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Genomic characterization of avian and neoavian orthoreoviruses detected in pheasants

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ABSTRACT

Avian reoviruses are well-known pathogens seriously affecting the productivity of poultry industry. Game birds represent a small segment of the agricultural sector and much remained to be learnt about factors affecting productivity. Here we show that reovirus infections might occur in pheasants and demonstrate that reoviruses of pheasants are of diverse origin. The complete or coding-complete genomic sequences of two Hungarian reovirus strains, D1996/2/1 and Reo/HUN/Pheasant/216/2015, have been determined in this study. The strain D1996/2/1 was isolated in 2012 from birds with gizzard erosion, whereas the other strain was isolated in 2015 from diarrheic pheasant poults. Phylogenetic analyses showed that none of the Hungarian isolates shared common origin with a pheasant reovirus detected recently in the United States. Additionally, we found that Reo/HUN/Pheasant/216/2015 is a multi-reassortant reovirus within the species *Avian orthoreovirus* that shared genetic relationship with turkey reoviruses (σC), partridge reoviruses (λA, σB), and chicken reoviruses (λB, λC, μA, σA, and σNS), in the respective gene phylogenies, whereas two genes (μB and μNS) did not reveal any possible common ancestors. The other isolate, D1996/2/1, was found to be distantly related to previously described reoviruses raising the possibility that it might represent a novel orthoreovirus species or a new genogroup within the newly accepted species, *Neoavian orthoreovirus*. The genetic diversity among pheasant reoviruses could raise challenges for virus classification as well as for development of molecular diagnostic tools and vaccine based prevention and control measures.

1. Introduction

Members of the genus *Orthoreovirus* are non-enveloped viruses with ten double-stranded RNA genomic segments encased in a double-layered icosahedral capsid shell. Orthoreoviruses are currently assigned into ten official species. These are *Mammalian orthoreovirus*, *Avian orthoreovirus*, *Nelson Bay orthoreovirus*, *Reptilian orthoreovirus*, *Baboon orthoreovirus*, *Piscine orthoreovirus*, *Mahlapitsi orthoreovirus* (Attoui et al., 2011; Markussen et al., 2013; Jansen van Vuren et al., 2016); in addition, the latest ICTV updates added three species which are as follows: *Broome orthoreovirus*, *Testudine orthoreovirus*, and *Neoavian orthoreovirus*. All currently known reovirus strains from chickens, turkeys, pheasants, partridges and waterfowls are considered as members of the species *Avian orthoreovirus*. Other, only distantly related avian orthoreovirus-like strains, including the Tvärminne avian orthoreovirus, the Bulbul orthoreovirus, the Steller sea lion reovirus, and the Psittacine reovirus strain Ge01 were originally proposed to be the first members of novel orthoreovirus species, respectively (Dandár et al., 2014; Ogasawara et al., 2015; Palacios et al., 2011). However, the newest ICTV update classified these strains into the species *Neoavian orthoreovirus* (https://talk.ictvonline.org/taxonomy).

Game birds have traditionally been bred and reared for centuries in Hungary for hunting purposes. Game birds are usually farmed semi-intensively or extensively before being released to the wild. Even though enteric diseases, often associated with intensive farming conditions, can lead to considerable losses for game bird breeders, there is still limited information available about the viral agents playing a role in the development of enteric disease syndromes in these species. Reoviruses have rarely been reported from pheasants. An example is a report from Turkey showing an association between reovirus infection and watery diarrhea (Mutlu et al., 1998).

In our previous study a group A rotavirus strain, RVA/pheasant-wt/HUN/216/2015/G23P[37], was identified in pooled stool samples from young, 7-week-old pheasant poults (*Phasianus colchicus*) with ruffled feathers, poor appetite, increased water consumption, diarrhoea, and

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slightly increased mortality in the flock (Gál et al., 2016). From the pooled stool samples virus isolation was attempted on LMH, Vero, and Marc-145 cell lines and an orthoreovirus strain, Reo/HUN/Pheasant/216/2015 could be isolated. Another pheasant origin reovirus strain, D1996/2/1, was detected in 2012 at a Hungarian pheasant breeding farm. In the ~9500 bird flock, increased mortality was seen (9.8% of young animals at ages 0–26 days died; half of the fatal cases were poults 0–7 days of age). Live poults (n = 2) and carcasses (n = 31) were sent for laboratory investigation. Gross and histopathological examination uncovered gizzard erosion and internal bleeding. Virological examination (that included adenovirus PCR) and bacteriological culturing gave negative results; however, a syncytium forming agent was isolated upon inoculation of the supernatant of suspension prepared from the bursa Fabricii and the gizzard on LMH cells (Palya, unpublished data).

The genomic organization of pheasant origin reoviruses is not well understood. The whole genome of a single strain, Reo/PA/Pheasant/13649/14, has been determined recently (Tang and Lu, 2016). That strain showed low sequence similarity to common chicken origin reovirus strains, such as S1133 or 176. Little is known about the conservation of genomic structure and phylogenetic relationship among pheasant reovirus strains. Therefore, it seemed to be of interest whether pheasant reoviruses from different geographic areas share common genomic features and evolutionary history, or, these viruses are genetically more heterogeneous. In this study, we aimed at investigating this issue by analyzing the complete genomic sequences of the recently identified Hungarian pheasant strains, Reo/HUN/Pheasant/216/2015 and D1996/2/1.

2. Materials and methods

2.1. Genome sequencing

Genomic characterization started with random primed amplification of the genomic RNA derived from the cell-culture-isolated strains using TRI Reagent combined with Direct-Zol RNA MiniPrep Kit (Zymo Research). Complementary DNA was prepared in the presence of 10 μM random hexamer tailed by a PCR primer sequence tag. Reverse transcription was performed with the AMV reverse transcriptase (Promega). PCR amplification was carried out using Taq DNA polymerase (Thermo Scientific). Sequencing was carried out on an Ion Torrent semiconductor sequencing equipment (Ion Torrent Personal Genome Machine; Life Technologies) according to the manufacturer's recommendations. Details are found elsewhere (Dóró et al., 2016; Homonnay et al., 2014). In brief, 100 ng of random PCR product was subjected to enzymatic fragmentation using the reagents supplied in the NEBNext Fast DNA Fragmentation & Library Prep Set for Ion Torrent™ kit (New England Biolabs). The adaptor ligation was performed using reagents from the same kit, whereas barcoded adaptors were retrieved from the Ion Xpress™ Barcode Adapters (Life Technologies). The library DNA was run on 2% precast gels, and then products between 300 and 350 bp were used in emulsion PCR using the Ion PGM Template kit on an One-Touch v2 instrument. Following enrichment of the templated beads, the sequencing protocol recommended for Ion PGM™ Sequencing 200 Kit v2 on a 316 v2 chip was strictly followed.

To obtain the terminal sequences of the genomic dsRNA segments of D1996/2/1, DNA oligonucleotides were ligated to the 3' ends (Lambden et al., 1992). Next, oligonucleotide primers were designed to amplify and sequence these parts of the genome by Sanger sequencing method (data not shown).

2.2. Bioinformatics analysis

Sequences were assembled and aligned using the CLC Genomic Workbench v7.0 software (https://www.qiagenbioinformatics.com/), using a combination of *de novo* assembly and reference mapping. After visual inspection of sequence mappings, a single consensus sequence was created for each gene of both strains. Sequence similarity search was performed through the BLAST server (https://blast.ncbi.nlm.nih.gov/). Codon-based multiple sequence alignments were generated using the Muscle algorithm within the http://translatorx.co.uk translation alignment option of Geneious Prime software (Kearse et al., 2012). Phylogenetic analysis was performed using the MEGA6 package (Tamura et al., 2013). Gene-specific substitution models were evaluated, and the best-fit models were selected based on the Bayesian information criterion (GTR + G λC, μA, μNS, σA, σB, σNS; GTR + G + I λA, λB, μB; K2 + G + I σC). Maximum-likelihood trees were generated, and tree topologies were validated by bootstrap analysis (100).

2.3. GenBank accession numbers

The complete and near-complete genomic sequences of the pheasant reovirus strains Reo/HUN/Pheasant/216/2015 and D1996/2/1 have been deposited to GenBank database under accession numbers MT423657 to MT423666 and MT423647 to MT423656, respectively.

3. Results and discussion

The consensus sequences of the two strains were assembled from short Ion Torrent sequence reads based on average sequencing depths ranging from 80X to 221X (pending on strain and genome segment). Genomic organization of the studied strains was similar to and corresponded with that of other avian orthoreoviruses of gallinaceous birds (Table 1.). With the exception of S1, which was tricistronic in both strains, all segments were found to encode a single open reading frame (ORF). Both genomes were predicted to contain 12 ORFs: λA, λB, λC, μA, μB, μNS, σA, σB, σC, σNS, p10 and p17 (Table 1). Where available, the 5' and 3' terminal sequences were conserved among the genomic segments.

Nucleotide (nt) and amino acid (aa) sequences of the coding regions of Reo/HUN/Pheasant/216/2015 showed the highest sequence identity values with reference chicken, turkey and partridge reovirus strains and were much lower when compared with waterfowl reoviruses (Table 2). Two Hungarian strains, a chicken and a partridge origin strain, T1781 (58.9–94% nt; 56.4–96.9% aa) and D1007 (66.5–95.5% nt; 72.5–98.2% aa), respectively, proved to be the closest relatives of Reo/HUN/Pheasant/216/2015. Similarity values fell in a wide range when the σB and σC genes and proteins were compared. In case of σB (61.3–95.5% nt; 62.2–95.9% aa) the calculations demonstrated the highest values with D1007. The BLASTn and pairwise distance analyses of the σC revealed the highest scores with turkey reovirus strains (e.g., NC/98, 94%), and indicated more distant relationship with other reovirus strains (range, 40.6–84.9% nt; 29.4–84.2% aa). Relatively low identity values were also observed when analyzing the μB gene (range, 63.0–66.8% nt; 69.2–73.4% aa), although calculations performed with sequences available in the GenBank database showed high nt (95%) and aa (97.7%) similarity values with the recently described Hungarian chicken reovirus strains, 16821-M-06, 924-Bi-05, and 3457-M-11 (data not shown), whose μB gene could not be linked to any currently known taxa (Farkas et al., 2016).

Phylogenetic analysis of the L, M and S class genes supported these results (Fig. 1). With the exception of the μB gene, Reo/HUN/Pheasant/216/2015 clustered together with reovirus strains originating from gallinaceous birds. In case of the λB, λC, and σC genes very clear clustering pattern was observed with chicken (λB, 90.3%; λC, 92.1%) or turkey (σC, 93.2%) origin reoviruses, respectively. In other phylogenies close genetic relationship could be observed with the Hungarian chicken and partridge reovirus strains, T1781 (μA, 94%; σA, 87.7%; σNS, 87.4%) and D1007 (λA, 87.9%; σB, 95.5%), respectively. Though in the μNS phylogeny Reo/HUN/Pheasant/216/2015 clustered together with reovirus strains of gallinaceous birds, it appeared as a separate branch in the group (maximum nt similarity, 75.3%). On the μB-gene-based phylogenetic tree Reo/HUN/Pheasant/216/2015 did not cluster

Table 1
General features of the pheasant orthoreovirus strain D1996/2/1 and Reo/HUN/Pheasant/216/2015.

Strain	Genome segment	Size (bp)	Length of the 5' end ORF 3' end	Sequence at the termini 5' end/3' end	Encoded protein	Protein size (aa)	Strain in GenBank (accession number): greatest nt sequence identity
D1996/2/1	L1	3924	24 - 3822 - 78	GCUUUU/UUCAUC	λA (Core shell)	1273	Chicken OS161 (AY641743): 73%
	L2	3903	12 - 3855 - 36	GCUUUU/UUCAUC	λC (Core turret)	1284	Steller sea lion (HM222979): 65%
	L3	3828	13 - 3780 - 35	GCUUUU/UUCAUC	λB (Core RdRp)	1259	Brown-eared bulbul (AB914761): 72%
	M1	2287	12 - 2214 - 61	GCUUUA/UUCAUC	μA (Core NTPase)	737	Brown-eared bulbul (AB914763): 68%
	M2	2156	29 - 2028 - 99	GCUUUU/UUCAUC	μB (Outer shell)	675	Brown-eared bulbul (AB914764): 74%
	M3	2036	29 - 1926 - 81	GCUUUU/UUCAUC	μNS (NS factory)	641	Brown-eared bulbul (AB914765): 67%
	S1	1651	32 - 291 - 33	GCUUUU/UUCAUC	p10 (NS FAST)	96	Passer montanus
			414		p17 (NS other)	137	ARV/Sparrow/Kagawa/22/2006 (LC035389)
			984		σC (Outer fiber)	327	74%
	S2	1325	14 - 1251 - 60	GCUUUU/UUCAUC	σA (Core clamp)	416	TVAV (KF692096): 72%
	S3	1204	24 - 1110 - 70	GCUUUU/UUCAUC	σNS (NS RNAb)	369	Brown-eared bulbul (AB914769): 68%
	S4	1198	29 - 1098 - 71	GCUUUU/UUCAUC	σB (Outer clamp)	365	Duck Ych (MK173037): 74%
Reo/HUN/Pheasant/216/2015	L1	3958	20 - 3882 - 56	GCUUUU/UUCAUC	λA (Core shell)	1293	Partridge D1007 (KR476798): 88%
	L2	3876 + n	12 - 3858 - 6 + n	GCUUUU/-	λC (Core turret)	1285	Chicken 3211-V-02 (KX398274): 94%
	L3	3829	13 - 3780 - 36	GCUUUU/CUCAUC	λB (Core RdRp)	1259	Chicken MS01 (KY860641): 91%
	M1	2283	12 - 2199 - 72	GCUUUA/CUCAUC	μA (Core NTPase)	732	Chicken T1781 (KC865789): 94%
	M2	2143 + n	17 - 2031 - 95	-/UUCAUC	μB (Outer shell)	676	Chicken 924-Bi-05 (KX398266): 96%
	M3	1996	24 - 1908 - 64	GCUUUU/UUCAUC	μNS (NS factory)	635	Chicken AVS-B (FR694196): 77%
	S1	1651 + n	24 - 300 - 30 + n	GCUUUU/-	p10 (NS FAST)	99	Turkey NC/98 (DQ995806): 94%
			453		p17 (NS other)	150	
			981		σC (Outer fiber)	326	
	S2	1324	15 - 1251 - 58	GCUUUU/UUCAUC	σA (Core clamp)	416	w

Strain	Genome segment	Size (bp)	Length of the 5' end ORF 3' end	Sequence at the termini 5' end/3' end	Encoded protein	Protein size (aa)	Strain in GenBank (accession number): greatest nt sequence identity
	S3	1202	30 - 1104 - 68	GCUUUU/UUCAUC	σB (Outer clamp)	367	Partridge D1007 (KR476806): 96%
	S4	1192	23 - 1104 - 65	GCUUUU/UUCAUC	σNS (NS RNAb)	367	Chicken 3457-M-11 (KX398291): 94%

with any reference avian or neoavian orthoreoviruses used in our calculations (maximum nt similarity, 66.8%). In the μB phylogeny performed with additional sequences available in GenBank, including the representative members of old and new orthoreovirus species, Reo/HUN/Pheasant/216/2015 formed a common group with the Hungarian chicken strains: 16821-M-06, 924-Bi-05, 3457-M-11 (data not shown). Although the first 8 nt of Reo/HUN/Pheasant/216/2015 was not determined, 17 nt of the 5' UTR showed 100% identity with these strains.

Comparison of the cognate genomic segments with other pheasant origin strains showed that Reo/HUN/Pheasant/216/2015 shared relatively low genetic identity (nt, 59.4–82.5%; aa, 55.8–96.9%) with Reo/PA/Pheasant/13649/14 collected and isolated in the USA, and was genetically distant from the other characterized strain, D1996/2/1 (nt, 47.6–69.7%; aa, 39–80%). Sequence analysis demonstrated that Reo/HUN/Pheasant/216/2015 might have acquired its current genomic composition through several homologous and, in case of the μB, a heterologous reassortment event. The origin of the μB gene could not be determined; so far, this sequence variant of μB has only been detected in Hungarian chicken strains 16821-M-06, 924-Bi-05, and 3457-M-11, which were collected in 2005, 2006 and 2011, respectively (Farkas et al., 2016). Interestingly, BLASTn analyses of the 5' terminus of the μB gene (200 nt) revealed 79% nt and 83% aa sequence identity with pteropine orthoreoviruses suggesting a possibly shared evolutionary origin of this genomic segment in these avian origin strains and pteropine orthoreoviruses.

The other Hungarian pheasant origin reovirus strain, D1996/2/1, shared moderate to low nt and aa sequence identity values with representative members of the officially established orthoreovirus species, including the *Avian orthoreovirus* and the *Neoavian orthoreovirus* species that comprise genetically heterogenous virus strains from different hosts (Table 3 and Fig. 2). Nonetheless, the highest degree of identity was observed with some previously unclassified reoviruses, currently classified in the *Neoavian orthoreovirus* species, such as Pycno-1 (nt, 48.2–73.4%; aa, 40.1–88.8%), Tvärminne avian orthoreovirus (nt, 43.7–71.5%; aa, 36.0–83.3%) and Steller sea lion reovirus (nt, 46.3–73.5%; aa, 37.6–87.5%). With classical avian reoviruses the similarity values were lower; in particular, 39.8–71.0% nt and 27.4–80.8% aa sequence identity values were observed. In the genus *Orthoreovirus* among other criteria, specific sequence identity cut-off values have been determined to classify members into species (Attoui et al., 2011). Greater than 75% nt sequence identity between homologous genes is the cut-off value to classify orthoreovirus strains into the same species, while a nt sequence identity less than 60% is considered to be the cut-off value to demarcate orthoreoviruses into different virus species (Kugler et al., 2016). Identity values of D1996/2/1 and S1133 were found to fall in between these cut-off values, or were lower, when analyzing the 10 major reovirus genes (λA, 71.2%; λB, 65.3%; μB, 67.5%; λC, 47.0%; μA, 56.3%; μNS, 40.2%; σA, 58.3%; σB, 48.4%; σC, 51.6%; σNS, 57.6%) (Table 3, Fig. 2). Different aa sequence identity cut-off values have been determined for the more divergent outer capsid proteins (σB and σC), and the more conserved core proteins (λA, λB, λC, μA, σA) and μB, i.e. aa identities greater than 55% (outer capsid proteins) and 85% (conserved core proteins) indicate that orthoreovirus strains belong to the same species, while less than 35% (outer capsid proteins) and 65% (conserved core proteins) identity is used to classify orthoreovirus strains into different species, respectively (Attoui et al., 2011). For the non-structural proteins no cut-off values have been defined (Kugler et al., 2016). When comparing D1996/2/1 and S1133, aa similarity fell into the demarcation zone in case of λA (81.4%), λB (73.1%), μB (75.9%), σB (44.6%) and σC (39.5%), and three proteins showed lower values (λC, 39.9%; μA, 57.2%; σA, 59.9%), while μNS and σNS showed 39.5% and 55.0% similarities with the homologous proteins of S1133. The values fell in the same ranges when comparing D1996/2/1 with the other Hungarian pheasant reovirus (proteins with similarity below the cut-off: λC, 40.2%; μA, 57.2%; σA, 58.6%; proteins in the grey-zone: λA, 80.0%; λB, 73.1%; σB, 44.2%; σC, 46.7 %)

In phylogenies performed with all genomic segments, D1996/2/1 was only distantly related to other known orthoreoviruses of poultry (Fig. 3) and known members of neoavian orthoreoviruses, but appeared in a common branch with these strains, as well as with Nelson Bay reovirus and Steller sea lion reovirus, suggesting that these orthoreoviruses share a common ancestor (see additional trees in the supplementary file).

Unfortunately, there is limited amount of reovirus sequences available from wild animals, therefore the exact origin of D1996/2/1 remained unknown. The high level of nt sequence identity found in the 5' UTR sequences (GCUUU[U/A][U/C]) with other avian orthoreovirus and neoavian orthoreovirus strains in case of most genomic segments may suggest the avian origin of this novel pheasant orthoreovirus strain.

All pheasant origin orthoreovirus strains (i.e. D1996/2/1, Reo/HUN/Pheasant/216/2015, Reo/PA/Pheasant/13649/14) included in the analyses belong to different genogroups indicating the high diversity of orthoreoviruses that are capable of successful replication in pheasants. Similar findings were described recently for waterfowl origin reoviruses (Farkas et al., 2018). It is currently unknown whether pheasants are true hosts for any of the recently characterized strains or serve as dead-end host after incidental infection. Game bird production in semi-intensive or extensive farming conditions might be more vulnerable to viruses emerging from their natural reservoirs. Moreover, co-infection with genetically distant viruses may result in novel variants through reassortment events (McDonald et al., 2016). Successful reassortment between strains requires compatible viral packaging signals and concordant RNA–RNA and/or RNA–protein interactions. Novel allele configurations might manifest in phenotypic changes (e.g., antigenic characteristics, host range, pathogenicity); those combinations that contribute to increased viral fitness might be selected in the host against reassortants with reduced viral fitness. Due to the high genetic diversity of orthoreoviruses detected in poultry and game birds, continuous surveillance is strongly recommended to avoid spreading of pathogenic viruses and reduce economic losses.

Table 2
Nucleotide (NT) and amino acid (AA) sequence identities (%) of genes between Reo/HUN/Pheasant/216/2015 and selected avian and neoavian orthoreoviruses.

	NT										AA									
	λA	λB	λC	μA	μB	μNS	σA	σB	σC	σNS	λA	λB	λC	μA	μB	μNS	σA	σB	σC	σNS
Pheasant D1996/2/1	69.73	64.77	47.56	56.87	64.71	47.73	58.31	50.11	52.96	55.62	80.00	73.13	40.27	57.28	67.37	39.00	58.66	44.22	46.70	53.04
Pheasant 13649/14	79.25	82.54	73.12	79.76	65.66	73.60	81.05	80.29	59.39	77.19	96.85	96.18	83.97	93.27	72.05	85.71	97.42	89.46	55.84	90.88
Pekin duck D2533/6	71.38	66.94	55.41	58.01	62.51	58.74	61.67	56.42	41.12	61.57	84.65	75.60	55.67	59.48	65.56	58.59	65.89	54.76	26.40	62.84
Muscovy duck D1546	77.15	74.78	70.07	74.18	63.01	69.91	76.49	62.61	41.62	76.74	94.96	90.62	78.42	85.44	69.34	79.78	91.21	64.29	30.96	90.54
Muscovy duck D2044	76.99	74.83	69.91	73.86	62.96	70.44	76.49	62.61	41.46	77.19	94.80	90.54	78.42	85.03	69.18	80.26	91.21	64.29	30.46	89.86
Muscovy duck 815-12	77.44	75.63	70.02	73.76	63.51	71.35	76.06	61.26	40.95	77.19	95.43	91.34	79.05	85.16	70.24	81.54	90.96	63.95	29.44	90.88
Muscovy duck J18	77.54	75.34	70.54	73.17	65.56	70.66	76.06	65.20	42.98	77.19	95.83	90.78	79.67	85.44	71.75	80.74	92.25	69.73	32.99	89.86
Pekin duck 091	77.49	75.36	70.70	73.58	65.41	70.82	76.66	65.54	43.32	76.85	95.28	91.18	79.83	85.85	71.30	80.74	91.99	69.39	32.49	89.19
Pekin duck TH11	77.44	75.05	70.46	73.90	65.21	70.71	75.97	65.43	43.99	77.08	95.59	91.02	79.91	85.71	71.75	80.74	91.47	69.73	32.49	89.53
Muscovy duck ZJ00M	77.99	75.15	70.59	73.53	65.46	70.82	76.06	65.54	43.49	76.74	95.75	91.02	79.91	85.44	71.60	80.90	92.25	70.07	31.98	89.19
GooseD20/99	77.33	75.05	69.81	74.04	63.11	71.57	76.31	61.26	40.61	76.29	95.20	91.34	79.05	84.34	69.18	80.10	90.44	62.24	29.44	89.86
Goose 03G	77.49	75.10	70.44	72.99	65.86	70.50	75.54	65.77	43.65	75.51	95.51	90.94	79.59	84.89	71.30	81.22	91.21	69.39	32.99	89.86
Goose SDPY	79.91	82.89	72.91	79.58	65.71	74.88	80.88	82.88	61.59	76.07	96.54	95.79	84.05	92.86	72.05	86.68	96.38	90.14	59.90	90.20
Chicken S1133	79.38	90.38	81.04	80.63	65.81	74.93	81.14	84.91	62.44	77.53	96.69	96.98	92.57	92.99	72.51	86.84	97.67	90.82	58.88	93.92
Chicken T1781	78.93	85.06	92.11	94.00	66.82	74.13	87.68	81.98	58.88	87.42	96.61	96.03	96.87	96.70	73.41	86.52	99.22	90.14	56.35	96.96
Chicken AVS-B	79.12	83.02	73.17	80.31	66.47	75.36	80.10	83.00	60.07	77.64	96.77	96.10	84.28	92.99	71.90	86.84	97.42	91.16	58.88	90.88
Turkey TERV-MN1	78.41	83.50	72.28	79.35	66.22	75.04	80.53	69.37	93.06	78.31	96.54	95.47	82.25	92.31	72.96	86.20	97.67	78.23	91.37	90.20
Turkey TARV-MN4	78.57	83.50	72.60	79.17	66.72	75.25	80.28	69.26	93.23	77.75	96.22	95.47	82.56	92.45	70.85	86.04	97.42	77.55	91.37	89.86
Turkey D1246	78.59	82.38	72.18	79.21	66.57	74.93	80.96	70.50	93.23	77.87	96.38	95.39	82.41	91.48	71.45	86.04	97.42	77.21	90.86	90.54
Partridge D1007	87.88	83.81	71.89	86.77	66.52	73.92	86.65	95.50	84.94	78.65	98.19	95.31	82.64	94.37	72.51	85.39	98.71	95.92	84.26	92.23
Crow TVAV	71.30	63.81	47.17	56.27	65.06	51.10	58.91	46.96	45.85	56.85	82.83	71.70	39.87	57.28	68.58	44.30	64.34	37.76	38.07	55.74
Bulbul Pycno-1	68.97	64.98	47.30	57.60	64.76	48.05	56.68	48.87	53.81	55.96	79.53	72.97	39.09	56.73	69.03	39.33	58.91	40.14	51.27	54.73

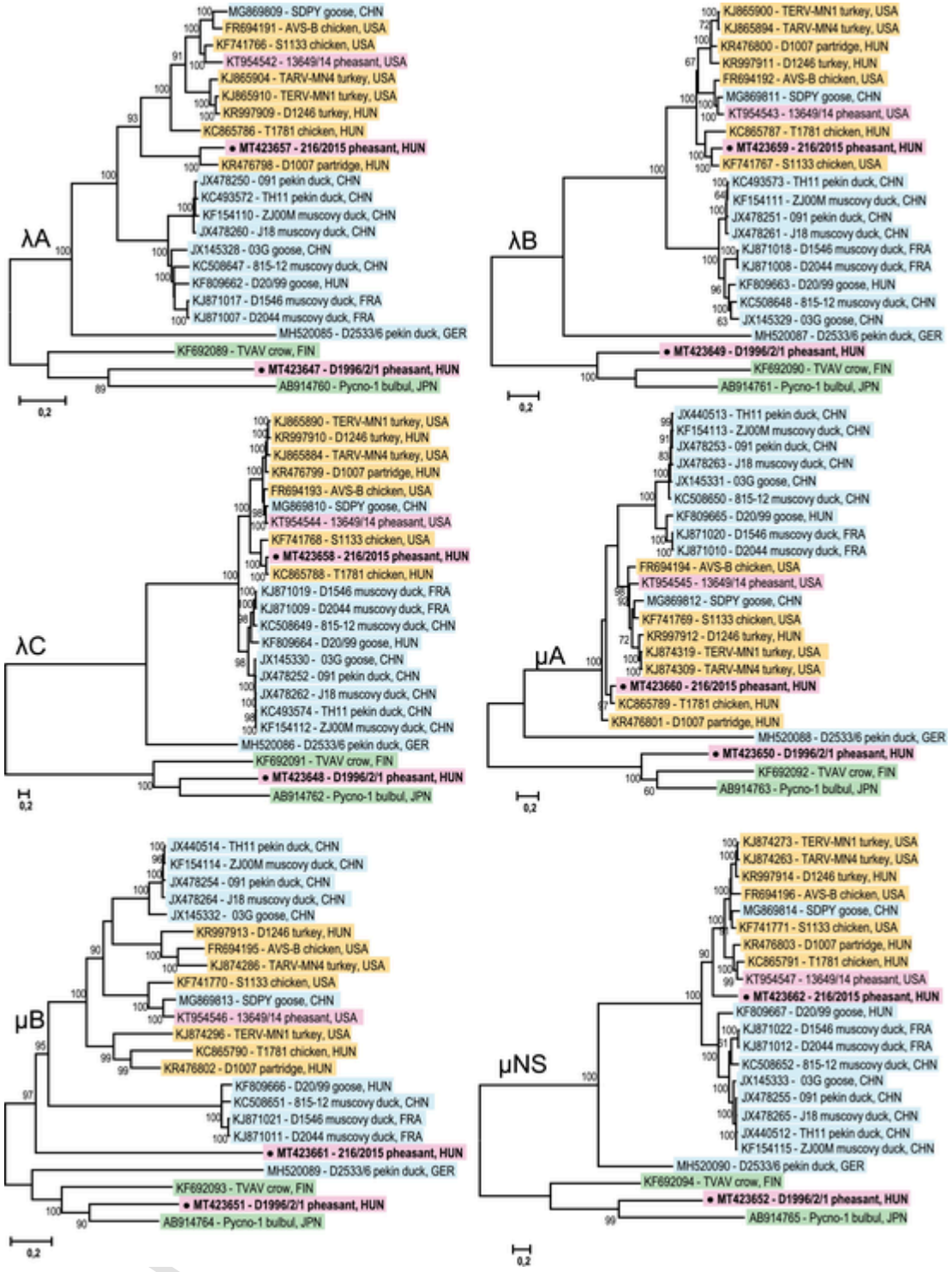

Fig. 1. Unrooted phylogenetic trees showing the clustering of avian origin orthoreoviruses based on the nucleotide sequences of the homologous genome segments. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. Orthoreoviruses of pheasants, other gallinaceous birds, waterfowls and wild birds are indicated with pink, yellow, blue, and green rectangles in the phylogenetic trees, respectively. Pheasant orthoreoviruses characterized in this study are indicated with bold letters. The scale bar is proportional to the genetic distance.

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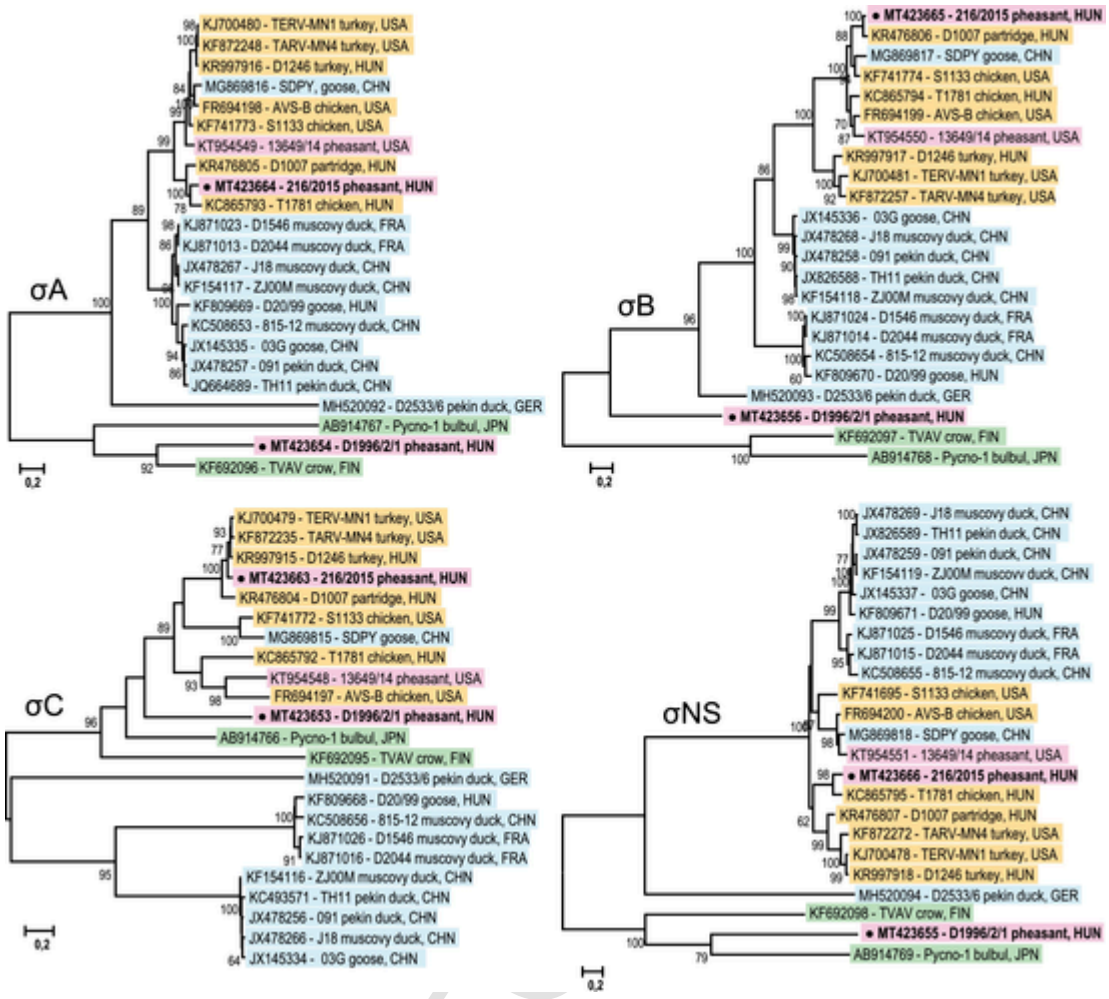

Fig. 1. *Continued*

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2021.198349.

Table 3
Nucleotide (NT) and amino acid (AA) sequence identities (%) of genes between D1996/2/1 and selected avian and neovian orthoreoviruses.

	NT										AA									
	λA	λB	λC	μA	μB	μNS	σA	σB	σC	σNS	λA	λB	λC	μA	μB	μNS	σA	σB	σC	σNS
Pheasant/216/2015	69.73	64.77	47.56	56.87	64.71	47.73	58.31	50.11	52.96	55.62	80.00	73.13	40.27	57.28	67.37	39.00	58.66	44.22	46.70	53.04
Pheasant 13649/14	70.86	64.61	47.96	57.19	68.42	48.96	56.16	48.87	51.27	56.52	80.31	73.37	40.89	56.46	74.77	40.13	58.91	45.58	46.19	54.39
Pekin duck D2533/6	68.18	65.03	47.98	54.26	65.91	48.96	57.28	55.29	40.95	55.28	76.14	70.67	41.13	52.34	73.72	41.89	54.52	48.30	27.41	52.03
Muscovy duck D1546	69.54	64.40	47.07	56.46	65.61	49.28	56.50	53.04	40.95	56.29	79.61	71.94	40.81	55.22	72.81	39.65	58.91	48.64	31.98	55.07
Muscovy duck D2044	69.54	64.61	46.94	56.27	65.66	49.33	56.59	53.04	40.44	56.74	79.37	72.02	40.66	54.81	72.51	39.81	59.17	48.64	31.47	55.74
Muscovy duck 815-12	69.99	63.95	47.10	56.46	65.76	47.41	57.45	52.48	39.76	56.18	79.84	72.42	40.81	54.53	73.11	39.81	59.17	47.96	31.47	55.41
Muscovy duck J18	69.62	64.85	47.67	56.46	68.62	47.57	56.59	51.24	42.47	56.29	79.92	71.94	40.81	54.81	75.38	38.84	59.43	46.60	32.99	56.76
Pekin duck 091	69.18	65.09	47.88	56.59	68.12	47.68	58.14	51.91	41.96	55.96	79.69	72.34	40.50	55.22	75.38	39.00	59.69	46.94	32.99	56.42
Pekin duck TH11	69.60	65.03	47.69	56.46	68.32	47.41	57.88	51.35	42.30	56.07	79.84	72.34	40.73	54.81	75.23	38.84	59.69	47.28	32.99	56.42
Muscovy duck ZJ00M	69.39	64.90	47.72	56.46	68.22	47.51	56.68	51.24	41.79	56.07	79.84	72.34	40.73	54.67	75.08	38.84	59.43	46.60	32.99	56.42
Goose D20/99	69.67	64.13	47.62	56.55	65.21	49.23	57.19	52.03	41.96	56.52	80.00	72.18	40.58	55.08	72.81	39.65	59.69	46.60	31.47	55.74
Goose 03G	69.10	64.56	47.59	56.50	68.22	47.89	57.28	50.90	42.30	55.84	79.84	72.02	40.73	54.67	74.62	39.00	59.17	45.92	33.50	56.08
Goose SDPY	70.25	65.54	47.80	56.96	68.77	47.94	57.45	49.10	51.61	56.85	80.24	73.69	40.66	56.46	75.38	39.33	58.14	44.56	49.75	55.41
Chicken S1133	70.75	65.19	47.02	56.50	66.92	48.53	56.59	49.10	53.81	57.30	80.39	72.81	39.87	56.18	74.77	39.97	58.66	45.58	50.25	54.73
Chicken T1781	70.86	65.25	47.51	56.96	68.17	49.55	57.97	50.68	51.10	55.62	80.71	73.13	40.27	56.46	75.98	40.45	58.66	45.92	45.69	54.05
Chicken AVS-B	70.41	64.82	48.09	56.32	68.57	48.48	57.28	48.76	50.93	56.07	80.39	73.21	40.73	56.87	76.44	39.00	59.69	45.24	46.19	55.07
Turkey TERV-MN1	70.91	64.85	47.28	56.78	66.57	47.84	57.71	52.14	53.30	56.29	80.79	72.42	40.27	56.32	75.08	39.81	59.17	45.92	45.69	54.39
Turkey TARV-MN4	70.72	64.74	47.69	56.27	67.57	47.89	57.80	52.25	53.30	57.19	80.39	72.42	40.50	56.46	75.98	39.81	59.17	46.60	45.18	55.07
Turkey D1246	70.99	65.01	46.91	56.09	68.17	48.21	57.54	52.70	52.79	55.73	80.47	72.58	40.27	56.04	75.68	39.65	59.17	45.58	44.67	54.39
Partridge D1007	71.01	64.56	47.46	56.82	67.52	48.00	57.11	50.45	52.62	55.96	80.08	72.18	39.80	56.32	76.74	39.97	58.14	44.90	46.70	55.07
Crow TVAV	71.54	70.30	54.60	63.32	70.43	55.75	70.28	47.07	43.65	60.67	83.31	82.83	53.87	70.19	82.02	54.41	77.26	37.76	36.04	67.57
Bulbul Pycno 1	71.69	71.31	56.99	66.16	73.38	59.49	60.29	48.20	52.45	64.27	84.09	82.75	57.23	72.94	88.82	56.50	63.31	40.14	48.22	67.91

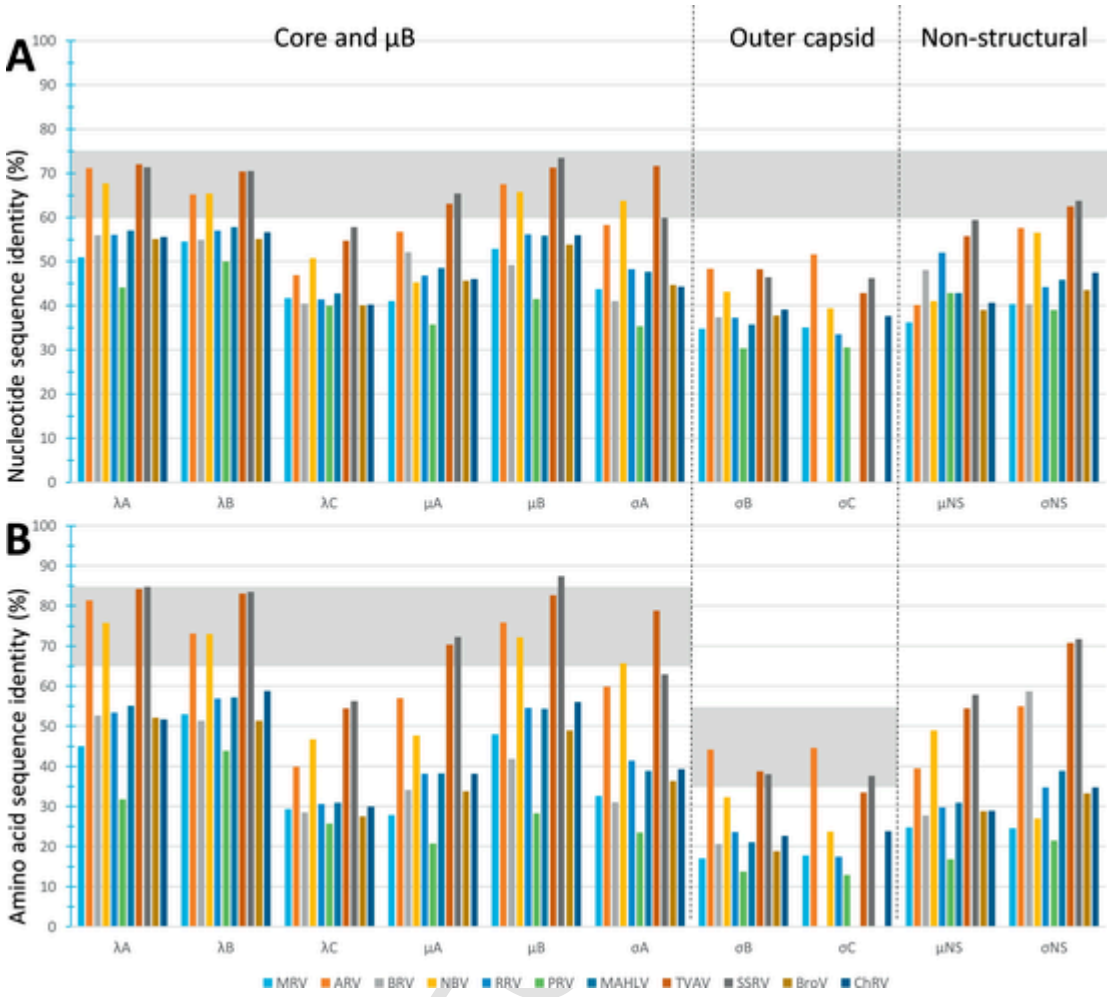

Fig. 2. Comparative diagram based on the percentile nucleotide and amino acid sequence identities of different genome segments between the pheasant strain D1996/2/1 and representative members of the ten established *Orthoreovirus* species (*Mammalian orthoreovirus,* MRV: Mammalian orthoreovirus 1 strain Lang; *Avian orthoreovirus*, ARV: Avian orthoreovirus strain S1133; *Nelson Bay orthoreovirus*, NBV: Nelson Bay virus; *Reptilian orthoreovirus,* RRV: Bush viper reovirus strain 47/02; *Baboon orthoreovirus*, BRV: Baboon orthoreovirus; *Piscine orthoreovirus*, PRV: Piscine orthoreovirus strain Salmo/GP-2010/NOR; *Mahlapitsi orthoreovirus*, MAHLV: Mahlapitsi virus strain 2511, *Broome orthoreovirus,* BRV: Broome virus; *Testudine orthoreovirus,* ChRV: Chelonian orthoreovirus strain CH1197/96; and *Neoavian orthoreovirus*, NeARV: Tvärminne avian virus), and the Steller sea lion virus (SSRV) not yet assigned to any orthoreovirus species. The bars are ordered according to the virus list at the bottom. (A) The grey area indicates the species demarcation cut-off values (60–75%). (B) The grey areas indicate the species demarcation cut-off values for the more conserved core proteins plus the μB protein (65–85%), and for the outer capsid proteins (35–55%), respectively. No cut-off values have been defined for the non-structural genes indicating the lack of consensus concerning their role in virus taxonomy.

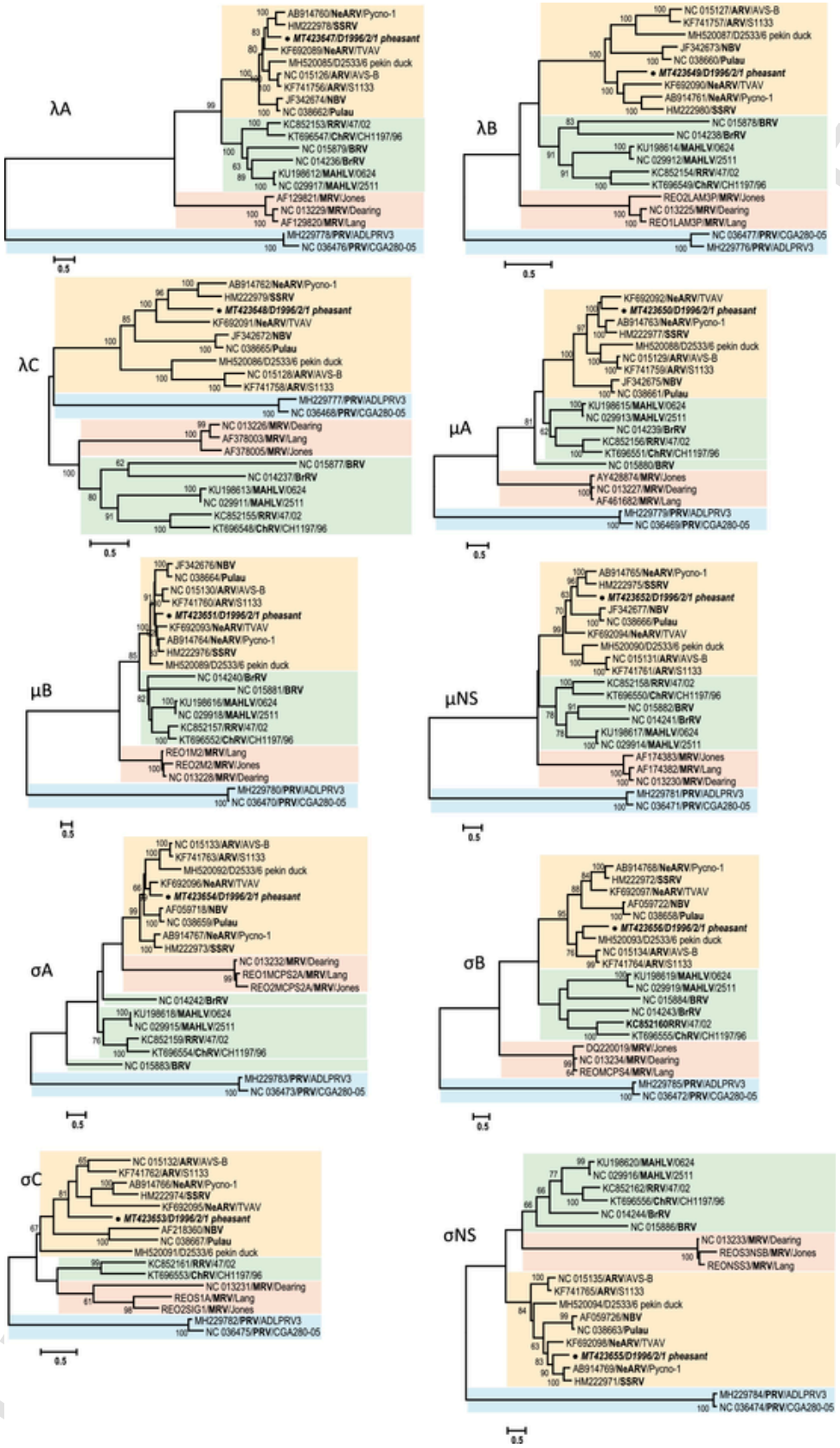

Fig. 3. Unrooted phylogenetic trees based on the nucleotide sequences of the corresponding genome segments of representative members of the ten established *Orthoreovirus* species (*Mammalian orthoreovirus* (MRV), *Avian orthoreovirus* (ARV), *Nelson Bay orthoreovirus* (NBV), *Reptilian orthoreovirus* (RRV), *Baboon orthoreovirus* (BRV), *Piscine orthoreovirus* (PRV), *Mahlapitsi orthoreovirus* (MAHLV), *Broome orthoreovirus* (BrRV), *Testudine orthoreovirus* (ChRV), and *Neoavian orthoreovirus* (NeARV), the Steller sea lion virus (SSRV) and Pekin duck or-

thoreovirus strain D2533/6 showing the clustering of orthoreoviruses. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. The scale bar is proportional to the genetic distance. The color code shows the major evolutionary lineages within the genus *Orthoreovirus*.

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