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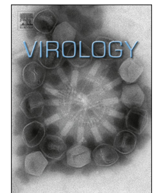

Complete genome sequences of pigeon adenovirus 1 and duck adenovirus 2 extend the number of species within the genus *Aviadenovirus*

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ABSTRACT

Complete genomes of the first isolates of pigeon adenovirus 1 (PiAdV-1) and Muscovy duck adenovirus (duck adenovirus 2, DAdV-2) were sequenced. The PiAdV-1 genome is 45,480 bp long, and has a gene organization most similar to turkey adenovirus 1. Near the left end of the genome, it lacks ORF0, ORF1A, ORF1B and ORF1C, and possesses ORF52, whereas six novel genes were found near the right end. The DAdV-2 genome is 43,734 bp long, and has a gene organization similar to that of goose adenovirus 4 (GoAdV-4). It lacks ORF51, ORF1C and ORF54, and possesses ORF55A and five other novel genes. PiAdV-1 and DAdV-2 genomes contain two and one fiber genes, respectively. Genome organization, G+C content, molecular phylogeny and host type confirm the need to establish two novel species (*Pigeon aviadenovirus A* and *Duck aviadenovirus B*) within the genus *Aviadenovirus*. Phylogenetic data show that DAdV-2 is most closely related to GoAdV-4.

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Introduction

The majority of adenoviruses (AdVs) isolated from birds are classified into the genus *Aviadenovirus* (Harrach et al., 2011). Before the era of DNA sequencing, serology was the principal means of identifying aviadenovirus types; hence, the term serotype was used. Subsequently, DNA sequencing of the hexon loop 1 (L1) gene region was frequently used for genotyping (Kajan et al., 2013; Marek et al., 2010; Meulemans et al., 2004; Raue and Hess, 1998). Now, high-throughput sequencing permits the rapid, relatively inexpensive and comprehensive analysis of complete aviadenovirus genomes.

Fowl adenoviruses (FAdVs) infect chickens and are grouped into five species in the genus *Aviadenovirus*, *Fowl aviadenovirus A* to *Fowl aviadenovirus E* (informally abbreviated to FAdV-A to FAdV-E), based on genome organization and phylogeny, and into 12 serotypes (FAdV-1 to FAdV-8a and FAdV-8b to FAdV-11), based on cross-neutralization tests (Harrach et al., 2011; Hess, 2000). Several additional aviadenoviruses infecting birds other than chickens have also been described (Bodewes et al., 2013; Hess et al., 1998; Luschow et al., 2007; Schrenzel et al., 2005; Smyth and McNulty, 2008; Wellehan et al., 2005). Several species have been accepted within the genus *Aviadenovirus* to accommodate these non-chicken AdVs: *Goose aviadenovirus A* (GoAdV-A; possibly five types, GoAdV-1 to GoAdV-5), *Falcon aviadenovirus A* (FaAdV-A; FaAdV-1) and *Turkey aviadenovirus B* (TAdV-B: TAdV-1) (http://www.ictvonline.org/virusTaxonomy.asp?version=2013; name of species includes the genus designation). *Pigeon aviadenovirus A* (PiAdV- A, PiAdV-1) and *Duck aviadenovirus B* (DAdV-B, DAdV-2) are potential additional species in the genus (Bouquet et al., 1982; Harrach et al., 2011; Hess et al., 1998; Marek et al., 2010).

Complete genome sequences are available for all FAdV species: FAdV-A (FAdV-1, also known as CELO virus), FAdV-B (FAdV-5 strain 340), FAdV-C (FAdV-4 strains ON1 and KR5), FAdV-D (FAdV-9 strain A-2A) and FAdV-E (FAdV-8 strain HG) (Chiocca et al., 1996; Grgic et al., 2011; Griffin and Nagy, 2011; Marek et al., 2012, 2013; Ojkic and Nagy, 2000). In addition, the whole genomes of the non-chicken aviadenoviruses TAdV-1 (TAdV-B) and GoAdV-4 (GoAdV-A) have been sequenced (Kajan et al., 2010, 2012). Recently, the whole genomes of TAdV-4 and TAdV-5 were sequenced, and were shown to belong to the potential species *Turkey aviadenovirus C* (TAdV-C) and *Turkey aviadenovirus D* (TAdV-D) in the genus *Aviadenovirus* (Marek et al., 2014).

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AdVs have been reported to infect pigeons, predominately younger birds. Several FAdV strains belonging to various serotypes have also been isolated from diseased or healthy pigeons (Goryo et al., 1988; Hess et al., 1998; McFerran et al., 1976; Naeem and Akram, 1995), and FAdVs have been reported in pigeons with inclusion bodies in liver cells. In addition, PiAdV-1 has been described, and was distinguished from the 12 FAdV serotypes by using cross-neutralization tests (Hess et al., 1998). DNA sequence studies confirmed the difference between FAdVs and PiAdV-1, and suggested the assignment of a new species (*Pigeon aviadenovirus A* (PiAdV-A)) in the genus *Aviadenovirus* (Marek et al., 2010; Raue et al., 2002). Little is known about the distribution of PiAdV-1 and its possible role in specific disease conditions of pigeons.

In 1977, a major outbreak of disease was seen in a replacement breeder facility for Muscovy ducks in France. Later, the virus was isolated and classified as an AdV based on its chemical and morphological properties. It was shown to share the aviadenovirus group antigen and to be serologically different from the 12 FAdVs, two TAdV serotypes and one avian AdV classified outside the genus *Aviadenovirus* (duck adenovirus 1 (DAdV-1) in the species *Duck atadenovirus A*, genus *Atadenovirus*) (Bouquet et al., 1982). As a result, it was proposed that this virus, which is currently listed as an unassigned virus in the genus *Aviadenovirus* (Harrach et al., 2011), should be assigned to serotype DAdV-2 in the species *Duck aviadenovirus B* (DAdV-B). However, in the absence of sequence data, this proposal has not yet been adopted by the International Committee on Taxonomy of Viruses.

The main purpose of this study was to apply high-throughput sequencing technology to obtain the complete genome sequences of reference strains for PiAdV-1 (strain IDA4) and DAdV-2. With the completion of these sequences, additional insights into the evolution of the genus *Aviadenovirus* were obtained.

Results

Genome organization

After filtering for contaminating chicken sequence reads, average coverage for the PiAdV-1 and DAdV-2 genomes was approximately 7000 and 26,000 reads per nucleotide, respectively. *De novo* assembly using 1–3% of these data was found to be optimal for assembly. Gap closure by PCR and Sanger sequencing resulted in final genome sequences of 45,480 and 43,734 bp for PiAdV-1 and DAdV-2, respectively. Nucleotide composition of the two genomes was 63.8 and 46.1% G+C, respectively. The percentage sequence identities to available aviadenovirus complete genome sequences are given in Table 1. The inverted terminal repeats (ITRs) were 56 and 721 bp long in PiAdV-1 and DAdV-2, respectively. A single mismatch (a C–T transition) exists within this region at position 50 in the PiAdV-1 genome.

PiAdV-1 has a relatively novel genome organization, and DAdV-2 has similar genome organization to that of GoAdV-4. In the region near the left end of the PiAdV-1 and DAdV-2 genomes, homologs were present to most ORFs in galliform aviadenoviruses and GoAdV-4, respectively. However, ORF0, ORF1A, ORF1B and ORF1C were lacking from PiAdV-1 compared to TAdV-1, and a homolog was identified to ORF52, which was described originally in GoAdV-4 and is also present in DAdV-2. However, compared with GoAdV-4 and DAdV-2, the PiAdV-1 ORF52 homolog is located on the opposite strand and may be spliced to the first exon of pTP. ORF51, ORF1C and ORF54 are lacking from the left genome end of DAdV-2 compared to GoAdV-4 (Table 2).

The central part of each genome (from IVa2 to fiber) shows an identical organization to the central region of other AdVs (Table 2), except that mastadenoviruses have a further gene, encoding protein V, in this region. Two and one fiber genes were identified in the PiAdV-1 and DAdV-2 genomes, respectively.

The region of the genomes near the right end showed more variations in predicted gene content. The PiAdV-1 genome contains six novel genes at the far right end (ORF58, a paralogous ORF58A, and ORF59 to ORF62). Two ORF19 homologs were found, and they are fused to each other. DAdV-2 contains a paralogue of ORF55, named ORF55A, and five other predicted novel genes (ORF63–ORF67). The order and orientation of homologs are conserved in this region among various aviadenoviruses. The conserved genes near the right end are ORF22, ORF20A, ORF20 and ORF19 in both genomes. In addition, PiAdV-1 also shares ORF8 with the FAdVs and TAdV-1, TAdV-4 and TAdV-5, while DAdV-2 shares ORF19B and ORF53 with GoAdV-4 (Table 2).

Global pairwise sequence alignment analyses identified areas of great interspecies diversity (Fig. 1). The PiAdV-1 and DAdV-2 genomes display high sequence conservation with other aviadenovirus genomes in the central region (from IVa2 to fiber).

Phylogeny

Phylogenetic analyses of the whole genomes (Fig. 2) or selected proteins (Fig. 3) of various AdVs supported the division of the genus *Aviadenovirus* into the currently recognized species. They also suggest the existence of two new lineages (proposed species) within the genus: *Pigeon aviadenovirus A* (PiAdV-A, for PiAdV-1) and *Duck aviadenovirus B* (DAdV-B, for DAdV-2). DAdV-2 is most closely related to GoAdV-4 (Fig. 2). In a similarity table of partial DNA polymerase sequences (data not shown), PiAdV-1 showed the highest identity to FAdV-1, FAdV-4 and TAdV-1 (74.44%), and DAdV-2 to GoAdV-4 and GoAdV-5 (82.22%).

Discussion

The family *Adenoviridae* is divided into five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus* (Harrach et al., 2011). Aviadenovirus genomes are much larger (at approximately 45 kbp) than those of avian atadenoviruses or siadenoviruses, and those of mammalian, reptilian and amphibian AdVs.

Whole genome sequence identities among members of the various officially accepted aviadenovirus species range from 42.4% (between TAdV-1 (TAdV-B) and GoAdV-4 (GoAdV-A)) to 72.2% (between FAdV-9 (FAdV-D) and FAdV-8 (FAdV-E)) (Marek et al., 2013). Sequence identities among members of the recently proposed species TAdV-C and TAdV-D and members of the recognized aviadenovirus species range from 45.3% (between TAdV-C and GoAdV-4

Table 1
Percentage sequence identities of the complete PiAdV-1 and DAdV-2 genomes to other aviadenovirus genomes.

	PiAdV-A PiAdV-1 IDA4	DAdV-B DAdV-2	FAdV-A FAdV-1 CELO	FAdV-B FAdV-5 340	FAdV-C FAdV-4 ON1	FAdV-C FAdV-4 KR5	FAdV-D FAdV-9 A2-A	FAdV-E FAdV-8 HG	TAdV-B TAdV-1 D90/2	TAdV-C TAdV-4	TAdV-D TAdV-5 1277BT	GoAdV-A GoAdV-4
PiAdV-1	100	41.8	48.6	48.9	48.6	48.6	49.2	49.8	61.8	47.1	47.4	41.9
DAdV-2		100	46.6	44.7	46.1	46.2	46.8	45.6	44.2	46.5	46.1	66.7

Table 2
Orientations, locations and amino acid (aa) sizes of predicted protein-coding regions in the PiAdV-1 and DAdV-2 genomes.

Gene	Strand[a]	PiAdV-1 location	No. of aa	DAdV-2 location	No. of aa
ORF52	[b]	411–1322 17,649–17,654	305	1642–2256	204
ORF1	R	2336–2851	171	1185–1649	154
ORF2	R	3432–4244	270	2517–3272	251
ORF14A	L	4279–4929 17,649–17,654	218		
ORF14	L	4838–5671 17,649–17,654	279	3300–4004 14,711–14,716	236
ORF13	L	5712–6608 17,649–17,654	300		
ORF12	L	6604–7536 17,649–17,654	312	4026–4928 14,711–14,716	302
IVa2	L	7496–8815	439	4894–6081	395
pol	L	8799–12,593	1264	6065–10,090	1341
pTP	L	12,575–14,452 17,649–17,654	627	10,092–11,891 14,711–14,716	601
52K	R	14,618–15,799	393	11,917–13,023	368
pIIIa	R	15,786–17,543	585	13,007–14,698	563
III	R	17,690–19,333	547	14,729–16,309	526
pVII	R	19,347–19,595	82	16,309–16,575	88
pX	R	19,717–20,271	184	16,616–17,155	179
pVI	R	20,347–20,991	214	17,208–17,921	237
hexon	R	21,065–23,929	954	17,956–20,778	940
protease	R	23,977–24,684	235	20,787–21,401	204
DBP	L	24,758–25,849 26,328–26,696	486	21,466–22,788 22,828–23,019	504
100K	R	26,900–29,620	906	23,043–25,874	943
22K	R	29,211–29,819	202	25,558–26,151	197
33K	R	29,211–29,616 29,786–30,084	234	25,558–25,903 26,088–26,335	197
pVIII	R	30,132–30,905	257	26,298–27,083	261
U	L	< 30,929–31,169	80	< 27,100–27,312	71
fiber-1	R	31,168–31,908	246	27,586–29,682	698
fiber-2	R	31,784–34,033	749		
ORF22	L	34,044–34,634	196	29,686–30,351	221
ORF20A	L	34,634–34,975 36,380–36,400	120	30,357–31,175 32,276–32,296	280
ORF20	L	35,359–36,288 36,380–36,400	316	31,154–32,113 32,276–32,296	326
ORF56	L			32,362–32,826 39,720–39,776	173
ORF55	L			32,842–33,246 39,720–39,776	154
ORF55A	L			33,297–33,698 39,720–39,776	152
ORF19	L	36,511–40,801 41,101–41,525	1571	33,721–35,442 39,720–39,776	592
ORF19B	L			35,449–39,078 39,720–39,776	1228
ORF62	L	42,082–42,195	37		
ORF61	L	42,229–42,537	102		
ORF8	R	42,464–43,393	309		
ORF58	R	43,571–43,981	136		
ORF58A	R	44,078–44,452	124		
ORF59	R	44,528–44,929	133		
ORF60	R	45,122–45,445	107		
ORF53	L			40,263–41,012	249
ORF63	R			41,011–41,283	90
ORF64	R			41,649–41,771	40
ORF67	L			41,845–42,021	58
ORF66	L			42,205–42,789	194
ORF65	L			42,756–42,968	70

[a] R – rightward-transcribed strand and L – leftward-transcribed strand.
[b] L in PiAdV-1, R in DAdV-2.

(GoAdV-A)) to 69.5% (between TAdV-D and FAdV- 1 (FAdV-A)) (Marek et al., 2014). Sequence identities between the PiAdV-A and DAdV-B members and members of other aviadenovirus species range from 41.8% (between PiAdV-A and DAdV-B) to 66.7% (between DAdV-B and GoAdV-4 (GoAdV-A)), and are clearly enough to support species separation. Importantly, phylogenetic analysis based on the amino acid sequence of the DNA polymerases of the members of the accepted and proposed aviadenovirus species show phylogenetic differences greater than 5–15% (Fig. 3a), which is presently the chief species demarcation criterion together with at least one further difference (host, genome organization, virus neutralization, pathogenicity etc.) (Harrach et al., 2011).

Nucleotide composition is usually a reliable species demarcation criterion for AdVs. Its value is quite similar in FAdV strains (53.8–57.9% G+C). However, TAdV-B and GoAdV-A have remarkably different G+C contents (66.9% and 44.7%, respectively) (Kajan et al., 2010, 2012). The G+C content of the PiAdV-1 and DAdV-2 genomes (63.8% and 46.1%, respectively) was most similar to that of TAdV-1 (TAdV-B) and GoAdV-4 (GoAdV-A), respectively, and still sufficiently different to support species separation.

In this study, phylogenetic and sequence analyses confirmed the present division of the genus *Aviadenovirus* into the recognized species. In addition, the validity of two putative species (PiAdV-A and DAdV-B) within the genus *Aviadenovirus* is strongly supported, based on phylogenetic, genome organization, nucleotide composition and host differences. DAdV-2 and GoAdV-4, the only fully sequenced aviadenoviruses having anseriform hosts, cluster together. Therefore, it seems probable that this branch of aviadenoviruses coevolved with the anseriform birds. Earlier, only a partial hexon gene sequence was determined by consensus PCR from DAdV-2 and six goose AdV strains (one of them now fully sequenced (Kajan et al., 2012)). The phylogenetic analysis of these short sequences indicated their monophyletic status (Papp et al., 2003).

The fibers of FAdVs are thought to play important roles in infectivity and pathogenicity (Pallister et al., 1996; Schachner et al., 2014). All FAdVs that have been examined have two fibers protruding from each penton base (Gelderblom and Maichle-Lauppe, 1982). FAdV-A and FAdV-C members have two fiber genes, while the sequenced FAdV-B, FAdV-D and FAdV-E members have a single fiber gene (Chiocca et al., 1996; Grgic et al., 2011; Griffin and Nagy, 2011; Marek et al., 2012, 2013; Ojkic and Nagy, 2000). TAdV-1, TAdV-5, GoAdV-4 and PiAdV-1 possess two fiber genes (Kajan et al., 2010, 2012; Marek et al., 2014), whereas TAdV-4 and DAdV-2 have a single fiber gene (Marek et al., 2014). It is not known how many fibers per penton base these AdVs possess.

Each penton base of FAdV-1 (FAdV-A) displays one long (fiber-1) and one short fiber (fiber-2). The short fiber is probably associated with the base in the same way as the single fiber associated with the penton base of mastadenoviruses, protruding out of the base in a straight manner, while the long fiber protrudes at a 90° angle and shows a curved bend (Hess et al., 1995). Twelve consecutive glycine residues located near the N terminus in FAdV-1 fiber-1 presumably facilitate the flexibility that would be required to accommodate the long and short fibers on the same penton base, and may be responsible for the fact that fiber-1 emerges from the viral surface at an angle (Guardado-Calvo et al., 2007). The poly(G) stretch was also identified in the tail region of fiber-1 of FAdV-C, TAdV-B, TAdV-D and PiAdV-A members. Fiber-2 of FAdV-A, FAdV-C, GoAdV-A, TAdV-B, TAdV-D and PiAdV-A members does not contain a poly (G) stretch. Although it is not known precisely how the fibers are attached to the penton base, we speculate that each FAdV-C penton base displays one copy of fiber-1 and one copy of fiber-2, based on the tail sequences and parallels to FAdV-A fiber-1 and fiber-2 (Marek et al., 2012). The same may be true for TAdV-B, TAdV-D and PiAdV-A. Shorter poly(G) stretches in fibers of FAdV-B, FAdV-D and FAdV-E could possibly provide the needed flexibility for accommodating two identical fibers on the same pentameric penton base. However, the single fiber gene of TAdV-C and DAdV-B has no poly(G) stretch within this region, similar to the two fiber genes of GoAdV-4. In addition, some human and Old World monkey AdVs (members of the species *Human mastadenovirus F*, *Human mastadenovirus G*, and putative species *Simian mastadenovirus B* and *Simian mastadenovirus C*)

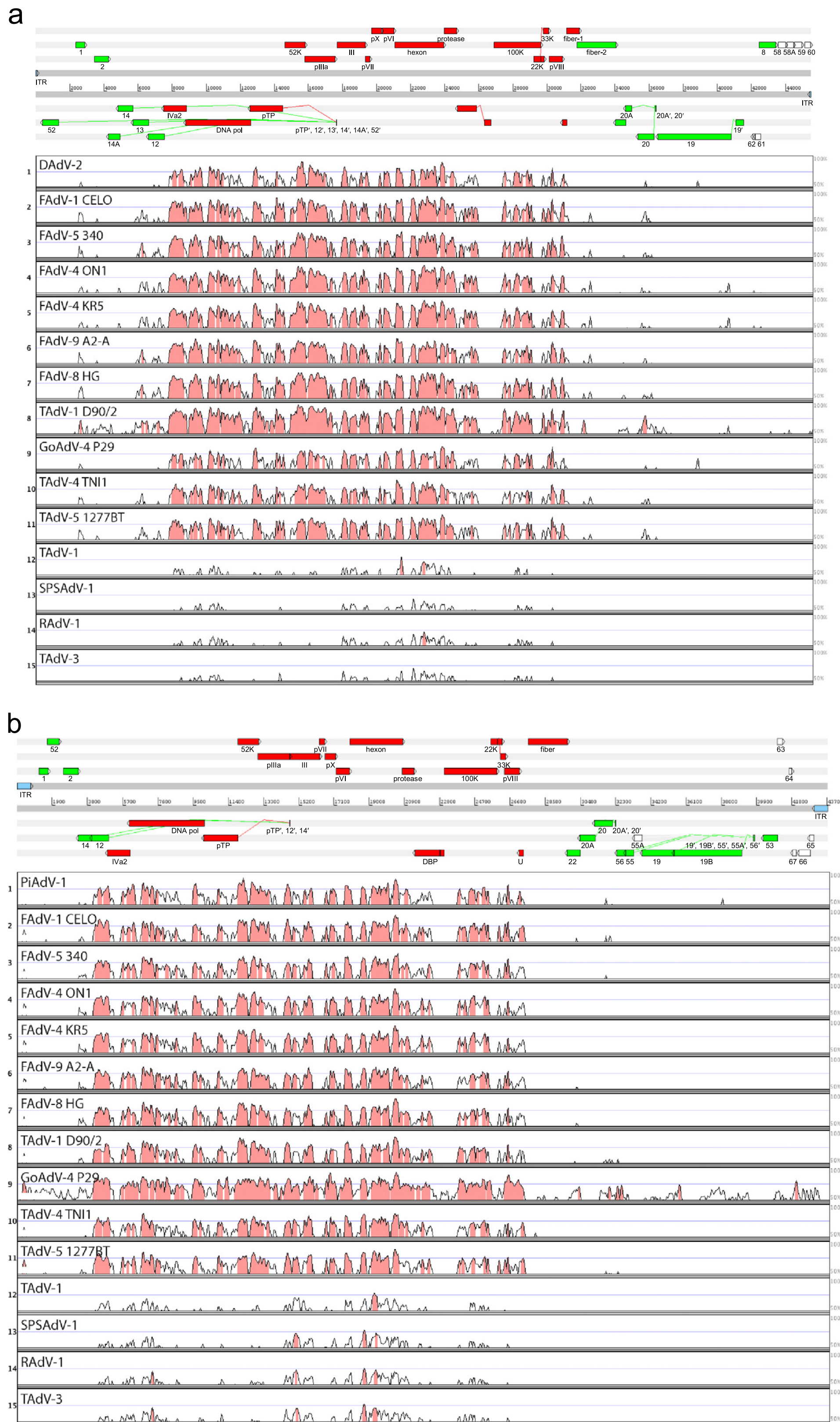

Fig. 1. Global comparisons of the genome sequences of (a) PiAdV-1 (strain IDA4) and (b) DAdV-2 with those of other AdVs. Peaks show regions having >50% sequence identity. At the top, the rightward and leftward strands of the genome are shown in dark gray with nucleotide positions indicated. The three rightward and three leftward reading frames are shown in light gray above and below the genome, respectively. Protein-encoding regions are depicted as colored arrows and bars (the ORF prefix omitted). Those colored red are conserved in every AdV sequenced to date, those colored green have orthologues only in other aviadenoviruses, and those shaded white are unique to PiAdV-1 or DAdV-2. Splicing between protein-encoding regions is indicated by diagonal lines. DBP, DNA-binding protein; DNA pol, DNA polymerase; ITR, inverted terminal repeat (colored blue); pTP, terminal protein precursor.

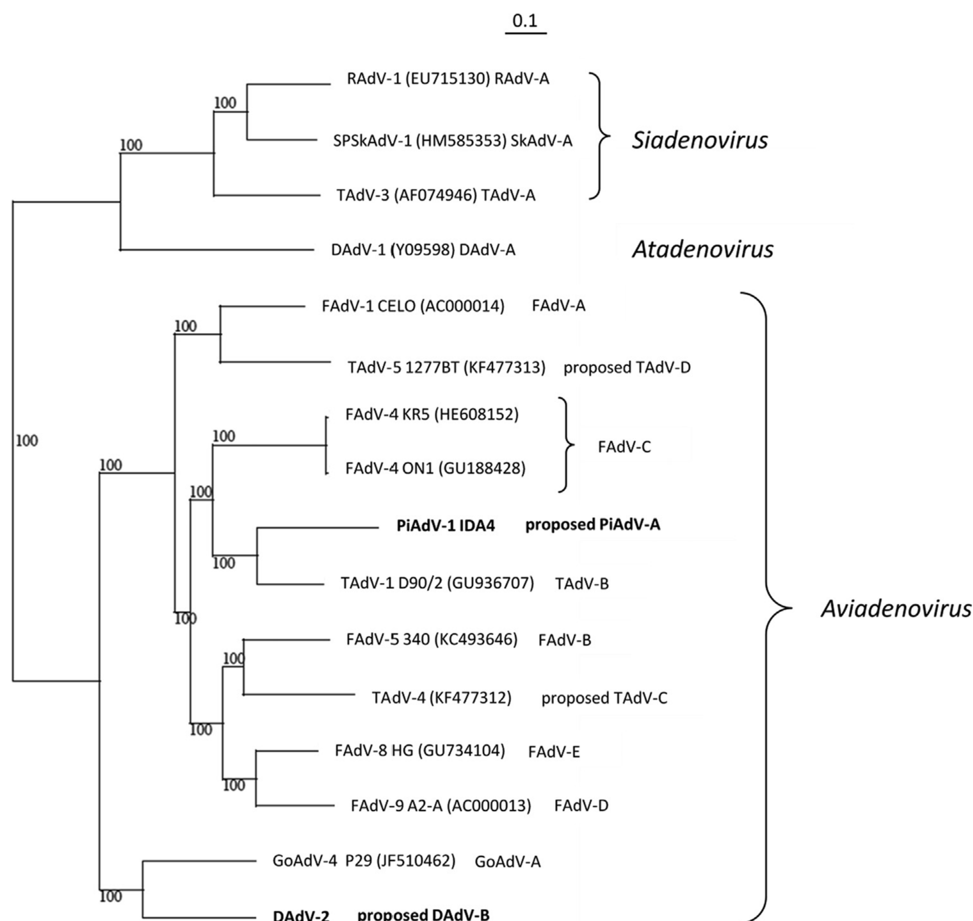

Fig. 2. Phylogenetic tree based on all available whole genome sequences of avian AdVs. PiAdV-1 and DAdV-2 were sequenced in this study and other avian AdV strains were previously sequenced (Chiocca et al., 1996; Grgic et al., 2011; Griffin and Nagy, 2011; Hess et al., 1997; Kajan et al., 2010, 2012; Kovacs and Benko, 2011; Marek et al., 2012, 2013, 2014; Ojkic and Nagy, 2000; Park et al., 2012; Pitcovski et al., 1998). Branch lengths are given in number of substitutions per site (see the scale). Bootstrap values are indicated for the major nodes. GenBank accession numbers of previously published AdV sequences are given. RAdV, raptor adenovirus; SPSAdV, South Polar skua adenovirus.

contain two fiber genes (without a poly(G) stretch), but (as proved for HAdV-40) have only one fiber per penton base (Chiu et al., 2013; Kidd et al., 1993; Kovacs et al., 2005; Roy et al., 2012). Consequently, it would be interesting to see whether these novel turkey and water fowl aviadenoviruses also have only one fiber per penton base.

Genes inherited by all modern AdVs from their last common ancestor are located centrally in the genome and additional, niche-specific genes have accumulated in each lineage, mostly near the genome termini. In general, many genus- and species-specific genes are involved in interactions with the host, presumably to promote survival in relevant biological niches, and a number appear to have been captured most recently, after the genera diverged (Davison et al., 2003). In this study, it was shown again that the genome terminal regions are among the most variable sequences among members of aviadenovirus species. However, it is still not clear which genetic features enable a virus to infect particular avian hosts or cause specific disease.

Viruses coevolving for long time with their host are thought to be well adapted and not markedly pathogenic. Thus, the high pathogenicity of a virus is often viewed as a consequence of a host switch (Benko and Harrach, 2003; Kohl et al., 2012). We hypothesize that host switches have happened relatively often during aviadenovirus evolution. This hypothesis is supported by the clustering of TAdV-D and FAdV-A. Also, PiAdV-A clusters with TAdV-B. Moreover, the primary host of FAdV-C members that are very pathogenic to chickens may perhaps be another avian species. Finally, the fact that the greatest serotype diversity is evident in FAdV-D and FAdV-E may indicate that viruses in these species have been coevolving with chickens for a long period.

Conclusion

The complete genome sequences of PiAdV-1 (strain IDA4) and DAdV-2 were obtained by Illumina sequencing. Phylogenetic and sequence analyses of the whole genomes support the division of the genus *Aviadenovirus* into species, and lead to the proposal of two new species: *Pigeon aviadenovirus* A (PiAdV-A for PiAdV-1) and *Duck aviadenovirus* B (DAdV-B for DAdV-2). Two and one fiber genes were revealed within PiAdV-1 and DAdV-2 genomes, respectively. The data suggest a common evolutionary origin of DAdV-B and GoAdV-A, thus indicating that this branch of the aviadenoviruses coevolved with anseriform birds. Complete genome sequence information of aviadenoviruses is important for taxonomy, diagnostics and further pathogenicity studies.

Materials and methods

Virus isolates

Plaque-purified reference strains of PiAdV-1 (strain IDA4) and DAdV-2 were propagated on confluent monolayers of chicken embryo liver cells as described previously (Marek et al., 2010).

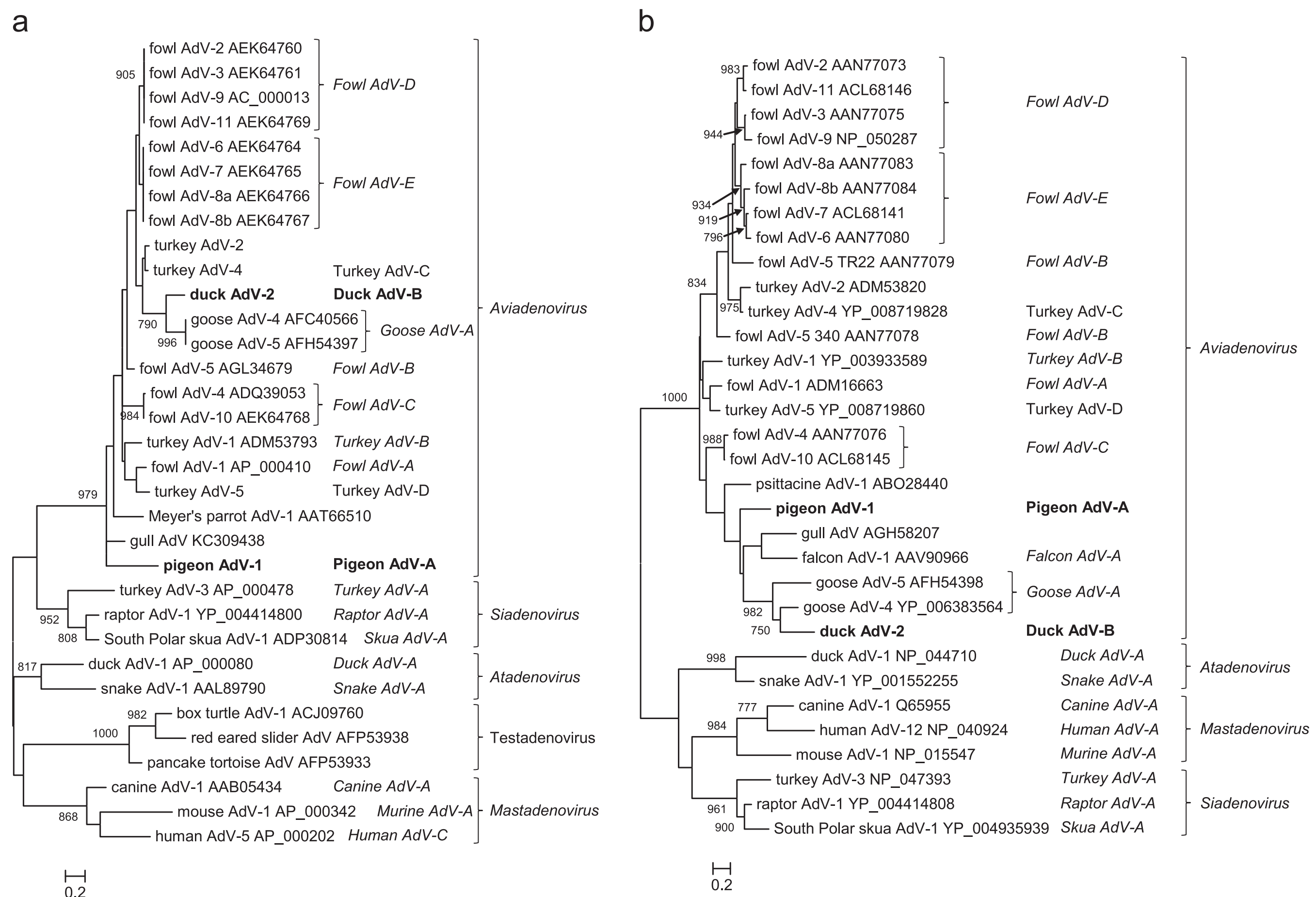

Fig. 3. Phylogenetic trees based on partial DNA-polymerase and hexon sequences originating from AdVs. Branch lengths are given in number of substitutions per site (see the scale). Bootstrap values are given for 1000 datasets if they exceeded 750. GenBank accession numbers are given after the names of virus types. The informal abbreviations for officially recognized (italic type) or proposed species are shown. The trees were rooted on the midpoint of the longest path between two taxa. (a) Tree based on partial DNA polymerase sequences and (b) tree based on partial hexon sequences.

DNA extraction

Cell culture supernatants were clarified by low speed centrifugation (10 min at 2000g). Cell-free virions were then pelletted by ultracentrifugation (3 h at 140,000g), and DNA was isolated (Marek et al., 2012). The presence of virus DNA in the sample was verified by PCR targeting the hexon gene (HexA/HexB or HexF1/HexR1) (Mase et al., 2009; Meulemans et al., 2004).

Genome sequencing

Whole genome sequencing was performed by using an Illumina system (GAIIx, Central Service Facility NGS Unit, Vienna, Austria for PiAdV-1, and HiSeq2000, BGI, Hong Kong for DAdV-2). Paired-end libraries were generated, and multiple virus samples were sequenced in a single lane. Sequence reads corresponding to the individual strains were separated. Since, due to propagation of the strains in chicken cells, contamination by chicken genome reads was anticipated, all reads were mapped initially against the available genome of *Gallus gallus* (v. 3.0) and the mitochondrial genome of *Gallus sonneratii* (AP006746.1). Only the unmapped reads were used for assembly of the virus genomes (Marek et al., 2012).

De novo assembly

The whole genome sequences were assembled by using CLC Genomics Workbench v. 4.0 (CLC bio, Aarhus, Denmark). Since excess coverage can result in a lower quality of the assembly, the read data were sub-sampled (Marek et al., 2013). The resulting contigs were ordered and oriented manually by comparison with sequences available for various complete aviadenovirus genomes and the left and right ends of several additional FAdV genomes (Marek et al., 2012). The contig sequences obtained were aligned by using Accelrys Gene version 2.5 (Accelrys, San Diego, CA).

Partial genome sequencing

Oligonucleotide primers were designed on the basis of the sequences at contig ends, and PCR was performed in order to close the gaps between contigs by Sanger sequencing. Based on obtained sequences from one genome end, primers were also designed for amplifying the sequences at the other genome end. Primer sequences are available from the authors on request. Sequencing services were provided by LGC Genomics, Berlin, Germany.

The complete genome sequences were confirmed by alignment with the read data (Marek et al., 2013). The PiAdV-1 and DAdV-2 genome sequences were submitted to GenBank under accession numbers FN824512 and KJ469653, respectively.

Sequence analyses

Percentage sequence identities of whole aviadenovirus genome sequences were calculated by using Lasergene software (DNASTAR Inc., Madison, WI). ORF identification was carried out by using

JavaScript DNA Translator 1.1 (http://www.annular.org/~sdbrown/dna/translator.html) and Artemis (Rutherford et al., 2000). ORFs specifying polypeptides > 30 amino acid residues in size were identified as potentially protein-encoding, and each was then assessed individually (Marek et al., 2013). Splice acceptor and donor sites were predicted manually by comparison with other AdV genomes (Davison et al., 2003; Kajan et al., 2012). Amino acid sequences of putative proteins were compared to proteins encoded by other aviadenoviruses by using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997), and the presence of homologous ORFs in other AdVs was used to confirm the existence of potential genes. These genes were named according to their homologs, and hypothesized novel ORFs were numbered by extending the series reported previously (Marek et al., 2013). Amino acid sequences were also searched for the presence of putative functional domains by using InterPro (Hunter et al., 2012). Global pairwise alignments were performed by using mVISTA LAGAN (Brudno et al., 2003).

Phylogenetic analyses

Complete genome sequences were aligned by using PRANK (Löytynoja and Goldman, 2010), and phylogenetic analysis was performed by using maximum likelihood (ML) methods within the RAxML software package (Stamatakis, 2006). We used a GTR+Γ model for the inference, and clade support was assessed by using non-parametric bootstrapping with 1000 replicates. The sequenced strains were compared to all published genome sequences of avian AdVs.

Further phylogenetic analyses were based on the partial amino acid sequences of the DNA polymerase (Kajan et al., 2011; Wellehan et al., 2004) and hexon (Meulemans et al., 2004) proteins. Multiple alignments were completed by using MAFFT and edited manually in BioEdit (Hall 1999; Katoh and Toh, 2008). The edited alignment lengths used for polymerase and hexon regions were 93 and 153 amino acid residues, respectively. ProtTest was used to predict the best evolutionary model for tree reconstruction (Darriba et al., 2011). Phylogenetic calculations were performed by using PhyML online (Guindon and Gascuel, 2003; Neron et al., 2009). The evolutionary model used for polymerase region analysis was LG and the gamma distribution for rate heterogeneity among sites (LG+G). The evolutionary model used for hexon region analysis was LG, gamma distribution and the empirical amino acid frequency (LG+G+F). To enhance the accuracy of the calculations, a distance matrix guide tree (obtained by using ProtDist) was applied in the case of the hexon-based tree. Robustness of the trees was determined by bootstrap calculations. A distance matrix-based similarity table was also calculated for the partial DNA polymerase sequences.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.033.

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