

Phylogenetic analysis of the hexon and protease genes of a fish adenovirus isolated from white sturgeon (*Acipenser transmontanus*) supports the proposal for a new adenovirus genus

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Abstract

The organisation of the central part of the genome of a fish adenovirus (AdV) isolated from white sturgeon (*Acipenser transmontanus*) was studied. The putative genes identified between those of the viral DNA polymerase and the pVIII protein showed no significant difference in size or localisation compared to other known non-mastadenoviral genomes. The complete nucleotide sequences of the hexon and the viral protease genes and the intergenic region in the white sturgeon adenovirus (WSAdV-1) were compared with members of the four official AdV genera. In the case of WSAdV-1, merely two nucleotides separated the hexon and the protease genes, while in the other AdVs certain genus-specific features were recognised. In distance analyses based on complete sequence of the hexon or the protease proteins, the clear separation of five groups was seen corresponding to the four accepted AdV genera and WSAdV-1. Although there were slight differences between the topologies of the phylogenetic trees, the results unambiguously confirmed the distinctness of WSAdV-1 thus supporting the establishment of a new, fifth AdV genus.

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1. Introduction

Adenoviruses (AdVs) infect a wide range of vertebrate animals (Russell and Benkő, 1999), but for almost five decades only mammalian and avian adenoviruses (classified into the *Mastadenovirus* and *Aviadenovirus* genus, respectively) were subjected to detailed studies. AdVs also occur in lower vertebrates (Essbauer and Ahne, 2001). The first molecular study on an AdV strain originating from a cold-blooded vertebrate was the analysis of the full genome of an isolate from leopard frog (*Rana pipiens*) (Davison et al., 2000). This frog adenovirus (FrAdV-1) proved to be a relative of an unusual bird adenovirus, turkey adenovirus type 3 (TAdV-3), also named turkey haemorrhagic enteritis virus (Pitcovski et al., 1998). FrAdV-1 and TAdV-3 share a similar genome organisation characterised by a putative

sialidase gene close to the left terminus, and are members of the newly accepted genus *Siadenovirus* (Davison and Harrach, 2002). More recently, the genome of an AdV isolated from corn snake (*Elaphe guttata*) was studied in detail (Farkas et al., 2002), and this virus (SnAdV-1) was found to be related to the members of *Atadenovirus* (Benkő and Harrach, 1998; Both, 2002), another genus, recently approved by the ICTV (Mayo, 2002).

The presence of AdV-like particles has been observed in several fishes, but only one isolate is available to date from white sturgeon (*Acipenser transmontanus*). Initial studies, based on phylogenetic analysis of a PCR-amplified portion of the viral DNA polymerase gene, have confirmed its adenoviral character and distinctness from other AdVs (Benkő et al., 2002).

The primary objective of the work presented here was to obtain further information about the genome of the WSAdV-1 and to conduct phylogenetic analyses based on complete genes. As our main purpose was to determine the taxonomic position of the WSAdV-1 within the family

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Mastadenovirus

HAdV-12 h P F S A G N A T T •
 p CCCTTCTCGGCGGGTAACGCTACCACCTAAGAAAGGCACCTCCAGACTGCTGTAATGGGTTCAAGCGAACAGGAGCTG M G S S E Q E L

EAdV-2 h P F S A G N A T T •
 p CCGTTTTCCGCGAGAAACGCAACTACTTAAACCTCACCTGTGGCGCAGTGTGCATGGGGAGTACGGAGACAGAAGCTG M G S T E T E L

CAdV-1 h P F S A G N A T T •
 p CCCTTCTCCGCCGCAACGCCACGACCTAACTAATATGGCGGAAGGAGGTTCATCAGAAGAAGAGCTG M A E G G S S E E E L

MAdV-1 h P F S A G N A T T •
 p CCGTTTTCAGCCGCAACGCCAACCTAATCGTATGGGTTCTGCTGAAACAGAGCTC M G S S E T E L

PAdV-5 h P F S A G N A T T •
 p CCATTCTCTGCCGGTAACGCGACCACATAATGGGTTCCACAGAAGAGGAAGCTA M G S T E E E L

Aviadenovirus

FAdV-1 h P F A T G N A V •
 p CCTTTTCGCCACAGGCAACGCTGTGTAAAAAAGACGGCTGGGATGTCGGGAACACCAGACCCAACTG M S G T T E T Q L

FAdV-9 h P F A T G N A V •
 p CCTTTTCGCTACAGGCAACGCGGTGTAAAAAACTGATTAATAATGTCGGGACCACGGAAGCCAGCTG M S G T T E S Q L

Atadenovirus

OAdV-7 h P F S A G S A A T •
 p CCTTTTTCAGCTGGTAGTGACGCAACATGAGCGGCACATCCGAAAGTGAGCTG M S G T S E S E L

BAdV-4 h P F S A G S A T T •
 p CCTTTTTCAGCTGGTAGTGCTACGACATGAGCGGAACATCAGAAAGTGAATTG M S G T S E S E L

DAdV-1 h P F A A G S A A T •
 p CCTTTTGCCCGGTAGTGACGCAACATGAGCGGCACATCCGAATCAGAGTTG M S G T S E S E L

SnAdV-1 h P F A S G S A A T •
 p CCGTTTGCTTCAGGATCAGCCGCGACATGAGCGGGAGCTCCGAGCAAGAGTTG M S G S S E Q E L

Siadenovirus

TAdV-3 h P F A T G T A S V •
 p CCTTTTGCTACTGGAAGTCTTTCAGTATAAAATGGCTGGAAGTTCAGTTCAGAATTG M A G T S S S E L

FrAdV-1 h P F A T G T A S A •
 p CCTTTTGCTACTGGAAGTCTTCTGCTTAATGGGAAGTTCAGGAGCTGACTTA M G T S G A D L

fifth genus

WsAdV-1 h P F N I G A N A • •
 p CCCTTCAACATTTGGAGCTAACGCCTAATAATGGGACCTCAAATAAAGAATTG M G T S N K E L

Fig. 2. The end of the hexon and beginning of the protease gene in different adenoviruses arranged according to the four official genera. Overlapping nucleotides are printed in bold, while intergenic sequences are underlined. h: hexon, p: protease.

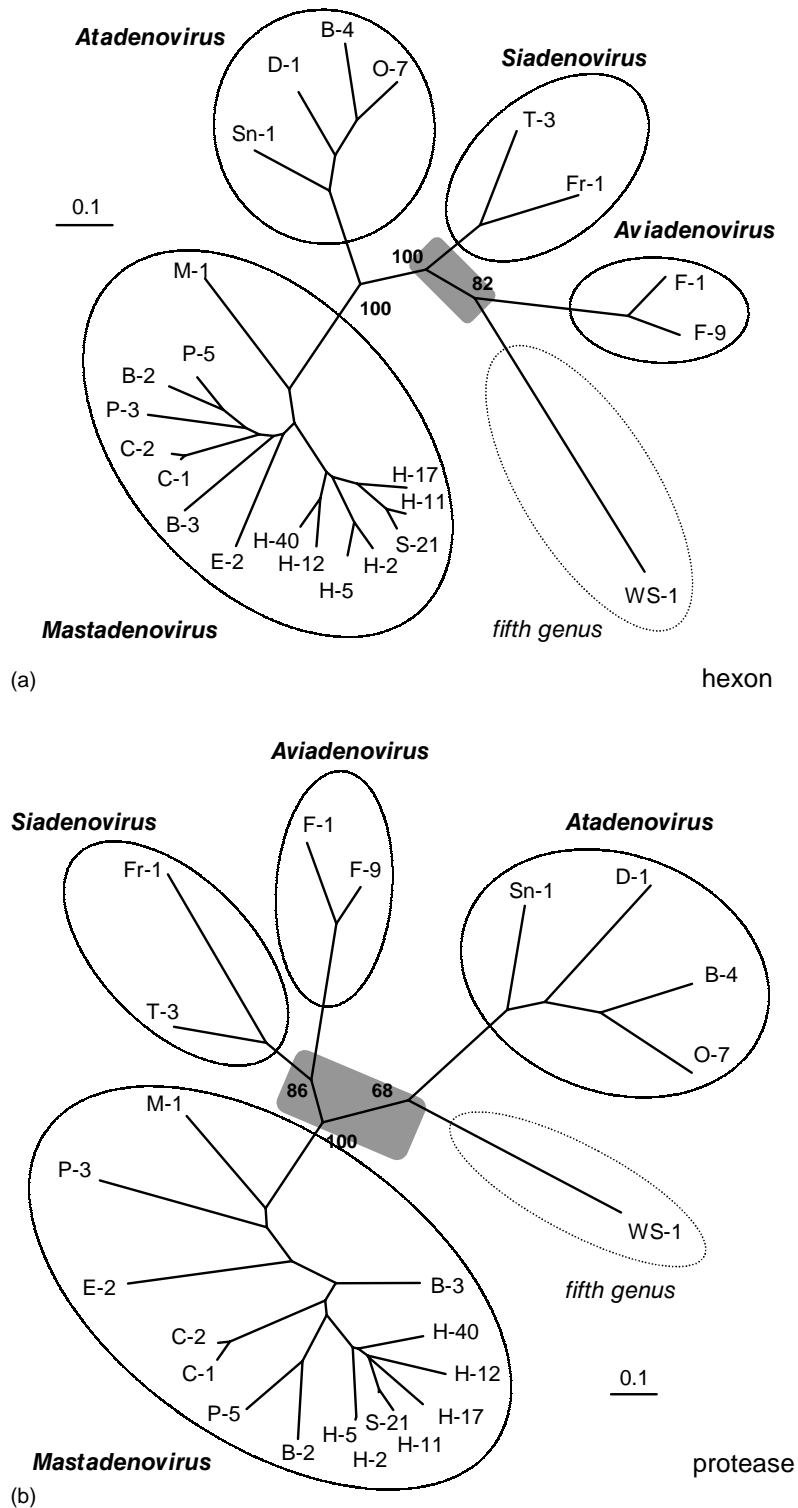


Fig. 3. Unrooted maximum likelihood trees of the 24 (a) hexon and (b) protease sequences. The trees were obtained comparing of 16 different relative positions of the five groups (see details in text). The relative positions of shadowed branching cannot be separated unambiguously; their different arrangements do not differ significantly from the ML tree. The bootstrap values were obtained from the NJ analysis (shown as percentages). The used sequences originated from the GenBank: HAdV-11 (H: human): AY163756; SAdV-21 (S: simian): NC_004001; HAdV-17: NC_002067; HAdV-12: NC_001460; HAdV-40: NC_001454; HAdV-2: NC_001405; HAdV-5: J01966; CAAdV-1 (C: canine): U55001; CAAdV-2: U77082; BAdV-2 (B: bovine): NC_002513 (the sequence was corrected); BAdV-3: NC_001876; PAdV-3 (P: porcine): NC_001997; PAdV-5: NC_002702; EAdV-2 (E: equine): L80007; MAdV-1 (M: murine): NC_000942; OAdV-7 (O: ovine): NC_004037; BAdV-4: NC_002685; DAdV-1 (D: duck; also called EDS virus): NC_001813; SnAdV-1 (Sn: snake): AY082603; FAdV-1 (F: fowl): NC_001720; FAdV-9: NC_000899; TAdV-3 (T: turkey; also called THEV): NC_001958; FrAdV-1 (Fr: frog): NC_002501. The “AdV” is omitted from the names.

map of WSAdV-1 could be further defined. In Fig. 1, the central region of WSAdV-1 is presented in comparison with the corresponding part of the FrAdV-1 genome (Davison et al., 2000). The genes identified thus far showed identical positions with, and similar size proportions to, those found in FrAdV-1. The G + C content of the individual clones ranged between 40 and 48%, and was calculated to be 45% for the entire sequence known to date. During the BLAST analyses, no tendency of WSAdV-1 to group with sequences originating from recognised adenovirus genera was noted.

Two adjacent genes (encoding the hexon and the protease) situated in a tandem arrangement on the *r* strand were chosen for further analyses. In WSAdV-1, there were only two nucleotides (nt) between these genes, and, interestingly, the hexon gene ended with two termination TAA codons. The hexon–protease intergenic sequences from AdVs representing the different genera were compared, and found to show certain genus-specific characteristics (Fig. 2). The sequences of the hexon and protease genes are available from a large number of different AdV types, and are therefore preferred subjects for phylogenetic analyses (Harrach and Benkő, 1998).

The hexon gene was 2835 bp in size, encoding 944 aa residues. In the phylogenetic calculations, 802 characters of an alignment for 24 different AdV types were studied. The intragenetic distances were significantly lower than the intergeneric ones, and the distances of WSAdV-1 from all four genera were higher than any intergeneric distances (Table 1). On the other hand, the highest intragenetic distance values were always consistently lower than the distance of WSAdV-1 from any member of a given genus. For example, the largest distance between two atadenoviruses (SnAdV-1 and ovine AdV-7) was 0.2985, while the smallest distance of WSAdV-1 (from SnAdV-1) was 0.7763.

The WSAdV-1 protease gene was 612 bp in size, encoding 203 aa residues. From an alignment for the same 24 AdV types, 195 characters were used in phylogenetic analyses. As with the results obtained with the hexon, the intergeneric distances were significantly higher than the intragenetic ones (Table 1), and the distinctness of WSAdV-1 from all four genera was unequivocal. The same conclusion was drawn using partial protein sequences from other genes (e.g. DNA polymerase, pIIIa, III, DBP and 100 K protein),

in that WSAdV-1 was unambiguously separated from the four genera (data not shown).

The topology of the distance-based NJ trees inferred from the hexon and protease sequences also showed the separation of the four adenovirus genera and WSAdV-1, although the genealogy of the two genes differed slightly (data not shown). The ML trees also showed slight differences (Fig. 3). The same relative order of the five lineages had the highest likelihood value with both genes as it was obtained by NJ analysis. Based on the hexon gene, the atadenoviruses seemed to be most closely related to mastadenoviruses (Fig. 3a), whereas on the tree inferred from the protease gene, the common branch of avi- and siadenoviruses was closer to mastadenoviruses (Fig. 3b). The bootstrap values obtained from distance analyses were in accordance with the ML analyses (Fig. 3). In the case of the hexon gene, two further topologies had similar likelihood values to the ML tree ($p > 0.050$), each with an alternative branching pattern within the shaded region in Fig. 3a. In one, WSAdV-1 branched from close to the root of the *Siadenovirus* genus, and in the other it branched from close to the root of the *Siadenovirus-Aviadenovirus* cluster. With the protease gene, there were 10 topologies with similar likelihood values ($p > 0.050$) to the ML tree (Fig. 3b), and the three hexon topologies mentioned above were among them.

4. Discussion

Based on microscopic studies, the presence of adenovirus-like particles has been reported from epidermal hyperplasia of cod (Jensen and Bloch, 1980) and dab (Bloch et al., 1986), as well as from a Japanese red sea bream with lympholeukemia (Miyazaki et al., 2000), but WSAdV-1 is still the only fish adenovirus isolate available (Hedrick et al., 1985). The characterisation of a fish AdV was of special interest because it was expected to contribute to further exploration of adenovirus evolution. The hypothesis that the four recognised adenovirus genera (*Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*) might correspond to viral lineages that have co-evolved with the four major vertebrate classes (mammals, birds, reptilians and amphibians) was recently published (Harrach, 2000, 2001; Benkő and Harrach,

Table 1

Comparison of distances calculated by MEGA2 for the hexon (below) and protease (above) amino acid sequences within (intra) and between each adenovirus genus

	<i>Mastadenovirus</i>	<i>Atadenovirus</i>	<i>Aviadenovirus</i> ^a	<i>Siadenovirus</i> ^a	WSAdV	Intra
<i>Mastadenovirus</i>	–	0.9122	0.8493	0.8313	0.9190	0.4087
<i>Atadenovirus</i>	0.5570	–	0.9634	0.8544	0.9048	0.5123
<i>Aviadenovirus</i> ^a	0.6520	0.6321	–	0.6864	0.9896	0.2826
<i>Siadenovirus</i> ^a	0.6088	0.5832	0.5587	–	0.8304	0.5456
WSAdV	0.8067	0.8004	0.7262	0.7419	–	–
Intra	0.2564	0.2668	0.1577	0.3503	–	–

^a The genus is represented by two species only.

2003). According to this assumption, fish are expected to have adenoviruses that are distinct from all the AdVs examined thus far.

The organisation of the central part of the WSAdV-1 genome presented in this paper deduced from partial sequences of cloned and PCR-amplified viral fragments proved that the number, approximate sizes and locations of genes are similar to those in avi-, at- and siadenoviruses. In this region, the mastadenovirus genome contains an additional gene, that of protein V, which is a core protein playing an important role during AdV infection (Matthews and Russell, 1998; Russell, 2000). The function of this protein is presumably replaced by other uncharacterised proteins or mechanisms in WSAdV-1 and the other genera. Since the sequences of the two ends of the WSAdV-1 genome are as yet unknown, we have focused our analyses on the region already sequenced.

The intergenic region between the hexon and protease genes showed interesting features seemingly preserved in certain genera (Fig. 2). In one siadenovirus (TAdV-3), a single nucleotide (A) separates the terminator TAA triplet from the ATG of the first methionine, while in the other (FrAdV-1), the last A of the hexon terminator TAA overlaps the A of the first codon of the protease (Fig. 2). In WSAdV-1 two nucleotide intergenic nucleotides are present, however the second TAA stop codon in the frame of the hexon overlaps the first nucleotide of the protease resembling the organisation of FrAdV-1. From aviadenoviruses, corresponding sequence data are available for only two genomes, showing that the hexon and protease genes are separated by 16 nt in each. In mastadenoviruses the junction of the two genes varies from overlap (PAdV-5) to relatively long intergenic distances (25 nt in HAdV-12). In the atadenoviruses, the presence of a four-nucleotide overlap of the two genes was reported in the egg drop syndrome virus (Harrach et al., 1997), in BAdV-4 (Dán et al., 1998) and in SnAdV-1 (Farkas et al., 2002). In every atadenovirus studied to date (Élő, 2002; Vráti et al., 1996), the same sequence (ATGA) joins the stop codon of the hexon and the start codon of the protease gene. This unique feature serves as further evidence for the common origin of atadenoviruses. This overlap likely appeared in the common ancestor of atadenoviruses, and as substitutions would change either the terminator or the start codon with deleterious effects, the ATGA sequence remained unchanged. It is noteworthy that all known non-atadenoviral hexon genes terminate with TAA.

Conserved aa residues H55-D72-C122 (forming the active triad) and C104 (McGrath et al., 2003) were identified in the protease of WSAdV-1. Interestingly, P137 (Rancourt et al., 1995) was also present, although it seems to be missing in aviadenoviruses (Chiocca et al., 1996; Ojkic and Nagy, 2000), in the majority of atadenoviruses (Harrach et al., 1997; Barbezange et al., 2000) and in TAdV-3 (Pitcovski et al., 1998). However, P137 is also present in FrAdV-1 (Davison et al., 2000), and in SnAdV-1 (Farkas et al., 2002).

The phylogenetic analyses based on the aa sequences of the hexon or protease genes showed the clear separation of WSAdV-1 from the four adenovirus genera, and confirmed previous results obtained from the pTP gene and a part of the DNA polymerase gene (Benkő et al., 2002; Benkő and Harrach, 2003). Interestingly, the distances obtained based on protease aa sequences were always larger than the corresponding distances of the hexon genes. The lower values characterising the distance matrix of the hexon seemed to be a paradox. The relatively large hexon protein is known to possess family-, genus-, species- and type-specific determinants, which show significant sequence variations (Benkő et al., 2000). On the contrary, the protease is a relatively small protein and contains highly conserved regions. The difference might be due to the different evolutionary rate of the two genes. Handling of the gaps in the alignment should not in principle influence this relation. However, the variable regions aligned with numerous gaps and therefore excluded from the calculations comprised more than 15% of the hexon sequence, whereas from the protease alignment only <4% was removed. This might have affected the difference between the obtained protein distances of the two genes.

Inclusion of the sequence data for WSAdV-1 in the phylogenetic trees has slightly changed the previously clear inference of the genetic relationships among the AdV genera. The analyses of different genes seemed to imply different genealogies. This could be a manifestation of the problem of the gene versus species trees (see e.g. Page and Charleston, 1998 and references therein). However, the ambiguity of the main old branching patterns of the main lineages of AdVs, especially in the case of the protease gene, where more possible topologies had non-significantly lower likelihood value than in the case of the hexon gene, indicate the limits of resolution available from these genes. Detailed analysis of these molecular evolutionary phenomena will be the subject of further studies.

It should be mentioned that the evolutionary relationships among the main fish lineages have not been resolved clearly as yet (Inoune et al., 2003). The white sturgeon is an acipenseriform fish classified into Chondrostei, one of the most ancient lineages of the ray-finned fish (Actinopterygii). Actinopterygii is by far the most diversified group of all vertebrates, and its two main lineages (Chondrostei and Teleostei, the bony fish) separated approximately 215 million years ago (Kumar and Hedges, 1998). Therefore, the analysis of an AdV originating from bony fish, such as cod or dab, would be especially interesting.

The results presented here firmly support the previous proposal, based on the analysis of a fragment of the DNA polymerase (Benkő et al., 2002) and the pTP sequence (Benkő and Harrach, 2003), that WSAdV-1 should be considered as the first representative of a prospective fifth adenovirus genus. The content and organisation of the central part of the adenovirus genome are well conserved in all members of the family, whereas the regions close to

the two ends contain characteristic genus-specific genes (Davison et al., 2003). Therefore, analysis of the genome termini of WSAAdV-1 would be essential for the definitive confirmation of a new AdV genus.

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