Arch Virol (2013) 158:2583–2588
DOI 10.1007/s00705-013-1739-y

BRIEF REPORT

Detection and characterization of a divergent avian reovirus strain from a broiler chicken with central nervous system disease

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Received: 3 April 2013 / Accepted: 17 April 2013 / Published online: 16 June 2013

Abstract Avian orthoreoviruses have been associated with a variety of diseases in chickens, including tenosynovitis, runting-stunting syndrome, hepatitis, myocarditis, osteoporosis, respiratory diseases, and central nervous system disease. The primary objective of our study was the molecular characterization of an avian reovirus strain, T1781, which was isolated from a broiler chicken with a central nervous system disorder in Hungary during 2012. The complete genome sequence was determined using a traditional sequencing method after cell culture adaptation of the strain. Sequence and phylogenetic analyses showed that T1781 shared only moderate nucleic acid sequence identity in several genes to previously analyzed reovirus strains from chickens, and each gene formed separate branches in the corresponding phylogenetic trees. The maximum nucleotide sequence identities of strain T1781 genes to reference avian reovirus strains ranged from 79 % to 90 %. Collectively, our analyses indicated that T1781 is a divergent chicken reovirus strain. The genetic background of this and other avian reoviruses associated with various disease manifestations needs further investigation.

Keywords Genome sequencing · Hungary · Phylogenetic analysis · *Reoviridae*

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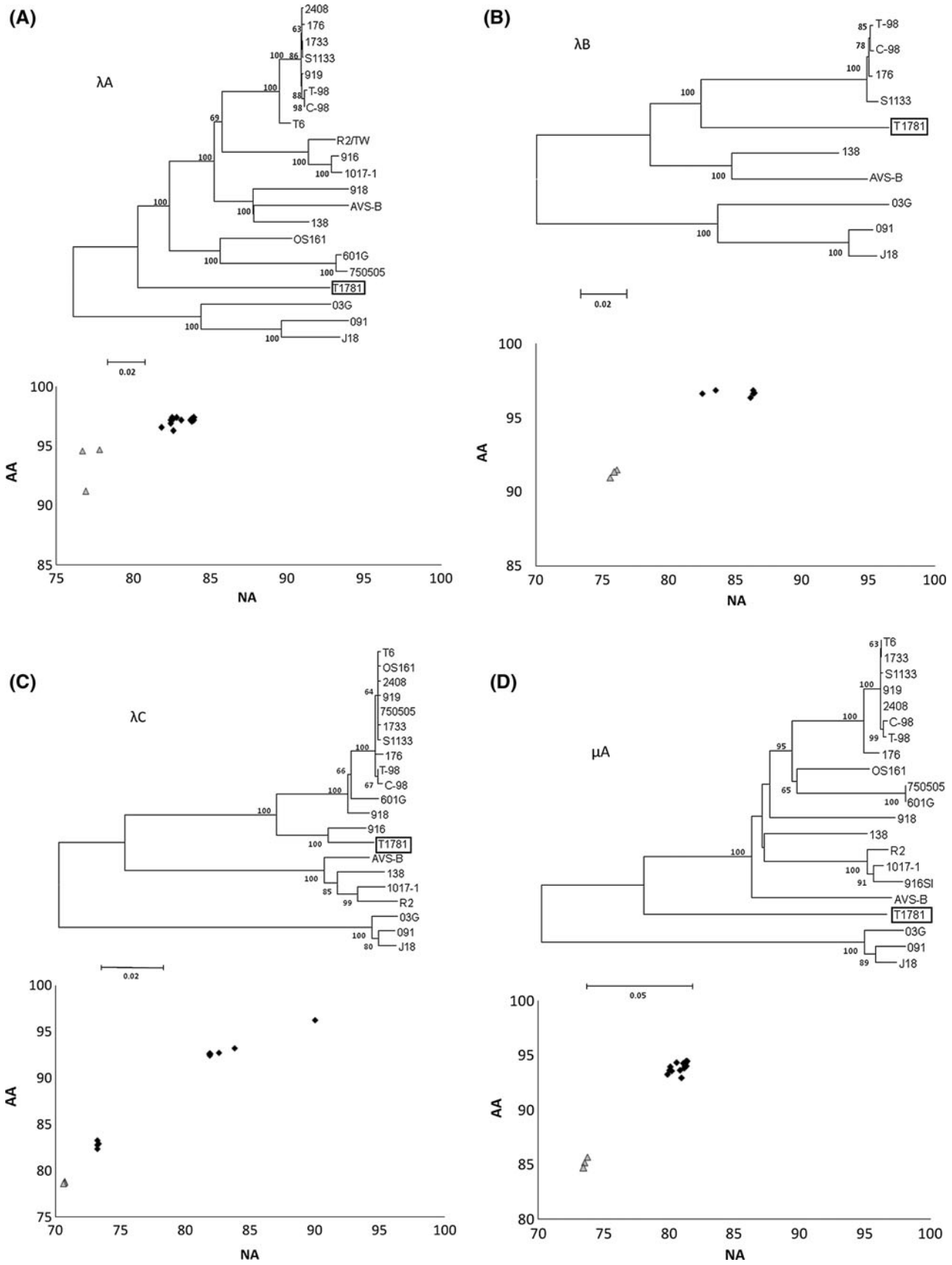
Avian reoviruses (ARVs) belong to the genus *Orthoreovirus* (family *Reoviridae*). The virion of ARVs is a 70-80 nm icosahedral, multilayered, non-enveloped particle [4]. The genome contains 10 linear double-stranded RNA segments, which are composed of three large (L), three medium (M), and four small (S) size-class segments, and encodes 11 or 12 open reading frames (ORFs). Each segment is monocistronic with the exception of the S1 or the S4 segment (depending on the strain), which encodes two or three partially overlapping ORFs [2, 4, 5].

ARV infections in chickens are often subclinical. The outcome of infection depends on several variables, such as the age, breed and immune status of the affected birds, the pathotype of the virus, the presence of co-infecting pathogens, the geographical location, the flock size and density, and other brooding conditions [9]. The most commonly seen disease associated with ARV in chickens is tenosynovitis; however, runting-stunting syndrome, hepatitis, myocarditis, osteoporosis, respiratory diseases and even central nervous system (CNS) infections have been reported to be associated with ARV infection of chickens [3, 10, 11, 13, 17].

During routine laboratory investigation of the carcass of an 18-day-old broiler chicken from a flock affected by CNS disease in eastern Hungary during February 2012, a novel


(A)
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◀ **Fig. 1** Phylogenetic trees plus percentage nucleotide and amino acid sequence identities of the L-class (panel **A** to **C**), M-class (panel **D** to **F**), and S-class (panel **G** to **J**) genes of T1781. Diamonds in the diagrams indicate coordinates for each pairwise comparison with available GenBank reference sequences when percentage nucleotide (NA) sequence identity is plotted against percentage amino acid (AA) sequence identity. Symbols indicate different animal hosts (black diamond, chicken; grey triangle, duck/goose; grey circle, turkey). Strains and GenBank accession numbers used in the analyses are as the follows: **λA-encoding gene:** 919, AY641739; T-98, EU616739; C-98, EU616735; OS161, AY641743; 2408, AY641742; 1733, AY641741; S1133, AY547458; 176, EU707934; 601G, AY641736; T6, DQ238094; AVS-B, NC_015126; 138, EU707933; 916, AY641737; R2/TW, AY641744; 1017-1, AY641740; 750505, DQ238093; 918, AY641738; 03G, JX145328; 091, JX478250; J18, JX478260.2; T1781, KC865786; **λB-encoding gene**: 138, EU707935; 176, EU707936; T-98, JN641889; C-98, JN641888; S1133, DQ534201; AVS-B, NC_015127; 03G, JX145329; 091, JX478251; J18, JX478261; T1781, KC865787; **λC-encoding gene:** 916, AY652701; 918, AY652700; 601G, AY652699; 176, EU707938; T-98, EU616738; 1733, AF384171; T6, AY652698; OS161, AY652696; 750505, AY652695; 2408, AY652694; C-98, EU616737; 919, AY652697; S1133, AY652693; 138, EU707937; 1017-1, DQ238096; AVS-B, NC_015128; R2, DQ238095; 091, JX478252.2; J18, JX478262; 03G, JX145330; T1781, KC865788; **μA-encoding gene:** S1133, AY639610; 176, AY557189; C-98, EU616740; T-98, EU616736; 138, AY557188; 919, AY639618; 2408, AY639613; T6, AY639621; 1733, AY639612; 750505, AY639615; 601G, AY639614; AVS-B, NC_015129; OS161, AY639619; 918, AY639617; R2, AY639620; 1017-1, AY639611; 916SI, AY639616; 091, JX478253.2; J18, JX478263.2; 03G, JX145331; T1781, KC865789; **μB-encoding gene:** AVS-B, NC_015130; 601G, AY635941; 750505, AY635942; 916SI, AY635943; NP03/CHN/2009, JF320801.2; O91, JX478254; 138, AY750052; J18, JX478264; OS161, AY635944; R2, AY635940; C-98, EU616741; 2408, AY635937; T6, AY635936; 03G, JX145332; T-98, EU616742; 919, AY635939; 176, AY750053; S1133, AY635934; 1017-1, AY635935; 1733, AY635938; TH11, JX440514; 918, AY635945; T1781, KC865790; **μNS-encoding gene:** T-98, EU616743; T6, AY573915; S1133, AY573904; R2, AY573914; OS161, AY573913; J18, JX478265; C-98, EU616744; AVS-B, NC_015131; 919, AY573912; 918, AY573911; 916SI, AY573910; 750505, AY573909; 601G, AY573908; 2408, AY573907; 176, AY557191; 1733, AY303993; 138, AY557190; 1017-1, AY573905; 091, JX478255; 03G, JX145333; T1781, KC865791; **σC-encoding gene:** GA41565/2005, DQ872798; ISR5233, FJ793549; 42563-4/2005, DQ872801; GEL13b98M, AF354227; ISR525, FJ793539; NLI02-98M, AF354229; 916, AF297214; GA40973/2005, DQ872797; NC/SEP-R108/03, DQ996604; GEL05-97M, AF354223; 1017-1, AF297216; TX/99, DQ996602; GA41560/2005, DQ872798; ISR522, FJ793540; MS42563-1/2005, DQ872800; GEL12-98M, AF354225; TX/98, DQ996601; 601G, AF297217; R2/TW, AF297213; GEI10-97M, AF354219; GA/12274/2012, JX983599; GA/12297/2012, JX983600; GA/12355/2012, JX983602; ISR5216, FJ793530; ISR5211, FJ793524; T1781, KC865792; **σA-encoding gene:** 138, AF059717; C-98, JN641886; 176, AF059716; AVS-B, NC_015133; S1133, AF104311; GX/2010/1, JN559376; 2408, AF247724; OS161, AF294770; 601SI, AF294769; 750505, AF294767; 919, AF294763; 1733, AF293773; 601G, AF311322; T6, AF294768; 918, AF294766; R2/TW, AF294765; 916, AF294764; 1017-1, AF294762; O91, JX478257; J18, JX478267; 03G, JX145335; T1781, KC865793; **σB-encoding gene:** AVS-B, NC_015134; 138, AF059721; T-98, EF030499; 176, AF059720; G-98, EF030497; C-98, EF030496; 1733, AF004856; S1133, U20642; B-98, EF030498; T6, AF208036; 2408, AF208038; 601SI, AF208037; 750505, AF208035; 919, AF208034; OS161, AF301471; 916, AY008383; 99G, DQ415659; 601G, AY008384; 918, AF301473; R2/TW, AF301472; 1017-1, AF301474; 03G, JX145336; O91, JX478258; J18, JX478268.2; T1781, KC865794; **σNS-encoding gene:** 1017-1, AF294771; R2/TW, AF294778; 916, AF294774; 918, AF294775; TARV-MN4, JQ954693; TK/MN/D-052725/07, GQ353319; 601G, AY008385; AVS-B, NC_015135; chicken/NC/SEP-836/05, EU400286; chicken/NC/SEP-837/05, EU400287; 138, AF059725; USP238-1, JF309123; USP337-1, JF309125; YJL, DQ198858; S1133, U95952; 1733, AY303992; T6, AF213469; OS161, AF294777; 750505, AF213470; 919, AF294776; C-98, JN641885; T-98, JN641884; 601SI, AF294773; 176, AF059724; J18, JX478269; O91, JX478259; 03G, JX145337; T1781, KC865795

ARV strain was identified. This strain was subjected to genetic characterization in this study.

Routine laboratory investigation of chicken carcasses sent from the affected broiler flock included testing for West Nile virus, Newcastle disease virus, avian encephalomyelitis virus (AEV), and avian influenza virus by virus-specific reverse transcription PCR, as well as virus isolation in eggs (yolk sac and allantoic cavity, respectively) and cell cultures. Some of the extracts prepared from the original clinical samples tested positive for AEV by RT-PCR [16]; however, they were negative for AEV by egg isolation. Based on the RT-PCR results, an etiologic role of AEV in the CNS disease was suspected.

However, simultaneously, ARV was isolated at the Budapest office of the Veterinary Diagnostic Directorate from a homogenate of intestine and pancreas by inoculation into the yolk sac of embryonated SPF eggs and subsequent transfer onto primary chicken embryonic kidney cells and the cultured chicken hepatoma cell line LMH. By day 3 postinfection of the second passage in both cell lines, virus-induced syncytia (data not shown), a feature that is characteristic of chicken reovirus infections in cultured cell lines, were observed [7]. The suspected reovirus infection was confirmed by an ARV-specific RT-PCR assay [15]. In addition, the Debrecen office of the Veterinary Diagnostic Directorate isolated an ARV strain from primary chicken embryonic liver cells inoculated with brain and intestinal homogenates of chickens with the same CNS disease.

Given that literature data indicate that ARV may be associated with CNS disease [17], we investigated the possible role of ARV T1781 in this disease manifestation using a supernatant of ARV T1781–infected cells to infect chickens. The supernatant had been previously tested for AEV by RT-PCR and shown to be negative. After intracerebral infection of one-day-old SPF broilers with this cell culture supernatant, 2 out of 6 animals died at days 6 and 7 postinfection, respectively. The brains of both animals tested positive for ARV but tested negative for other viruses including AEV. Histopathological examination of the brain showed lymphocytic encephalitis with perivascular infiltration in the cerebellum, and these lesions were not consistent with those induced by AEV. Of interest, animals infected orally remained healthy, while those

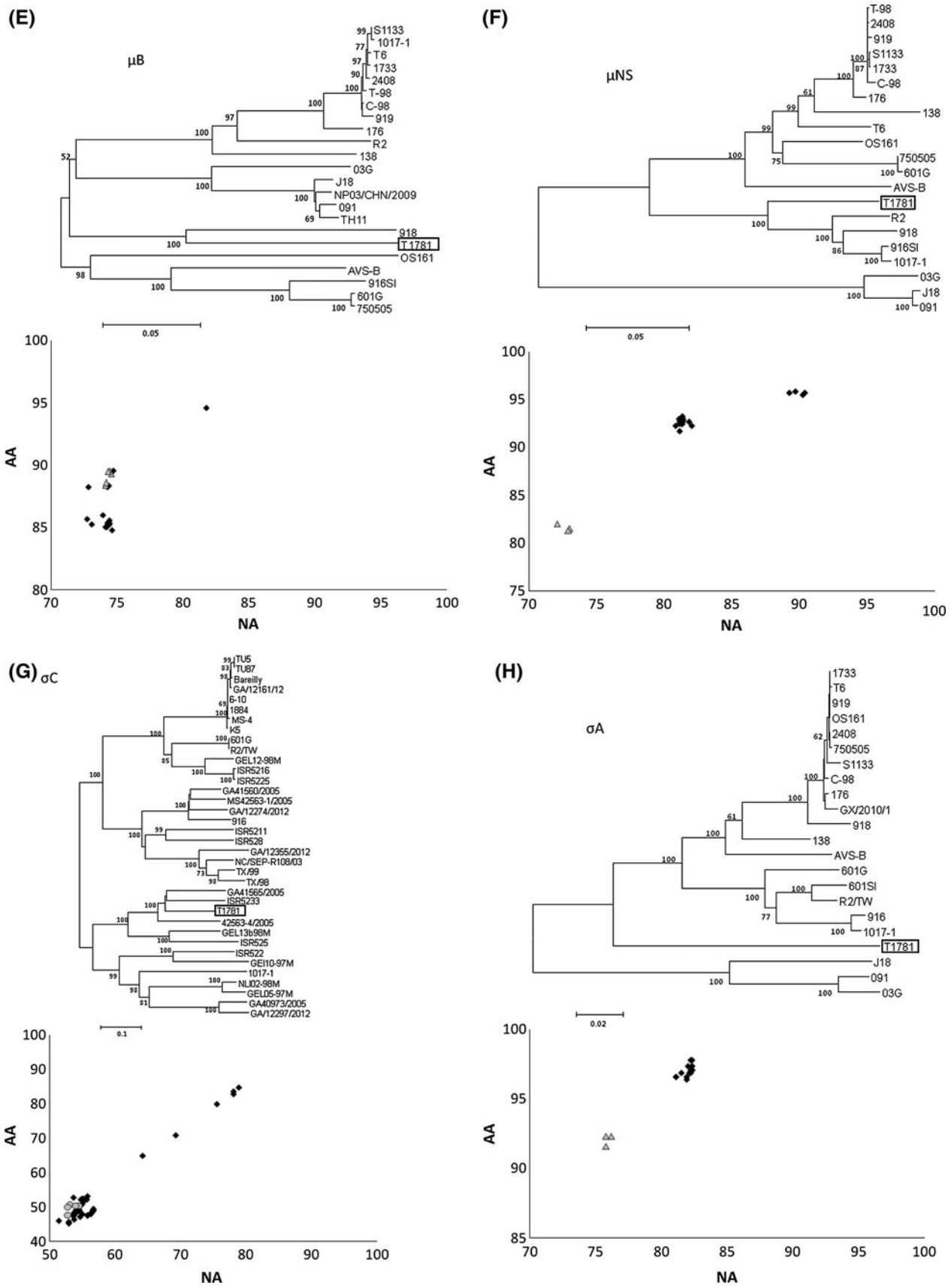

Fig. 1 continued

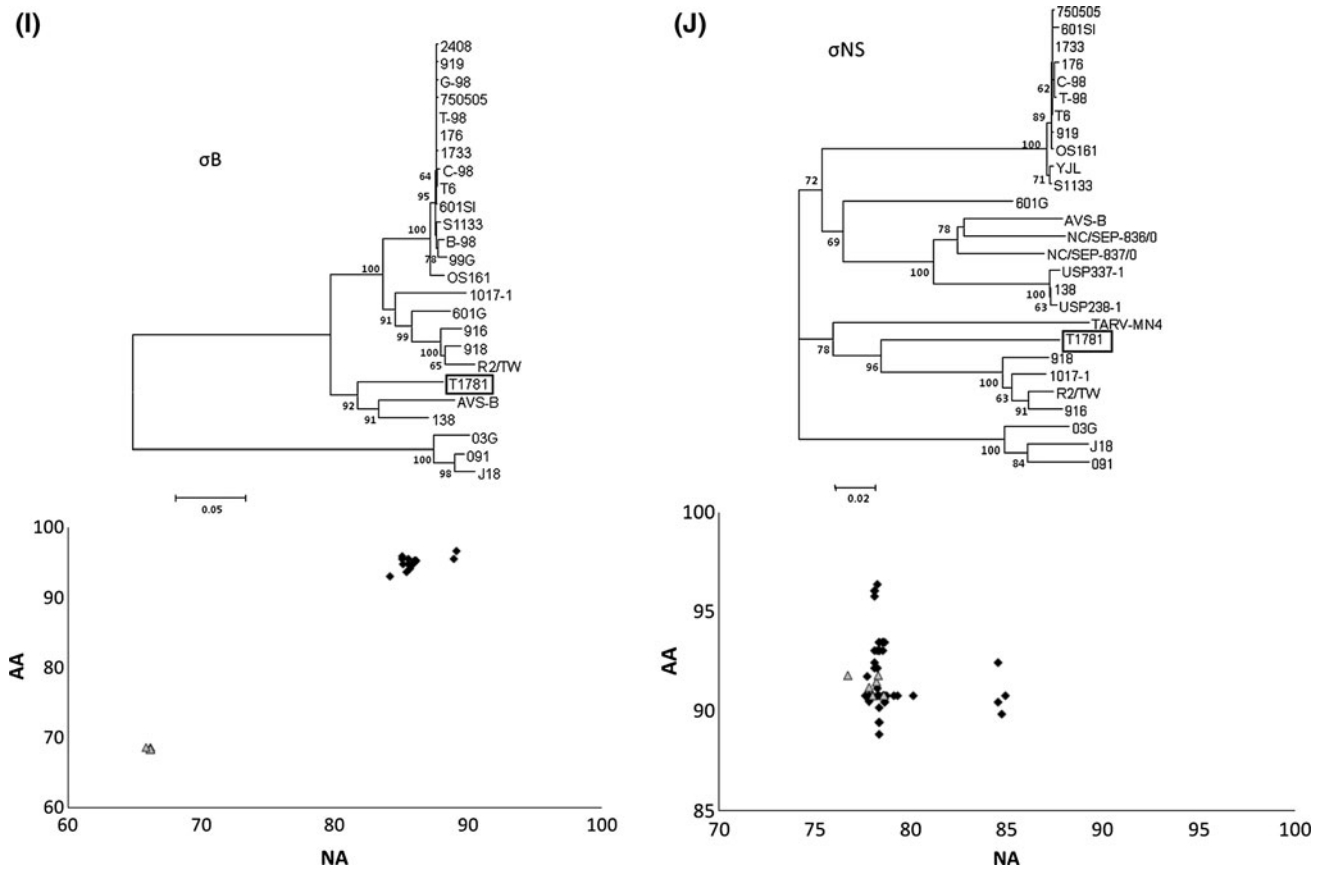

Fig. 1 continued

infected through the footpad developed tenosynovitis-like manifestations beginning on day 3.

Based on these findings, we speculate that ARV contributed to the CNS disease observed in the affected broiler flock, although infection experiments indicated that it might have had only a minor role in the clinical manifestations. Given that sequence information about CNS-associated ARV has not been published, we felt that determining the genetic composition of the isolated ARV would be useful to better understand possible virus sequences associated with this disease condition. For this study, the ARV strain T1781 isolated in Budapest was available for genetic analysis. Laboratory methods were described earlier [3]. Several freely available bioinformatics software tools were used to clean, edit and analyze sequence data (i.e., BLAST, BioEdit, GeneDoc, Multalin, MEGA5 [1, 6, 8, 12, 14]).

A total of 17, 6, and 17 full-length λA-, λB-, and λC-encoding genes of chicken ARV strains were available for comparison, respectively. The maximum nt and aa sequence identities of T1781 to cognate genes of reference chicken strains were 83.9 % and 97.4 % for λA (Fig. 1A), 86.4 % and 96.9 % for λB (Fig. 1B), and 90 % and 96.3 % for λC (Fig. 1C). Nucleotide-sequence-based phylogenies of these genes revealed that T1781 formed separate branches in trees of the λA and λB genes and formed a common cluster with strain 916 in the λC gene tree (Fig. 1A-C).

Similar to L-class genes, only a limited number of full-length gene sequences of chicken ARV strains could be used in sequence analysis and phylogenetic inference. The greatest nt and aa sequence identities of T1781 to cognate genes of reference chicken strains, respectively, were 81.3 % and 94.5 % for μA (Fig. 1D), 81.8 % and 94.6 % for μB (Fig. 1E), and 90.4 % and 95.7 % for μNS (Fig. 1F). Of interest, sequence similarity values for the μB-encoding gene was comparable for chicken ARVs (range, nt: 72.7 %-81.3 %; aa: 84.8 %-94.5 %; Fig. 1E) and goose/duck origin ARVs (range, nt: 74.3 %-74.7 %; aa: 88.4 %-89.6 %; Fig. 1E). The μA gene of T1781 represented a distinct genetic lineage. In the μB gene phylogeny, T1781 clustered with ARV strain 918 even though these two strains shared only moderate sequence similarity. Phylogenetic analysis of the μNS-encoding gene revealed that T1781 forms a common lineage with 918 and other closely related reference strains (Fig. 1D-F).

Concerning the S-class genes, sequencing results showed that the S1 genome segment of T1781 was tricistronic with partially overlapping open reading frames

(p10, nt 23–323; p17, nt 295–735; σC, nt 632-1612; the S1 genomic segment of AVS-B served as a reference sequence to designate nt positions [3]). The σC coding region shared low similarity with the majority of strains (nt, <79; aa, ≤85 %; Fig. 1G), and formed a common cluster with Israeli and US strains even though it shared only moderate sequence similarities with these strains. T1781 represented novel lineages in the σA and σNS gene trees and clustered with reference strains AVS-B and 138 in the σB gene tree (Fig. 1H-J).

In summary, the results from sequencing and phylogenetic analyses demonstrated that T1781 differs considerably at the nucleotide sequence level from other chicken ARV strains with available sequence data in GenBank. In contrast, similarities in the deduced amino acid sequences, which ranged from moderate to high, indicated that most changes in the genomic sequence are under structural or functional constraints acting against extensive protein diversification. Unfortunately, sequence data of ARVs in GenBank are restricted mainly to the S-class genes, and currently, the number of complete-chicken origin ARV genomes is very low. Therefore it was not possible to compare the complete genomic configuration of our strain to all those strains where greater genetic similarities were seen in selected gene phylogenies.

To the best of our knowledge, this is the first study to describe the genetic structure of an ARV strain where a potential association with CNS disease has been implicated. Future routine laboratory investigations should consider the inclusion of ARV in the laboratory diagnosis of CNS disease in broilers and provide detailed strain characterization to help uncover potential new disease associations and identify any putative markers responsible for neurotropism.

Acknowledgments We are grateful to Jon R. Gentsch for reading the manuscript and giving valuable suggestions. Financial support was given by the Hungarian Academy of Sciences (Momentum program).

Conflict of interest The authors declared that they had no conflicts of interest with respect to their authorship or the publication of this article.

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