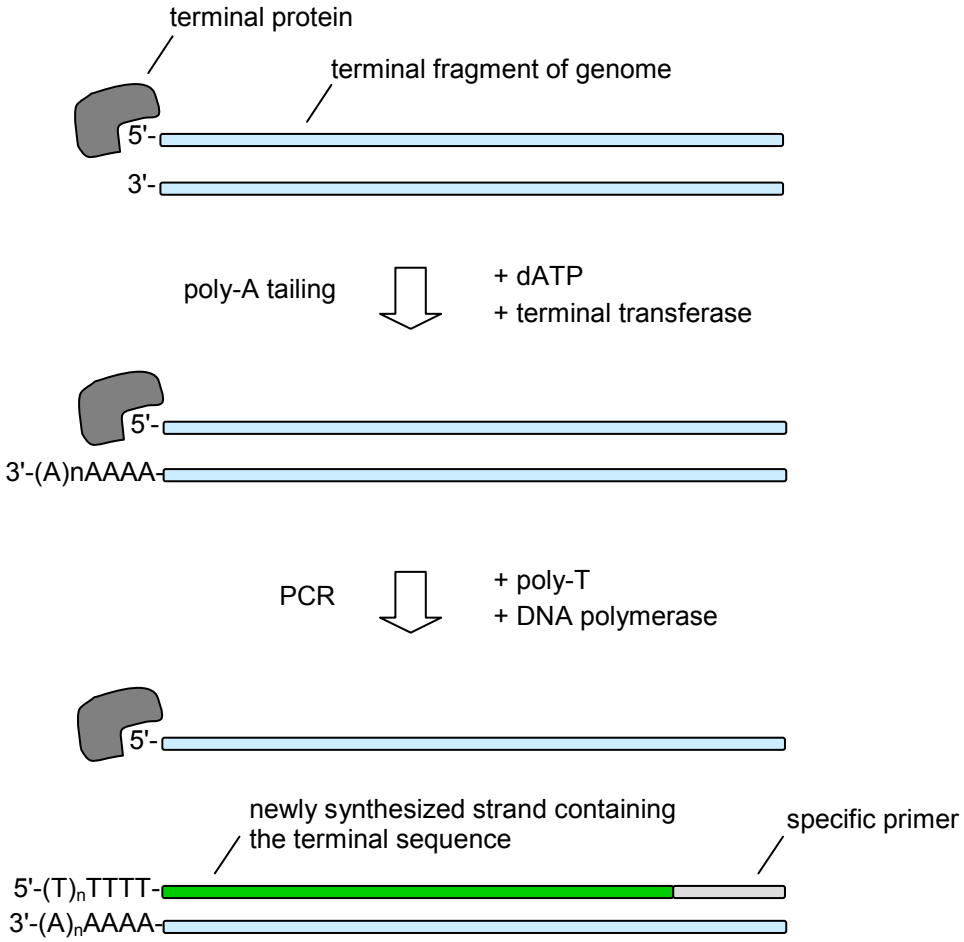


Figure 5. Determination of the genome ends by means of poly-A tailing. The 5'-end of the adenoviral genome is capped by the terminal protein which prevents effective cloning. However, adenines can be ligated to the 3'-end using the terminal transferase enzyme. The poly-A tail then can be matched with a poly-T primer which serves as a starting point for the DNA polymerase in a subsequent PCR. The newly amplified fragment will contain the terminal sequence of the viral genome.

2.8 SEQUENCING

Small DNA fragments were sequenced directly with the PCR primers. Long (>0.8 kb) products were subjected to primer walking. The identity of each sequence was verified by BlastX (Altschul *et al.*, 1990) searches.

DNA sequencing was carried out with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the following temperature profile:

- initial denaturation: 96ºC, 1 min;
- denaturation: 96ºC, 10 sec;
- hybridization: 50ºC, 5 sec;
- elongation: 60ºC, 4 min.

The reactions were subjected to electrophoresis on ABI 373A or 3100 automated DNA sequencers (Applied Biosystems) by commercial service providers.

2.9 SEQUENCE ANALYSIS

The following softwares were used for the *in silico* analysis of the acquired sequences. Raw sequence data was viewed with the BioEdit Sequence Alignment Editor v5.0.7 (Hall, 1999) and/or the free software FinchTV v1.4 (http://geospiza.com/finchtv, Geospiza Inc., Seattle, WA, USA). For the determination of splice sites, a comparative approach was used. Wherever splicing was expected (*e.g.* pTP, DBP or 33K), the splice donor and acceptor sites were determined manually.

2.9.1 Staden

The sequence (trace) files were processed and assembled by the Staden package using the programs Pregap4 v1.5 and Gap4 v4.10 (Bonfield *et al.*, 1995; Staden, 1996) (Figure 6).

Figure 6. Screenshot of the Gap v4.10 user interface (clockwise from bottom left): output window, contig selector, contig editor, trace display window.

Before entry into a Gap4 database the raw data from sequencing instruments needs to be passed through several processes, such as screening for vectors, quality evaluation, and conversion of data formats. Pregap4 was used to pass the readings through these steps. Gap4 is a genome assembly program. In general the assembly algorithms build the largest contigs they can by finding overlaps between the readings. Generally the 3' ends of readings from

sequencing instruments are of too low a quality to be used to create reliable consensus, but they can be useful, for example, for finding joins between contigs. The final result from the sequencing project was a consensus sequence.

2.9.2 Artemis

The genome was annotated by Artemis Release 10 (Rutherford *et al.*, 2000), a free genome viewer and annotation tool that allows visualisation of sequence features and the results of analyses within the context of the sequence, and also its six-frame translation. The entire genome can be viewed in one screen.

2.9.3 Similarity Plotting

SimPlot (Lole *et al.*, 1999), a widely used software for recombination analysis, calculates and plots the percent identity of the query sequence to a panel of reference sequences in a sliding window, which is moved across the alignment in steps. The window and step sizes are adjustable. Each curve is a comparison between the genome being analyzed and a reference genome. SimPlot was used to demonstrate the distance across siadenovirus genomes.

2.10 PHYLOGENETIC ANALYSIS

2.10.1 Nucleotide and protein alignments

The assembled nt sequence was translated *in silico* with the JavaScript DNA Translator 1.1 (Perry, 2002) accessible online at http://nbc11.biologie.uni-kl.de/framed/left/menu/auto/right/JDT. Multiple aa alignments from the predicted aa or nt sequences were made either by MultAlin v5.4.1 (Corpet, 1988) with Blosum 62-12-2 score matrix, or by ClustalX v2.0 (Larkin *et al.*, 2007) was also used with the Blosum series for protein weight matrix.

2.10.2 Maximum likelihood

Phylogenetic tree reconstructions were done using sequences of prominent representatives of every major adenoviral lineage. Maximum likelihood (ML) analysis was carried out through the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py; Néron *et al.*, 2009) using PhyML (Guindon & Gascuel, 2003).

2.10.3 Bayesian inference

Bayesian inference was computed by MrBayes v3.1.2 (Huelsenbeck *et al.*, 2001; Huelsenbeck & Ronquist, 2001) using the following settings. The amino acid model was set to mixed,

allowing the MCMC (Markov chain Monte Carlo integration) (Larget & Simon, 1999) to sample all the ten available fixed rate models without extra computational burden. This is roughly equivalent to model testing: by the end of the run the program determines which particular model contributed most to the posterior distribution. The WAG (Whelan & Goldman, 2001) and rtREV (Dimmic *et al.*, 2002) models contributed the most to the posterior probabilities (pp) of trees inferred from aa alignments. For nucleotide alignments, the GTR (general time reversible) (Tavare, 1986) model was preferred. Rate variation across sites was allowed according to a gamma distribution. Topology was not constrained, all sample trees were associated with equal prior probability. Outgroup (WSAdV-1) was specified as a prior. Branch lengths were computed in accordance with a uniform molecular clock. Two independent runs were computed for 10^6 generations. Every 100th tree was sampled, out of which 25% were discarded as burn-in. The average standard deviation of split frequencies fell below 0.01 indicating the convergence of the two runs onto the stationary distribution.

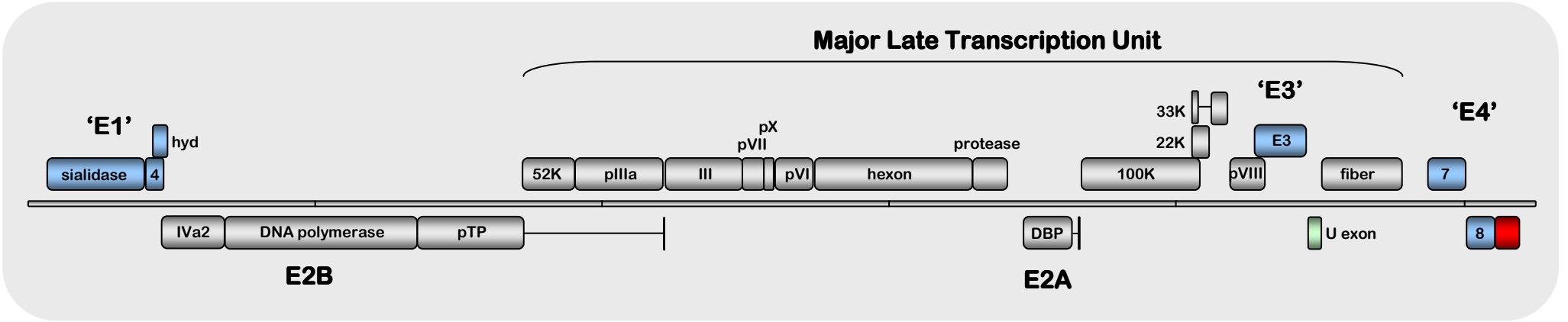
3 RESULTS

3.1 RAPTOR ADENOVIRUS 1

3.1.1 General features

The genome organization and genetic content of RAdV-1 is consistent with those in both known extant siadenoviruses. It contains 25 ORFs, 18 of which are located in the E2A, E2B, and L (late) transcriptional units and are essential for replication (Table 6).

The nucleotide (nt) sequence of the viral genome was acquired entirely by PCR performed on total nucleic acid extract of virus-containing organ samples and by DNA sequencing. The sequence comprises 26,284 nt, it was deposited in GenBank under the accession no. EU715130 constituting one of the shortest adenoviral genomes to date. The G+C content of the genome is 38.5%. The orientation and proportional size of all 25 ORFs are presented on the schematic genome map in Figure 7. Non-coding regions make up a mere 6.2% (excluding the ITRs), half of which is restricted downstream of the fiber gene (see below). The ITR sequences were found to be 29 nt long. The genetic complement of the transcriptional units E2A, E2B, and L (the constituents of which are highly conserved throughout the family) contains 18 genes in RAdV-1. These three regions are further characterized by the spliced nature of the genes of pTP and DBP. The 5' exon of pTP (a mere 5 aa) is located between the genes of pIIIa and III, more than 2 kbp upstream of the 3' exon. The 5' exon of 33K is spliced into the 5' end of 22K, whereas its 3' exon is located 5 nt downstream of the stop codon of 22K. Neither pol nor IVa2 are spliced in RAdV-1. A considerable overlap of 149 nt exists between pVIII and E3. The U exon is present but its 3' exon could not be located. This exon is highly conserved within the genus, however, similarities are not so apparent when compared to U exons from other genera. The gene of the histone-like core protein precursor VII has an elevated G+C content (49.3%) relative to the rest of the genes.



Conserved across *Adenoviridae*

Conserved within *Siadenovirus*

Present across *Adenoviridae*, conserved only within *Siadenovirus*

novel ORF

Figure 7. Genome layout of raptor adenovirus 1. The central horizontal line represents the double-stranded DNA marked at 5 kb intervals. Coloured blocks represent viral genes or ORFs proportional in size. Those above the DNA line are transcribed rightwardly, those below leftwardly. Grey blocks represent mainly structural proteins transcribed from the major late promoter into one long pre-mRNA, later to be processed through alternative splicing. Genes in the E2A and E2B transcriptional units take part in DNA replication. pTP, DBP and 33K are translated from two exons. Blue blocks represent ORFs specific to siadenoviruses. The assignment of the E1, E3, and E4 transcriptional units to these regions is based on their location only. However, homologs of all five can be found in the other two members of the genus. The green block represents an incomplete exon present in almost all adenoviruses but conserved only within the genera. Its 3' exon could not be located in any siadenovirus. The red block is presumed to be an ORF previously unidentified in any adenovirus.

Table 6. List of ORFs/genes found in RAdV-1. Transcription units E1, E3 and E4 are hypothetical, not confirmed in siadenoviruses. The description of genes in the E2 and L units are taken from Russell (2000 and 2009) and are based mainly on studies conducted on human AdV-C.

Location (nt)	ORF, strand	Description
1–29	ITR	inverted terminal repeat, shortest in family
335–2,023	sialidase, *r*	E1, non-structural, bacterial xenolog, function unknown
2,049–2,366	ORF4, *r*	E1, genus-specific, absent from FrAdV-1
2,149–2,391	hyd, *r*	E1, genus-specific
2,429–3,523	IVa2, *l*	E2B, core protein, binds to DNA, partakes in packaging
3,516–6,848	DNA polymerase, *l*	E2B, 3'–5' proofreading exonuclease, catalyses strand-displacement synthesis
6,848–8,680; 11,079–11,093	pTP, *l*	E2B, terminal protein precursor, 2 copies in virion, binds to genome end
8,662–9,567	52K, *r*	L, non-structural
9,557–11,077	pIIIa, *r*	L, minor structural protein, 60 monomers
11,099–12,445	III, *r*	L, homopentameric penton base, in complex with fiber
12,445–12,831	pVII, *r*	L, highly basic, histone-like core protein, >800 copies
12,833–13,009	pX, *r*	L, core, μ protein, ~100 copies
13,027–13,692	pVI, *r*	L, minor structural protein, ~360 copies
13,701–16,436	hexon, *r*	L, structural, homotrimeric, principal capsid protein, 240 copies, major antigen determinant
16,439–17,047	protease, *r*	L, virus-encoded cystein protease, cleaves at least 6 precursors
17,296–18,177; 18,250–18,276	DBP, *l*	E2A, DNA binding protein, spliced, functions in replication
18,322–20,373	100K, *r*	L, scaffolding structural protein
20,255–20,554	22K, *r*	L, non-structural
20,255–20,368; 20,560–20,844	33K, *r*	L, non-structural, 5' exon spliced into 22K
20,934–21,542	pVIII, *r*	L, minor structural protein, 120 copies
21,394–22,284	E3, *r*	E3, genus-specific gene with unknown function
22,302–22,538	U exon, *l*	3' exon(s) unknown
22,537–23,931	fiber, *r*	L, homotrimeric attachment protein; consists of tail, shaft and knob
24,382–25,038	ORF7, *r*	E4, genus-specific genes with unknown function
25,047–25,544	ORF8, *l*	
25,531–25,959	ORF9, *l*	E4, specific to RAdV-1, no homologs in entire family
26,255–26,284	ITR	E4, inverted terminal repeat, shortest in family

A non-coding stretch of 449 nt is discernible between the fiber gene and ORF7. This region is similar to that found in TAdV-3, but shows no resemblance to the corresponding region in FrAdV-1 (Davison et al., 2000) in as much as TAdV-3 and RAdV-1 do not contain direct repeat sequences to any extent. In RAdV-1, this part is characterized by short, alternating runs (4-5) of the same nt, just as in TAdV-3. It is also of interest to observe that on the opposite strand, upstream of ORF8, a unique ORF was detected in RAdV-1 (see section *3.1.4* for further details). However, in the corresponding stretch of ~700 nt in TAdV-3, there is a repeat region similar to that between the fiber gene and ORF7, in which no ORF was detected.

3.1.2 Sialidase

The left-hand end of the genome, upstream of IVa2, accomodates two or three ORFs. The leftmost gene is named sialidase (Davison et al., 2003) on account of its high similarity to the cellular sialidase encoded by certain bacteria such as *Clostridium* (TAdV-3) and *Akkermansia* (FrAdV-1). The sialidase in RAdV-1 is most similar to that of the Gram-negative bacillus *Bacteroides vulgatus*. Within the bacterial sialidases a conserved, recurring sequence motif can be detected, called the aspartic acid (Asp) box: S/T-X-D-[X]-G-X-T-W/F, which occurs three to five times along the sequences (Crennell et al., 1993; Roggentin et al., 1989). Three repeats of the motif are present in all three siadenoviral sialidases (Figure 8). An additional, slightly diverged form (S-X-D-X-G-X-*S*-W) of the Asp box can be found in the sialidase of FrAdV-1.

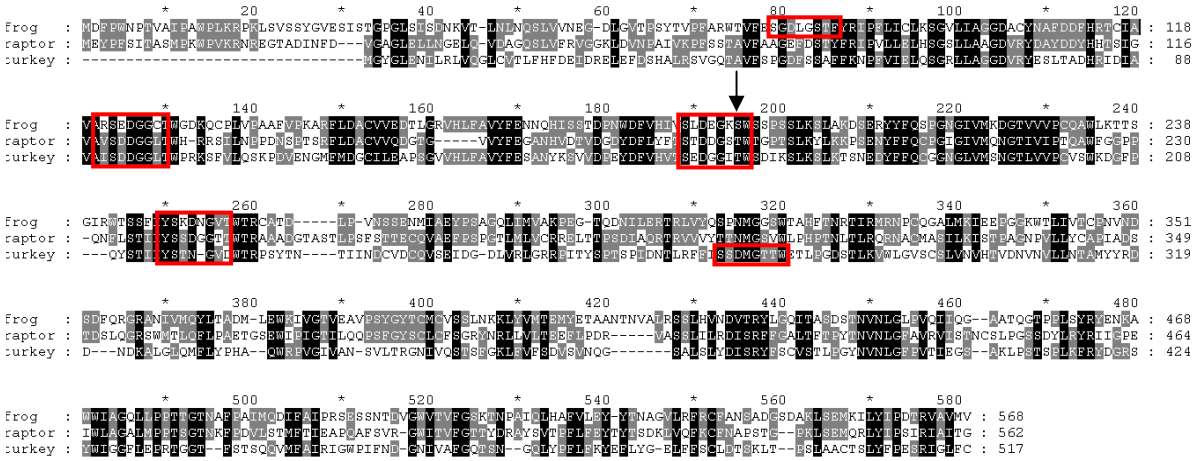


Figure 8. Recurring bacterial Asp box motifs in the adenoviral sialidase. The 8-aa-long sequences within the red rectangles follow the general pattern S/T-X-D-[X]-G-X-T-W/F. It occurs three times in the sialidase of RAdV-1 and THEV, and four times in FrAdV-1. The third motif in the frog adenovirus sialidase diverged from the above pattern at position 195 (residue 193, S in place of T, indicated by an arrow). Presence of the Asp boxes supports the bacterial origin of the gene. The three levels of shading indicates different degrees of conservation. Aligned by ClustalX, edited in GeneDoc.

3.1.3 ORF4 and hydrophobic protein

Between the genes of sialidase and IVa2 resides ORF4, with a homologous counterpart only in TAdV-3. This finding is consistent with what Pitcovski *et al*. (1998) reported. In FrAdV-1, however, Davison *et al*. (2000) identified a shorter ORF largely in overlap with the formerly identified ORF4, and named it "hydrophobic protein" on account of the high prevalence of strongly hydrophobic aa in the predicted protein. It is present in RAdV-1 and TAdV-3 but shows little similarity to one another or to that in FrAdV-1, as it is apparent from Figure 9.

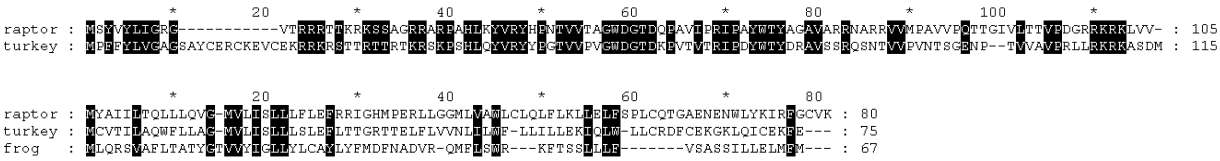


Figure 9. ClustalX alignments of the putative proteins coded by ORF4 and hyd edited in GeneDoc. The alignment on the top shows the conservation of ORF4 in raptor AdV-1 and turkey AdV-3. ORF4 is not present in the frog AdV-1 genome. Bottom alignment: the putative hydrophobic protein shows lower level of conservation across the three siadenoviruses. The alternating asterisks and numbers indicate intervals of 10 amino acids. Shading represents the degree of conservation, residues in the black columns are highly conserved.

3.1.4 Regions "E3" and "E4"

In RAdV-1, this region contains a single ORF eponymously named E3. Orthologous sequences are present in TAdV-3 and FrAdV-1, but in no other species throughout the family. No studies have been conducted regarding its function.

Three apparent ORFs reside in the putative E4 transcriptional unit, namely ORF7, ORF8, and ORF9. Homologs of ORF7 and ORF8 can be detected in the two closest relatives, TAdV-3 and FrAdV-1, whereas ORF9 appears to be unique not only within *Siadenovirus* but across all adenoviruses. In the corresponding region of FrAdV-1, a direct repeat of 608 nt is identifiable supposedly due to recombination between the sequenced virus and the initial virus stock (Davison *et al*., 2000). The function of siadenoviral E4 genes is unknown and requires further study.

3.1.5 Similarity plotting

The SimPlot tool is routinely applied to detect recombination events between various human AdV types within a particular species. Interspecies mosaicism is scarcely detected within *Adenoviridae* (though it has undeniably occurred), however, we used the software here to demonstrate the degree of divergence across the three siadenovirus genomes, as well as to bear out the status of RAdV-1 as a distinct species within the genus (Figure 10). The similarity is greater (up to 80%) in the central, conserved region, but at the genome ends, the genus specific regions, similarities expectedly drop strikingly, even below 50%. No traces of homologous recombination were detected among the three types.

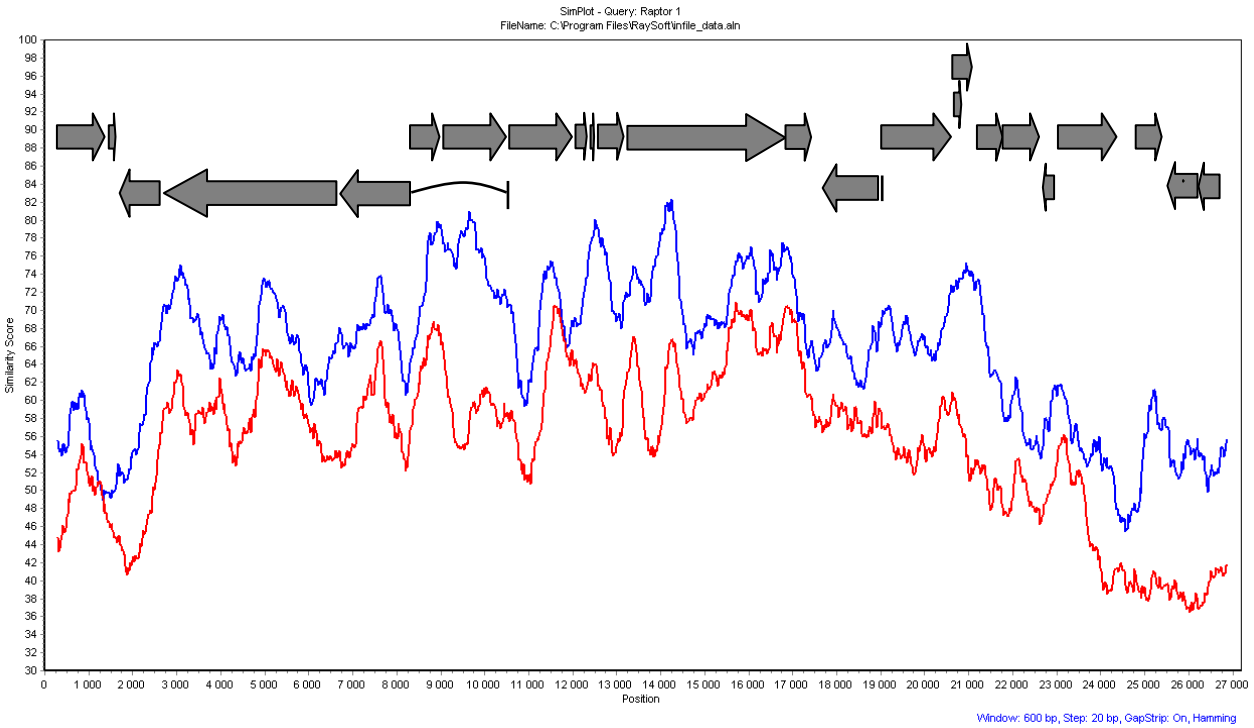


Figure 10. Similarity plotting of three siadenovirus genomes. The query sequence is RAdV-1, against which the TAdV-3 (blue) and FrAdV-1 (red) genomes were plotted. No recombination was detected among the three genomes.

3.2 GREAT TIT ADENOVIRUS 1

3.2.1 Genome features

A stretch of 13,628 nt sequence of the central part of the genome of the putative GTAdV, encompassing eight full (DNA polymerase, pTP, 52K, pIIIa, III, pVII, pX, pVI) and two partial (IVa2 and hexon) genes, was acquired by IMDA, PCR, and sequencing. The sequence was deposited to GenBank under the accession no. FJ849795. Considering the size of the three full genomes from siadenoviruses known to date, supposedly half of the DNA sequence of GTAdV is determined. The genetic content and arrangement of genes in the fragment are consistent with what had been found in siadenoviruses described earlier (Davison and Harrach, 2002) and in RAdV-1. In mastadenoviruses, this genome region contains one additional gene that is missing from the members of the other four genera. Accordingly, a homolog of protein V could not be found in GTAdV. Moreover, the remarkably biased G+C content (37.5%), a characteristic feature of siadenoviruses, could be observed as well.

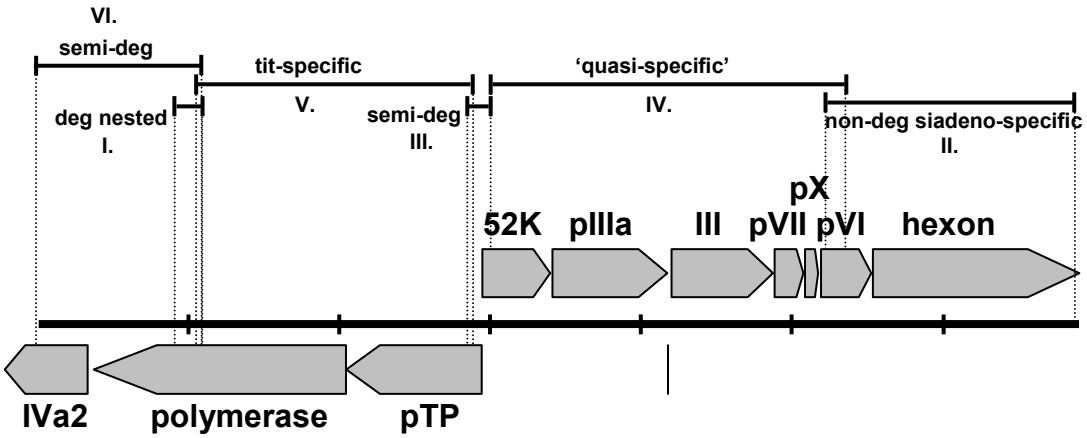


Figure 11. Schematic presentation of the genome fragment of GTAdV-1 amplified by means of 6 successful PCRs with various primer types. Approximately 50 attempts were made until the above fragment was acquired. The central horizontal line represents 13.7 kb of continuous sequence marked at 2 kb intervals. Arrows denote individual genes, with two incomplete ones (IVa2 and hexon) at the ends. The 5' exon of pTP is represented by a vertical line between pIIIa and III. The six reactions, resulting in six overlapping PCR products, are projected on the map and are labelled by Roman numerals. Occasional ambiguities at all joining regions were resolved by bridging PCRs.

The sequence of the genome fragment was derived from amplicons obtained by six successful PCRs out of about 50 attempts with various primer combinations. A partial genome map of the putative GTAdV is presented schematically in Figure 11. The amplicons are labelled with ascending Roman numerals in the chronological order of their acquisition. Due to the different annealing temperature requirements of the primers, the temperature profile of the PCRs was adjusted accordingly. The amplification strategy with details concerning primer sequences and specific PCR conditions are summarized in Table 4.

The initial detection of GTAdV was through amplicon I (300 nt) obtained by a nested PCR using adenovirus family-specific degenerate consensus primers (Wellehan et al., 2004). Amplicon II (3,251 nt in length) was generated using non-degenerate siadenovirus genus-specific primers. The target sequences upon which these PCR primers were designed are identical in all three siadenoviruses examined earlier (Kovács and Benkő, 2009). This amplicon covers almost the entire hexon gene except for a few triplets at the 3' end. It also includes a large 3' portion of pVI.

Amplicon III was acquired by a semi-degenerate PCR. The left end was primed by a genus-specific degenerate consensus primer, whereas the right end by a genus-specific non-degenerate primer. Amplicon III was found to be 273 nt long covering the initial codons of the 3' exon of pTP (transcribed leftward) and the 5' end of 52K (transcribed rightward). As in other siadenoviruses, the 5' exon of pTP that codes for five amino acids (MNQEL) is located in the short (20 nt long) intergenic sequence between pIIIa and penton base (protein III)

contained in amplicon IV. The sequence of this short amplicon provided a suitable starting point, from which bridging PCRs could be primed in both directions.

In order to join amplicons II and III, the siadenovirus-specific non-degenerate primer (si52Krev) from the previous reaction was reversed (si52Kfo) and matched with a species-specific primer (5957pVI) derived from amplicon II. The reaction resulted in the longest fragment (Amplicon IV of 4,660 nt) spanning through six genes (52K, pIIIa, III, pVII, pX, and pVI). These ORFs along with the hexon gene constitute the late transcription unit (L region) initiated by the major late promoter. The unit consists of genes encoding structural and core proteins (Benkő et al., 2005).

Amplicons I and III were joined by a PCR with species-specific primers. The fragment (Amplicon V of 3,619 nt) completed the sequence of pTP and contained a large part of the viral pol. Both genes are leftward-oriented and constitute part of the E2B transcription unit.

To amplify the leftmost fragment, a genus-specific degenerate consensus primer was designed on the basis of the IVa2 sequences of TAdV-3 and FrAdV-1, both retrieved from GenBank. This primer was matched with a specific primer (Cpoloutward) and the semi-degenerate PCR resulted in Amplicon VI, 2,201 nt in length. It covers the 5' two-thirds of IVa2 and completes the sequence of the DNA polymerase gene.

Further attempts at the amplification of the genome parts leftward from IVa2 and rightward from hexon for the time being remained unsuccessful.

3.3 PHYLOGENY OF *SIADENOVIRUS*

Bayesian inference and ML analysis resulted in phylogenetic trees with essentially the same topology. Slight topological variations may be present within the genera. However, relations of the five genera invariably show the same pattern regardless of the phylogenetic method used or the gene upon which it was run. Calculations with other genes such as DNA polymerase, penton base or protease resulted in the same tree no matter what method was used (ML or Bayesian). The relationship of the five known AdV genera is depicted in Figure 12. The phylogenetic tree reconstruction was based on the complete sequence (~900 aa) of the principal capsid protein, hexon. In general, phylogenetic calculations indicate that, if *Ichtadenovirus* is seen as an outgroup taxon, then the genera *Aviadenovirus* and *Siadenovirus* represent ancient clades, whereas *Atadenovirus* and *Mastadenovirus* contain more derived taxa. All five genera represent distinct, monophyletic lineages, out of which *Siadenovirus* is a scarcely populated one including three species originating from birds and one from a frog.

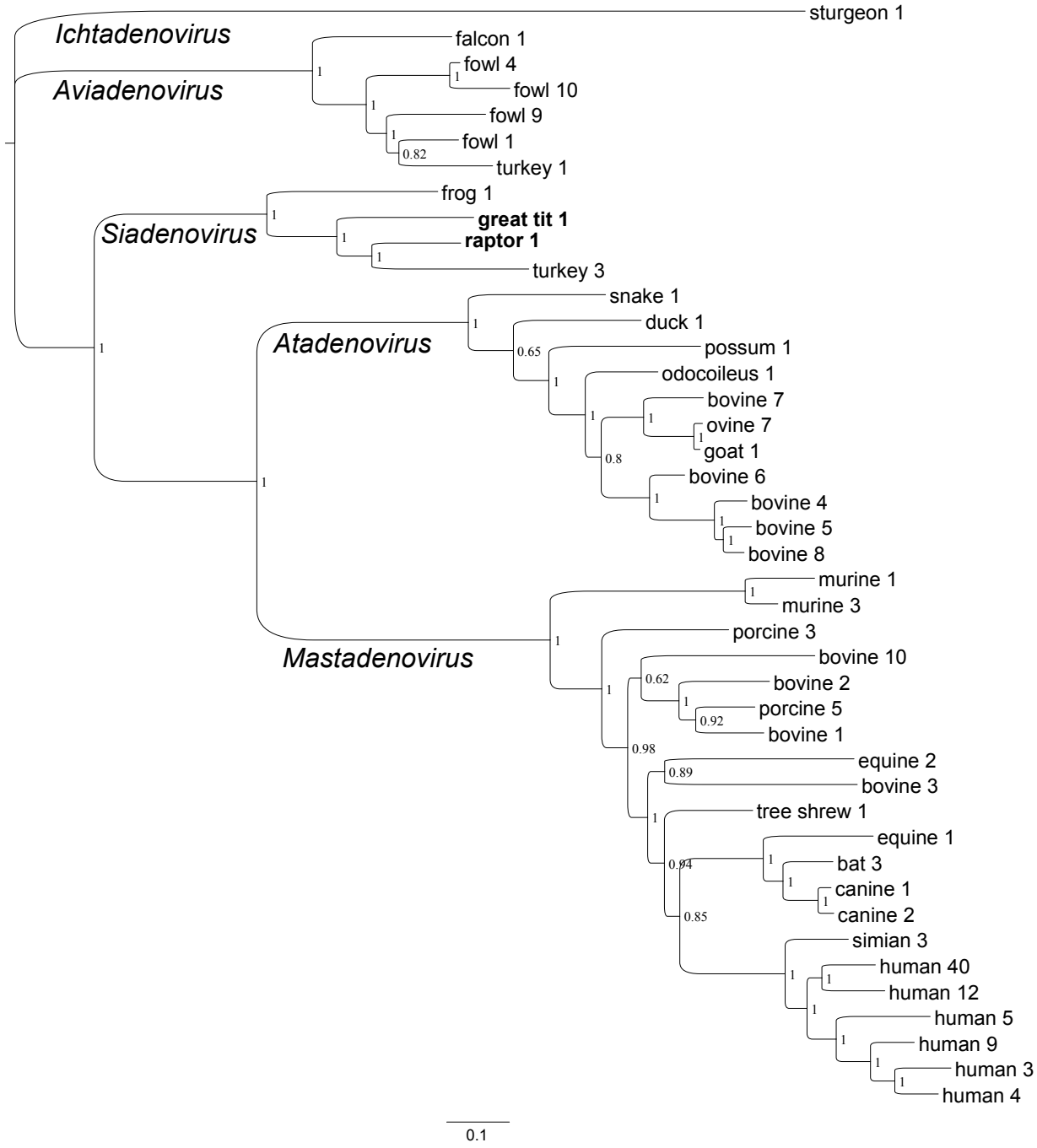


Figure 12. Bayesian phylogeny inferred from the alignment of the principal capsid protein hexon (~900 aa). All five lineages represent distinct, monophyletic genera. White sturgeon adenovirus1 is used as an outgroup. Siadenoviruses derived from birds are clustered on the same branch.

Figure 13 is intended to illuminate relationships within the genus *Siadenovirus*. The Bayesian inference here was based on the partial (267 nt) viral DNA polymerase gene because it allowed for two additional novel types to be included in the tree, namely the psittacine AdV-2 (Wellehan *et al*., 2009) and the sulawesi tortoise AdV-1 (Rivera *et al*., 2009). When the inference was made from nucleotide alignments (Figure 13a), and turkey AdV-1 (an aviadenovirus) was used as an outgroup, two sister clades were apparent, with GTAdV-1, RAdV-1, and TAdV-3 on one branch, and psittacine AdV-2, sulawesi tortoise

AdV-1, and FrAdV-1 on the other. However, the clustering of psittacine AdV-2 with the tortoise and frog AdVs was supported by a relatively low pp value (0.56). When the calculation was based on aa alignments (Figure 13b), all four bird-related siadenoviruses were grouped on the same branch, whereas the tortoise and frog AdVs were separated on a different branch. In this case, the relationship among RAdV-1, GTAdV-1 and TAdV-3 was not resolved and is depicted as a trifurcation, but their monophyly was strongly supported by a high pp value (0.93); whereas the clustering of the psittacine AdV-2 was again weakly supported (0.66). From these two trees the following conclusions can be drawn with regard to the relationships within *Siadenovirus*. Firstly, the monophyly of the three bird-related siadenoviruses (RAdV-1, GTAdV-1, TAdV-3) was strongly supported. Secondly, the monophyly of the tortoise and frog AdVs was invariably supported by a maximum pp value (1.00). The relationship of the psittacine AdV-2 to the above two sister groups within the genus could not be resolved most probably due to the shortness of the sequence upon which the inference was based, as well as to the low number of representatives.

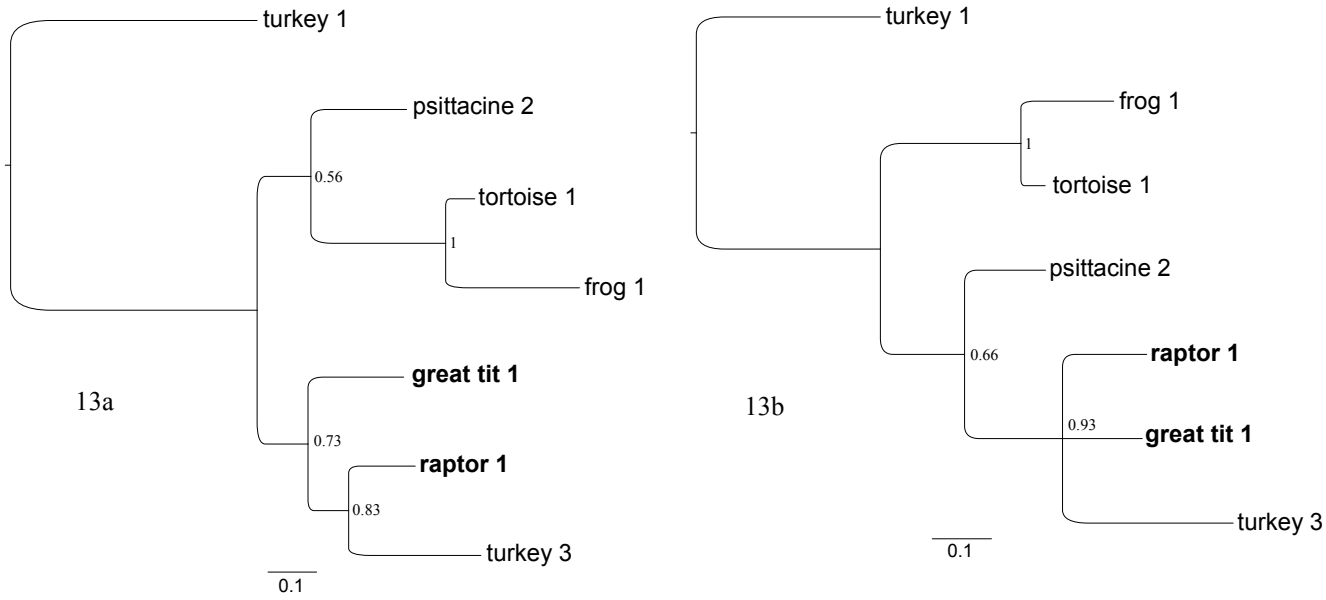


Figure 13. Phylogenetic relationships within the genus *Siadenovirus* based on partial DNA polymerase sequences calculated by MrBayes. 13a is inferred from nucleotide alignments and 13b is from amino acid alignments. Frog AdV-1 and tortoise AdV-1 invariably show monophyly, supported by maximum posterior probabilities, whereas raptor AdV-1, turkey AdV-3 and great tit AdV-1 occupy a different, common branch. The affiliation of psittacine AdV-2 is not resolved due to low posterior probabilities. Turkey AdV-1 from the genus *Aviadenovirus* was chosen as an outgroup.

4 DISCUSSION

4.1 CURRENT STATE OF *SIADENOVIRUS*

TAdV-3 for years had been considered an avian AdV out of the ordinary. When its genome sequence became available, however, the differences from the prototype avian AdV, fowl AdV-1, were greater than expected. The genome of the latter was significantly longer (~45 kb), whereas that of TAdV-3 was as much shorter (~26 kb), than the genome size characteristic to mastadenoviruses (~36 kb) considered universal at an early stage. A couple of years later the genome sequence of FrAdV-1 showed that TAdV-3 was not a unique, very special virus, but the two constituted a distinct group, phylogenetically separate from aviadenoviruses. In the meantime, another completely sequenced, special avian adenovirus, duck adenovirus 1 (that is the EDS virus), turned out to possess a fourth type of genome organization, which on the basis of phylogenetic calculations belonged to a fourth clade, the newly established *Atadenovirus* genus.

The new genome sequence, that of RAdV-1, entirely consolidated the genome features characteristic to siadenoviruses. The genomes of these AdVs probably represent the shortest, minimal AdV genome sufficient for propagation.

Thanks to the augmented sensitivity of the consensus, nested PCR described a few years ago (Wellehan *et al.*, 2004), animal-related adenovirus detection seems to have been gathering pace, and with that several novel siadenoviruses have been reported. While, despite targeted attempts, no new adenoviruses were found in amphibians, siadenoviruses were detected in the representatives of several bird species. One of the subject matters of the present study, RAdV-1, was reported in three different species of raptorial birds (Zsivanovits *et al*., 2006). TAdV-3 infection based on electron microscopy and serology was reported from Chukar partridges (*Alectoris chukar*) (Shivaprasad, 2008). Japanese scientists identified a new siadenovirus in Budgerigar (Katoh *et al*., 2009). All three instances involved disease signs consistent with adenovirus infection. Another new type in a new species, psittacine AdV-2 (PsAdV-2), was detected by consensus PCR from a Plum-headed parakeet (*Psittacula cyanocephala*) and an Umbrella cockatoo (*Cacatua alba*) (Wellehan *et al*., 2009). No extensive molecular investigation (*i.e.* genome sequencing) was conducted in either of the above cases, only short sequences of the genes of the DNA polymerase and the hexon are available from the budgerigar AdV and from PsAdV-2, respectively. An entirely unexpected occurrence of siadenovirus took place in Sulawesi tortoises (*Indotestudo forstenii*) (Wellehan *et al*., 2009), the first adenovirus detected by PCR in a non-squamate reptile. This was indeed

surprising, given that every AdV previously detected in reptilian hosts proved to be atadenoviruses. Even more interesting was a report of adenovirus from another chelonian, the ornate box turtle (*Terrapene ornata*) (Farkas & Gál, 2009). This turtle genus happens to be the same in the cell culture of which FrAdV-1 was isolated (Clark *et al*., 1973). This new adenovirus cannot be classified in either of the recognized genera, and most likely represents an additional, the sixth, genus.

At present, nt sequence from a total of seven siadenoviruses is retrievable from GenBank (summarized in Table 7), almost all representing distinct species as well. If we ignore the avirulent strain of TAdV-3, which is basically the same sequence-wise as the virulent TAdV-3, three different siadenovirus types are sequenced completely, TAdV-3, FrAdV-1 and RAdV-1. Approximately 50% of the great tit AdV genome is sequenced, whereas further three types are represented only by a couple of hundred nt sequence from the DNA polymerase or the hexon gene. Given that the genus had been represented by only two types (in two species) so far, the analysis of RAdV-1 and GTAdV-1 represents a significant step in the confirmation of the characteristic genome organization of siadenoviruses.

It is not invariably possible to make predictions as to future prospects, but perhaps it is safe to say that the proportion of isolated viruses is bound to decrease drastically in the near future. It is certainly the case with AdVs coming from wild and exotic animals — current tendency indicates that the existence of an ever increasing number of AdVs can be proven only by PCR. Representatives of the school of "classical virology" as often as not find it difficult to approve of non-isolated viruses, as it was indeed the case with RAdV-1, however, in recent years the tide seems to be turning in favour of molecular virology. These two major fields of study complement each other nicely for the time being, but in the foreseeable future molecular techniques will predictably supersede the classical virological methods for detection and characterization.

The main problem is that in case of wild and exotic animals proper cell lines are seldom available. Aviadenoviruses used to be routinely isolated from chicken embryo cells or from embryonated eggs, but TAdV-3 was only isolated successfully in a special B-lymphoblastoid cell line (MDTC-RP16). RAdV-1 could not be propagated either, not even in the cell line in which TAdV-3 replicates. FrAdV-1, isolated from the Vermont leopard frog, was propagated in a cell line (TH-1) derived from the heart tissue of the Common box turtle (*Terrapene carolina*). Difficulties with isolation seem to confirm the host switches, since viruses that replicate in their original host will easily replicate in homologous cell lines.

It seems that the small genome of siadenoviruses is sufficient for replication *in vivo*, but not sufficient *in vitro*.

4.2 COMPARATIVE GENOME ANALYSIS

4.2.1 Divergent adenovirus genomes

Siadenovirus genomes represent the most compact and probably the shortest possible genome organization in the adenovirus family. With an approximate 26 kb, their genome is significantly shorter than that of a typical aviadenovirus. General characteristics of avi-, at- and siadenovirus genomes are compared in Table 8 in order to demonstrate the differences between three divergent adenoviral genome types occurring in birds.

Table 8. Comparison of avi-, si- and atadenovirus genomes. [a]FAdV-1 and 4 contains 2 fiber genes.

Feature	***Aviadenovirus***	***Siadenovirus***	***Atadenovirus***
Genome size	~43–45 kb	~26 kb	~33 kb
Number of conserved genes	17(18[a])+1	17+1	17+1
Number of genus-specific ORFs	20	6–7	14
ORFs in 'E1'	9	2–3	3
ORFs in 'E3'	–	1	–
ORFs in 'E4'	11	2–3	11
G+C content	53–54%	34–38%	43%
Complete genomes	FAdV-1, FAdV-9, FAdV-8, TAdV-1, FAdV-4	TAdV-3, FrAdV-1, RAdV-1	DAdV-1

Completely sequenced avi- and siadenoviruses are relatively few in number (when compared to mastadenoviruses), however, certain general observations can be made on the basis of the eight genomes available now in GenBank. The genome of only one atadenovirus from birds is sequenced, that of DAdV-1 (EDS). With respect to the genes of the E2A, E2B, and L transcription units, representatives of the three genera show little difference apart from an additional fiber gene in FAdV-1. In addition, electron microscopic evidence attests that in all three fowl AdVs, two fibers (in FAdV-1, the two are different in size) extend from each vertex of the assembled capsid. Siadenoviruses follow the general plan of the conserved transcriptional units characteristic to most AdVs. Differences between the three genome types are most manifest in the gene composition of the flanking regions. Especially the number of ORFs in place of the mastadenoviral transcriptional units E1 and E4 is particularly high in

aviadenoviruses with 9 and 11, respectively, whereas it is extremely low (2–3) in the corresponding region of siadenoviruses. The EDS genome features only 3 ORFs in the E1 unit, but it has numerous ORFs in E4, similarly to aviadenoviruses. It is also of interest to note that aviadenoviruses and atadenoviruses completely lack the E3 unit, but siadenoviruses consistently feature a single ORF (of unknown function) in this region. The G+C content of the aviadenovirus genome is balanced, whereas that of the EDS virus and siadenoviruses is biased towards A+T. The genes of two structural proteins (V and IX) present only in mastadenoviruses are expectedly absent from these two siadenoviruses.

For the grouping of adenoviruses, the nucleotide composition of the genome, that is the proportion the bases G+C and A+T, has been used for quite some time. In members of the three genera (*Mast-, Avi-* and *Ichtadenovirus*) whose host origin is considered to be clarified, the proportion is usually balanced (45–55%) or slightly biased in favour of G+C. Contrarily, a strikingly high (>60%) A+T content was discernible in the initially characterized atadenoviruses (isolated from ruminants, birds and a marsupial). The name of the genus testifies of the early recognition of this fact. However, adenoviral sequences from lizards and snakes possessed a surprisingly balanced base composition, albeit phylogenetic reconstructions clearly suggested their inclusion in a common clade with atadenoviruses. In accordance with an early hypothesis, increase in the genomic A+T content may be linked to host switches (Benkő & Harrach, 2003). Certain amount of empirical evidence is available in favour of this hypothesis in other virus families such as fish herpesviruses (Doszpoly *et al.*, 2011), feline lentiviruses (Poss *et al.*, 2006), and canine parvoviruses (Shackelton *et al.*, 2006). The A+T content in the sequence of every siadenovirus so far known is above 60%.

4.2.2 Siadenovirus genomes

Besides the genomes of TAdV-3, FrAdV-1, RAdV-1 and GTAdV-1, several short sequences of novel siadenoviruses (see Table 7) are also deposited in GenBank that can be particularly useful in reconstructing phylogenies within the genus (see Figure 9).

Table 9 summarizes the size and G+C content of siadenoviral genes. The central part of the siadenoviral genomes are conserved and extremely compact across the four representatives.

Table 7. Summary of siadenovirus types described to date. Apart from the avirulent strain of THEV, all represent distinct species.

Type	Species	Accession no.	Host	Sequence	G+C	Reference
Turkey adenovirus 3 (THEV)	*Turkey adenovirus A*	AF074946	Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*), Pheasant (*Phasianus colchicus*)	complete, 26,263 nt	34.9%[a]	Pitcovski et al. (1998), Davison et al. (2003)
Avirulent turkey hemorrhagic enteritis virus strain Virginia	*Turkey adenovirus A*	AY849321	Turkey	complete, 26,266 nt	34.9%[a]	Beach et al. (2009)
Frog adenovirus 1	*Frog adenovirus A*	AF224336	Northern leopard frog (*Rana pipiens*)	complete, 26,163 nt	37.9%	Clark et al. (1973), Davison et al. (2000)
Raptor adenovirus 1	*Raptor adenovirus A*	EU715130	Harris hawk (*Parabuteo unicinctus*), Bengal eagle owl (*Bubo bengalensis*), Verreaux's eagle owl (*B. lacteus*)	complete, 26,284 nt	38.5%	Zsivanovits et al. (2006), Kovács & Benkő (2009), Kovács & Benkő (2011)
Great tit adenovirus 1	unassigned	FJ849795	European great tit (*Parus major*)	partial, 13,628 nt[c]	37.5%	Kovács et al. (2010)
Budgerigar adenovirus 1	unassigned	AB485763	Budgerigar (*Melopsittacus undulatus*)	partial, 528 nt[d]	40.5%[b]	Katoh et al. (2009)
Psittacine adenovirus 2	unassigned	EU056825	Plum-headed parakeet (*Psittacula cyanocephala*), Umbrella cockatoo (*Cacatua alba*)	partial, 269 nt[e]	37.9%[b]	Wellehan et al. (2009)
Sulawesi tortoise adenovirus 1	unassigned	EU056826	Sulawesi tortoise (*Indotestudo forsteni*)	partial, 272 nt[e]	35.3%[b]	Rivera et al. (2009)

[a] 49 nucleotide differences between the two strains (14 putative amino acid changes in sialidase, E3 and fiber knob)
[b] Value may not be representative of the whole genome
[c] Partial IVa2 and hexon, complete DNA polymerase, pTP, 52K, pIIIa, III, pVII, pX, and pVI
[d] Partial hexon sequence
[e] Partial DNA polymerase sequence

The following characteristics are common in all four. It consists of 17 genes, three of which are made of two exons, namely pTP, DBP and 33K. These genes are arranged in three transcription units. All 12 structural genes are driven by the MLP and are transcribed rightwardly: 52K, pIIIa, III, pVII, pX, pVI, hexon, 100K, 22K, 33K, pVIII, and fiber. The protease gene is also located in this late unit.

Table 9. Length and G+C content of siadenoviral genes/ORFs. aa: number of amino acid residues.

ORF	RAdV-1		TAdV-3		FrAdV-1	
	aa	G+C (%)	aa	G+C (%)	aa	G+C (%)
sialidase	562	44.8	517	38.7	568	45.8
ORF4	105	46.2	115	42.2	–	–
hyd	80	46.1	75	43.0	67	33.8
IVa2	364	35.1	361	31.4	361	34.0
DNA pol	1,110	35.7	1,112	33.1	1,108	34.9
pTP	601	36.5	598	33.2	625	34.9
52K	301	37.4	300	35.1	297	37.6
pIIIa	506	37.7	505	33.7	484	36.6
III	448	36.5	448	33.8	445	35.1
pVII	128	48.4	120	44.4	149	49.3
pX	58	32.2	58	37.3	57	36.8
pVI	221	40.6	231	38.4	217	39.1
hexon	912	38.1	906	34.4	920	40.3
protease	202	32.5	214	31.0	204	35.6
DBP	380	42.9	380	37.0	378	40.8
100K	683	37.8	630	33.2	631	38.3
22K	99	38.0	89	35.6	107	36.4
33K	132	34.3	120	33.1	129	39.0
pVIII	202	47.8	200	43.6	193	46.7
E3	325	34.4	300	34.1	305	35.9
U exon	79	42.2	85	37.0	74	33.9
fiber	464	38.1	454	34.1	429	38.7
ORF7	218	43.5	218	37.9	187	37.6
ORF8	165	41.5	165	39.0	184	36.8
ORF9	142	44.3	–	–	–	–

The E2A transcription unit contains only one gene, that of DBP, whose mature mRNA is assembled from one short and a longer exon. The E2B unit contains the genes of pTP, DNA polymerase and IVa2. The 5' exon of pTP (a mere 5 aa) is located in the very short (~20 nt) intergenic sequence between pIIIa and III. The DNA polymerase and IVa2 genes are not spliced, as opposed to mastadenoviruses. Both E2 transcription units are transcribed leftwardly. The region between pVIII and fiber, the E3 transcription unit in mastadenoviruses, contains a single ORF of unknown function.

Due to the shortness and compactness of the genome, as well as the low number of genus specific genes, there is little room for variation in the flanking regions ("E1" and "E4")

across the siadenovirus genomes. Slight differences can be observed nonetheless. First of all, the validity of two short ORFs in the "E1" region is worth discussing. Upon completion of the TAdV-3 genome, Pitcovski *et al.* (1998) had listed four ORFs in this transcription unit. ORFs 1, 2 and 3 were later merged into one single ORF, and were given the name sialidase on account of its similarity to bacterial sialidases. This gene is present in all three completely sequenced siadenoviruses.

ORF4 is a short putative gene coding for ~100 aa, located immediately downstream of sialidase. This ORF is absent from FrAdV-1. However, another shorter ORF can be detected in another reading frame a couple of nt downstream in all three siadenoviruses, largely in overlap with ORF4. Davison *et al.* (2000) named it hydrophobic protein. It is discernible in all three viruses, but similarities are superficial, as it is apparent from the aa alignments in Figure 9. Accordingly, the "E1" region of FrAdV-1 is constituted from 2 possible genes, whereas that of TAdV-3 and RAdV-1 contains three ORFs. In the course of a preliminary study, mRNAs transcribed from the sialidase gene during the early phase of infection were detected in cells infected by TAdV-3.

But as to the other two ORFs, it would be premature to draw such conslusions until further mRNA mapping studies are conducted, because arbitrary decisions as to the validity of such short ORFs may not invariably reflect the actual biological properties of the virus.

A possible explanation for the existence of sialidases in siadenoviruses can be theorized as a gene capture event through horizontal gene transfer before the last common ancestor of siadenoviruses started to split, therefore they can be considered xenologs of the bacterial sialidases. Although the function of the adenoviral sialidase is unknown, it may be relevant to note that certain human adenovirus types such as HAdV-8, 19 and 37, causative agents of epidemic keratoconjunctivitis, use sialic acid residues instead of CAR as a port of entry (Arnberg, 2009; Kaneko *et al*., 2009).

4.3 PHYLOGENY

Adenoviruses within double-stranded DNA viruses, probably due to their relatively low rate of evolution, represent a more or less reliable model for the investigation of virus–host mirror phylogeny or possible coevolution. Currently, five distinct clades (the number of which is likely to rise in the future), all representing separate genera, are discernible within the family *Adenoviridae*. In phylogenetic reconstructions, the genus *Siadenovirus* is consistently placed near the base of the tree, intimating a relatively ancient offshoot in the adenovirus family tree.

This clade is a rather poorly represented genus, and one is in a difficult position if one wishes to shed light on its evolutionary history and original hosts. It is relatively easy to establish mammals and birds as the original hosts of mast- and aviadenoviruses, respectively, since no representatives of these two genera have ever been found in other vertebrate hosts different from mammals or birds. The situation is somewhat more nebulous with atadenoviruses. A cursory look at the relationship between the genus and hosts would suggest a general reptilian origin for this clade, but the case is not as simple as that. The first atadenoviruses have been recognized in various, predominantly bovine ruminants (Vrati *et al.*, 1996; Dán *et al*., 1998; Lehmkuhl *et al.*, 2001; Both, 2004), but soon an atadenovirus of avian, as well as one of marsupial origin were characterized. The previous one was the duck AdV-1 (Harrach *et al*., 1997; Hess *et al*., 1997), whereas the other virus was detected in brushtail possums (*Trichosurus vulpecula*) in New Zealand (Thomson *et al*., 2002). The lineage was named after the strikingly high A+T content of their genomic DNA (Benkő & Harrach, 1998). In a search for the original host of atadenoviruses, attempts have been made to find and characterize AdVs occurring in lower vertebrates (Harrach, 2000). The complete genome analysis of the first reptilian AdV originating from a corn snake (*Elaphe guttata*) essentially confirmed the hypothesis on the reptilian origin of atadenoviruses (Farkas *et al.*, 2008). The genomic organization of snake adenovirus 1 (SnAdV-1) corresponded to that of other atadenoviruses characterized previously including the presence of genus specific genes such as LH, RH, and p32K (Both, 2002). The only significant divergence found was the equilibrated nucleotide composition (51% G+C) (Farkas *et al.*, 2004). Interestingly, this feature has been found to be characteristic of a number of atadenoviruses isolated from, or detected in, a number of other squamate reptiles (Squamata), including various lizards (Wellehan *et al*., 2004; Papp *et al*., 2009) and additional snakes (Farkas & Gál, 2008). It has been assumed, that the biased base composition of the genomic DNA might reflect the fact that the virus had switched hosts (Benkő & Harrach, 2003). The high (~60%) A+T content coupled with an elevated level of virulence may be an indicator of a recent host switch, in as much as these factors assist the virus in coping with a different intrahost environment (Wellehan *et al*., 2004). In theory, a gradual shift in the nucleotide composition from G+C to A+T may be an adaptive response in the continuous ‘arms race’ that takes place within a host; and may provide the virus with a significant advantage in the face of a competitive environment when the virus enters a new host for the first time. As a corollary, any AdV with a balanced G+C has likely become host adapted, while those with a biased G+C content probably represent recent (new) host invasions. The recent finding of a siadenovirus in a tortoise, also possessing an A+T rich

genome (Rivera *et al*., 2009), has further challenged the hypothesis of the general reptilian origin of atadenoviruses. Now it is more probable that only the squamate reptiles (*i.e.* in the order Squamata) are the original source of this virus lineage.

The genus *Siadenovirus* was originally thought of as the lineage that had cospeciated with amphibians (Davison *et al*., 2003), but the results of the investigation aimed at the verification of this hypothesis seem to act as a refutation. In spite of targeted attempts, no other frog AdV has yet been found. On the other hand, the number of siadenoviruses identified in birds has increased significantly from one to five, thus predominating in number. Siadenoviruses of avian origin include the highly pathogenic TAdV-3 (Pitcovski *et al*., 1998) from poultry, RAdV-1 (Zsivanovits *et al*., 2006; Kovács & Benkő, 2009) from birds of prey, great tit AdV (Kovács *et al*., 2010) from a perching bird, and two additional siadenoviruses from psittacines (Katoh *et al*., 2009; Wellehan *et al*., 2009). This fact together with the most recently demonstrated siadenovirus in Sulawesi tortoises (Rivera *et al*., 2009) implies that amphibians may not represent the original hosts of siadenoviruses but perhaps another reptilian order different from Squamata. Although birds are considered a separate class, but in actual fact, reptiles cannot be regarded monophyletic without the inclusion of birds. Adenoviruses of birds (*Aviadenovirus*) constitute a distinct clade, so it would not be surprising if the rest of the reptilian orders had their own characteristic adenovirus group. Most recently in the United States and in Hungary at almost the same time, adenoviral sequences from various sliders and box turtles were acquired, which proved to be distinct from those in the other five genera (Farkas & Gál, 2009; Doszpoly, Wellehan, personal communication). The at least four new types of turtle adenoviruses form a sixth clade, which probably merits the establishment of a new genus. In addition, every siadenovirus described thus far is rich in A+T regardless of the taxonomical status of the host species, implying that the real origin of this virus lineage is yet to be discovered.

Thus, according to current knowledge, at least four AdV lineages are present in reptiles: atadenoviruses in lizards and snakes (Squamata), aviadenoviruses in birds, siadenoviruses and members of a new group in various turtles. On the other hand, birds harbour representatives of three adenoviral genera. It appears that in case of certain adenoviruses, host switches are not uncommon between hosts species of close evolutionary relationship. Notably, adenoviral infection in worm lizards (Amphisbaenia) and in tuatara (Rhynchocephalia) has not been reported yet. Adenoviral particles observed by light and electron microscopy have been reported in crocodiles (Jacobson *et al.*, 1984), but no sequence

data are available. Thus this ancient reptilian lineage cannot be excluded as the original hosts of siadenoviruses or another AdV genus yet to be discovered.

There seems to be a certain level of incongruity in the theory of adenoviruses codiverging with their hosts. Assuming a long-term codivergence, aviadenoviruses and atadenoviruses are expected to form a clade. The problem is that they do not. Firstly, even though the ichtadenovirus is considered the most ancient clade in the family and used as an outgroup, the phylogenetic calculations are still unrooted, and we can not be certain of the exact temporal divergence of the particular clades. Their relations to one another can not be resolved clearly simply due to the immense amount of time involved, and because viruses do not fossilize. All we have at our disposal is the genetic material of extant viruses, and it is nearly impossible, even with state-of-the-art phylogenetic methods, to elucidate events that took place so deep in time hundreds of millions of years ago. That said, we are not in a position to resolve complex evolutionary matters for the time being, our intended purpose was to report the sequence of two non-isolated adenoviruses, and to demonstrate their genus affiliation. At any rate, certain hypotheses arise regarding this matter, which call for further disquisition:

The possibility of several host switches must be taken into consideration. This indeed seems a plausible explanation and is one that we have been advocating for quite some time. To be more specific, atadenoviruses present in avian and mammalian hosts are accounted for by recent host switches from squamate hosts. We say recent because the genome organization of all AdVs within *Atadenovirus* are essentially identical. Another important factor that can be relied on is base composition. Atadenoviruses from squamate hosts exhibit balanced nt compositions, whereas atadenoviruses in mammals or birds show biased nt compositions. This is invariable. We are aware that A+T bias may not be reliable from an evolutionary point of view, and character polarity must be taken into account in differentiating between ancestral, derived, or convergent character states. But still, a biased nt composition is quite reliable in ascertaining whether the virus in question is in its original host or not.

Another possibility is that divergent adenoviral lineages were present in amniote ancestors. We do not know much about this contingency at this point. We may even venture to say that it can not be resolved with current knowledge and phylogenetic methods. But new adenoviruses found in the above mentioned animals may take us closer to this goal. Chances are improving because as exotic reptiles and amphibians are gaining more and more popularity as pets, they are frequently taken to the veterinarian, allowing for the possibility of examining their viruses as well.

5 NEW SCIENTIFIC RESULTS

1. The complete sequence of the first non-isolated adenovirus was determined. The RAdV-1 genome represents the first non-isolated adenovirus and the third completely sequenced siadenovirus. It is 26,284 nt in length and contains 25 ORFs, 18 of which are conserved in all adenoviruses, 6 are specific to the *Siadenovirus* genus, and 1 seems type-specific. The G+C content is 38.5%. The inverted terminal repeats (ITR) at the genome ends are 29 nt long. Three ORFs are present in the hypothetical E1 transcription unit. In the leftmost position is the putative sialidase, after which the genus was named. In it, three repeats of the aspartic acid (D) box motif of bacterial sialidases (S/T-X-D-[X]-G-X-T-W/F) are discernible. ORF4, originally described in the genome of TAdV-3, and the so-called hydrophobic protein are both present as opposed to the genome of FrAdV-1, where no homolog of ORF4 could be identified. Between the genes of pVIII and fiber, a homolog of the E3 gene of TAdV-3, as well as the U exon were identified. Close to the right end of the genome, three ORFs were found. ORF7 and 8 have their counterparts in TAdV-3 and FrAdV-1. However, ORF9 is unique in the adenovirus family. The complete genome sequence of RAdV-1 is retrievable from GenBank under the accession no. EU715130.

2. Approximately half of the genome sequence of another non-isolated adenovirus, GTAdV-1, was determined. A total of 13,628 nt was acquired from the central, conserved genome part of GTAdV-1. Eight full (DNA polymerase, pTP, 52K, pIIIa, III, pVII, pX, pVI) and two partial (IVa2 and hexon) genes was acquired by 6 successful PCRs out of about 50 attempts. The genetic content and arrangement of these genes in the fragment are consistent with what had been found in siadenoviruses described earlier. The G+C content of the genome fragment is 37.5%. The partial genome sequence of GTAdV-1 was deposited in GenBank under the accession no. FJ849795.

4. The two new members of Siadenovirus occupy a common branch with TAdV-3 in the phylogenetic tree, separate from FrAdV-1. The siadenovirus clade represents a relatively ancient offshoot in the adenovirus family tree. Phylogenetic calculations imply that RAdV-1 and GTAdV-1 share a recent common ancestor with TAdV-3. FrAdV-1 is consistenty placed on a separate branch with another novel siadenovirus, tortoise AdV-1.

5. The increasing number of siadenoviruses found in birds along with a high A+T content in each member of the genus challenges the hypothesis of siadenoviruses having originated from amphibian hosts.

6 SUMMARY

The genus *Siadenovirus* in the *Adenoviridae* family is poorly represented. Prior to the present study, only two members were included in the clade, turkey adenovirus 3 (TAdV-3) and frog adenovirus 1 (FrAdV-1). The genus is now enriched with the genome sequence of two further, non-isolated types. Raptor adenovirus 1 (RAdV-1) was found in three different species of birds of prey, these included a Harris hawk (*Parabuteo unicinctus*), a Bengal eagle owl (*Bubo bengalensis*) and a Verreaux's eagle owl (*B. lacteus*). Great tit adenovirus 1 (GTAdV-1) was detected in a sample containing extracted nucleic acid from a dead Great tit (*Parus major*), screened for avian influenza virus beforehand. The viral DNA was acquired entirely by PCR using various primer types. The quantity of the nucleic acid in the sample containing GTAdV-1 was augmented in a non-specific manner prior to PCR. The complete genome sequence of the first non-isolated adenovirus, RAdV-1, was determined. It is 26,284 nt in length and contains 25 ORFs, 18 of which are conserved in all adenoviruses, 6 are specific to the *Siadenovirus* genus, and 1 seems type-specific. In the sialidase gene, three repeats of the aspartic acid box motif of bacterial sialidases are discernible, which suggests an early gene capture event. The inverted terminal repeats are 29 nt long. The G+C content is 38.5%. Approximately half of the genome sequence of GTAdV-1 was determined. A total of 13,628 nt was acquired from the central, conserved genome part. Eight full (DNA polymerase, pTP, 52K, pIIIa, III, pVII, pX, pVI) and 2 partial (IVa2 and hexon) genes was amplified by 6 successful PCRs out of about 50 attempts. The genetic content and arrangement of these genes in the fragment are consistent with what had been found in siadenoviruses described earlier. The G+C content of the genome fragment is 37.5%. The siadenovirus genome is relatively short and highly compact, leaving little room for variation in the terminal genome regions. The sequence of RAdV-1 bears out the hypothesis that siadenoviruses represent the shortest functional AdV genome. The two new members of Siadenovirus occupy a common branch with TAdV-3 in the phylogenetic tree, separate from FrAdV-1. The siadenovirus clade represents a relatively ancient offshoot in the adenovirus family tree. The increasing number of siadenoviruses found in birds along with a high A+T content in each member of the genus challenges the hypothesis of siadenoviruses having originated from amphibian hosts.

7 SUMMARY IN HUNGARIAN (ÖSSZEFOGLALÁS)

A *Siadenovirus* nemzetség meglehetősen alulreprezentált az *Adenoviridae* családon belül. Jelen tanulmány megkezdése előtt csak két tagja volt, a pulyka-adenovírus 3 (TAdV-3) és a béka-adenovírus 1 (FrAdV-1). A nemzetség mostanra két további, nem izolált típusból származó genomszekvenciával gazdagodott. A ragadozómadár-adenovírus 1-et (RAdV-1) három különböző vágómadárfajban találták, ezek: Harris-ölyv (*Parabuteo unicinctus*), bengáli uhu (*Bubo bengalensis*) és Verreaux-uhu (*B. lacteus*). A széncinege-adenovírus 1-et (GTAdV-1) egy elhullott széncinege (*Parus major*) kivont nukleinsavát tartalmazó mintájában mutattuk ki, melyet előzőleg madárinfluenzára teszteltek. A vírus DNS-t teljes egészében PCR-rel nyertük ki, különböző primertípusokat használva. A GTAdV-1-et tartalmazó minta nukleinsavának mennyiségét nem specifikus módon növeltük meg a PCR megkezdése előtt. Elsőként határoztuk meg egy nem izolált adenovírus (RAdV-1) teljes genomszekvenciáját. A genom 26 284 bázispár hosszú, 25 ORF-et tartalmaz, melyek közül 18 minden adenovírusban megőrzött, 6 a *Siadenovirus* nemzetség tagjaira specifikus, egy pedig típus-specifikusnak tűnik. A szialidáz génben megtalálható a bakteriális szialidázok aszparaginsav-motívuma, ami egy korai horizontális géntranszfert sejtet. A genomvégi fordított terminális ismétlődések hossza 29 nt. A G+C tartalom 38,5%. A GTAdV-1 genomjának hozzávetőleg a felét határoztuk meg. Összesen 13 628 bázispárnyi szakaszt nyertünk ki a genom középső, megőrzött régiójából. Nyolc teljes (DNS polimeráz, pTP, 52K, pIIIa, III, pVII, pX, pVI) és 2 részleges (IVa2 és hexon) gént erősítettünk fel a kb. 50 kísérlet mindössze 6 sikeres PCR-je során. Ezen gének elrendeződése megegyezik a korábban leírt siadenovírusok megfelelő régiójával. A genomrészlet G+C tartalma 37,5%. A siadenovírus-genom relatíve rövid és nagymértékben kompakt, ennélfogva a genom végi régióiban kicsi a lehetőség a variálódásra. A RAdV-1 szekvenciája megerősíti a feltevést, miszerint a siadenovírusok rendelkeznek a legrövidebb működő genommal az adenovírusok családján belül. A *Siadenovirus* nemzetség két új tagja a TAdV-3-mal közös ágat foglal el a törzsfán, elkülönülve a FrAdV-1-től. A siadenovírusok kládja egy viszonylagosan ősi leágazást képvisel az adenovírusok törzsfáján. A madarakban talált siadenovírusok növekvő száma, illetve a nemzetség minden tagjának magas A+T tartalma megkérdőjelezi a siadenovírusok kétéltű-eredetét.

8 ABBREVIATIONS

AdV	adenovirus
AIDS	acquired immunodeficiency syndrome
AIV	avian influenza virus
Asp	aspartic acid
BAdV	bovine adenovirus
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAR	coxsackie-adenovirus receptor
CELO	chicken embryo lethal orphan virus (FAdV-1)
DAdV	duck adenovirus
dATP	deoxyadenosine triphosphate
dT	deoxythymidine
DBP	DNA binding protein
EDS	egg drop syndrome
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
FAdV	fowl adenovirus
FrAdV	frog adenovirus
GON	group of nine hexons
GTAdV	great tit adenovirus
GTR	general time reversible nucleotide substitution model
HAdV	human adenovirus
HE	hemorrhagic enteritis
HEV-A	hemorrhagic enteritis virus, avirulent strain
HEV-V	hemorrhagic enteritis virus, virulent strain
HIV	human immunodeficiency virus
hyd	hydrophobic protein
ICTV	International Committee on Taxonomy of Viruses
IMDA	isothermal multiple displacement whole genome amplification
ITR	inverted terminal repeat
IUPAC	International Union of Pure and Applied Chemistry
kbp	kilo base pair

MCMC	Markov chain Monte Carlo integration
MLP	major late promoter
MLTU	major late transcription unit
MSD	marble spleen disease
MSDV	marble spleen disease virus
MTOC	microtubule organizing centre
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pol	adenoviral DNA polymerase
pp	posterior probability
PsAdV	psittacine adenovirus
pTP	terminal protein precursor
pX	μ protein precursor
RACE	rapid amplification of cDNA ends
RAdV	raptor adenovirus
rFPV	recombinant fowl poxvirus
RGD	arginine-glycine-aspartate motif
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SnAdV	snake adenovirus
TAdV	turkey adenovirus
THEV	turkey hemorrhagic enteritis virus (TAdV-3)
TP	terminal protein
UXP	U exon protein
VA RNA	virus associated RNA
vxHEV	commercial HEV vaccine
WAG	Whelan-Goldman fixed rate protein substitution model
WSAdV	white sturgeon adenovirus

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11 PUBLICATIONS

11.1 RELATED PUBLICATIONS

Kovács, E.R., Benkő, M. (2011). Complete sequence of raptor adenovirus 1 confirms the characteristic genome organization of siadenoviruses. *Infect. Genet. Evol.* 11, 1058–1065.

Kovács, E.R., Jánoska, M., Dán, Á., Harrach, B., Benkő, M. (2010). Recognition and partial genome characterization by non-specific DNA amplification and PCR of a new siadenovirus species in a sample originating from *Parus major*, a great tit. *J. Virol. Methods* 163, 262–268.

Kovács, E.R., Benkő, M. (2009). Confirmation of a novel siadenovirus species detected in raptors: Partial sequence and phylogenetic analysis. *Virus Res.* 140, 64–70.

11.2 PRESENTATIONS AT INTERNATIONAL CONFERENCES

Kovács, E.R., Harrach, B., Benkő, M. Complete sequence and genetic features of raptor adenovirus 1: a novel, non-isolated species in the genus Siadenovirus. *ESVV 8th International Congress of Veterinary Virology*, Budapest, Hungary, 23–26 August, 2009.

Kovács, E.R., Harrach, B., Benkő, M. Genome analysis of raptor adenovirus 1: a novel, non-isolated type, first member of a proposed new species in the genus Siadenovirus. *9th International Adenovirus Meeting*, Dobogókő, Hungary, 26–30 April, 2009.

Kovács, E.R., Benkő, M. Two novel siadenoviruses: molecular and phylogenetic analysis. *Adenoviruses: Basic Biology to Gene Therapy*, Zadar, Croatia, 23–24 September, 2008.

Kovács, E.R., Benkő, M. Two novel siadenoviruses: molecular and phylogenetic analysis. *14th International Congress of Virology*, Istanbul, Turkey, 10–15 August, 2008.

Kovács, E.R., Zsivanovits, P., Benkő, M. Genome sampling of a novel siadenovirus suspected to cause fatalities among raptors. *8th International Adenovirus Meeting*, Zürich, Switzerland, 30 August–02 September, 2006.

Kovács, E.R. Biodiversity of animal adenoviruses. *DNA Tumor Viruses 2006 Meeting*, Salk Institute for Biological Studies, La Jolla, CA, USA, 11–16 July, 2006.

11.3 UNRELATED PUBLICATIONS

Doszpoly, A., Kovács, E.R., Bovo, G., LaPatra, S.E., Harrach, B., Benkő, M. (2008). Molecular confirmation of a new herpesvirus from catfish (*Ameiurus melas*) by testing the performance of a novel PCR method, designed to target the DNA polymerase gene of alloherpesviruses. *Arch. Virol.* 153, 2123–2127.

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