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Highlights

- The genome sequence and phylogenetic analysis of two novel orthoreovirus strains are described.
- Strain 2533/4/1-10 is probably a triple reassortant.
- Strain 2533/6/1-10 might have been acquired from an unknown natural host species.

Summary

Complete genomic sequences of two orthoreovirus strains, D2533/4/1-10 and D2533/6/1-10, isolated from Pekin ducklings in Germany have been determined. Pairwise sequence comparisons and phylogenetic analyses indicated that strain D2533/4/1-10 might have acquired its genomic segments from three different origins, from classical and novel waterfowl reoviruses, and a yet unknown orthoreovirus strain. D2533/6/1-10 proved to be only distantly related to previously described orthoreoviruses. Reassortment, host species transmission events, and successful adaptation of novel variants may signify a challenge for animal health and maintenance of economic production.

Keywords

Waterfowl orthoreoviruses; Reassortment; Host species transmission event; Phylogenetic analysis

1. Introduction

Members of the genus *Orthoreovirus* possess multilayered non-enveloped virion particles that enclose the dsRNA genome of 10 segments (large: L1-L3; medium: M1-M3; small: S1-S4). Orthoreoviruses are currently assigned into seven official species: *Mammalian orthoreovirus* (MRV), *Avian orthoreovirus* (ARV), *Nelson Bay orthoreovirus* (NBV), *Reptilian orthoreovirus* (RRV), *Baboon orthoreovirus* (BRV) (Attoui et al., 2011), *Piscine orthoreovirus* (PRV) (Markussen et al., 2013)and *Mahlapitsi orthoreovirus* (MAHLV) (Jansen van Vuren et al., 2016). Currently known RVs of waterfowl (WRVs) are members of the ARV species and based on their genetic and biological properties these viruses are classified into two categories, the “classical” and “novel” WRVs (Chen et al., 2012). Outbreaks caused by classical viruses affect young Muscovy duck or geese flocks usually between 10 days to 10 weeks of age (Palya et al., 2003). Clinically lethargy, weakness,

diarrhoea, later lameness and stunting can be observed due to the infection in up to 60 % of the flock. Mortality rate is low (ranging between 2 to 20 %) but is generally higher in younger animals. Histopathological examinations reveal involvement of the liver and the spleen with enlargement and multiple small disseminated necrotic foci in both organs, pericarditis and epicarditis, arthritis and tenosynovitis are frequently observed. In classical WRVs the S4 segment is bicistronic, encoding the p10 and σC (cell attachment outer fiber) proteins, and lacking the FAST protein responsible for giant cell formation in cell cultures. The novel type of WRVs that emerged first in China in 1997 are associated with broader host spectrum (Muscovy duck, Pekin duck, mallard duck and goose), more severe clinical picture and related pathological findings (extensive haemorrhagic-necrotic pathologic lesions in the spleen), and significantly higher mortality rate (up to 60%) (Chen et al., 2012; Farkas et al., 2014; Ma et al., 2012; Yun eta al., 2012). In novel WRVs the S1 segment, the counterpart of S4 of classical WRVs, similarly to other ARVs is tricistronic; beside the p17 and σC proteins, the FAST protein, responsible for giant cell formation and consequentially more rapid virus spread, is encoded by this segment.

In case of semi-intensive housing of waterfowl, birds have access to natural or artificial swimming facilities and can be in close contact with free-living bird species, including migratory aquatic birds flying long distances. This condition favors the transmission of various pathogens between domestic waterfowl and wild birds. Currently there are no commercial vaccines available providing protection against WRVs. Continuous surveillance is required to obtain information about the circulating and emerging field WRV strains in poultry to elaborate effective preventive strategies. Complete genomic sequencing helps researchers in these efforts and is also crucial to delineate major mechanisms responsible for generation of genetic diversity of these viruses.

2. Materials and Methods

Two novel WRV strains, D2533/4/1-10 and D2533/6/1-10, were isolated in 2014 from the bursa of 10 and 28 day-old Pekin ducklings (*Anas platyrhynchos*) originating from a flock with approximately 10.000 birds in Germany. In the flock, locomotor disorder was observed in 12-36 day-old animals with a peak incidence among 26-32 day-old ducklings, while feather picking and bleeding was detected in ducklings at the age of ≥21 days. Post mortem examination of the affected birds revealed mild to moderate tenosynovitis and leg deformities, air-sacculitis, and lymphocyte depletion in the follicles of the bursa. Cryptosporidiosis was diagnosed in 18 day-old ducklings. From the brain and air-sac exudate of some animals *Coenonia anatine* could be isolated during the bacteriological investigations. In bursa specimens collected from 18 and 28 day-old birds the presence of circoviruses was confirmed while duck parvovirus could be excluded by PCR.

Both WRV strains were further propagated on duck embryo liver cell cultures. After one freezing/thawing cycle, 250 µl cell culture supernatant of both strains was subjected to RNA extraction using TRIzol reagent according to the manufacturer's instructions. Purified RNA was then directly used in sample preparation for next generation sequencing (NGS) applying sequence independent single primer amplification method (Rosseel et al., 2012). NGS was carried out on a 316 chip using Ion Torrent semiconductor sequencing equipment (Ion Torrent Personal Genome Machine, Life Technologies) as described previously (Bányai et al., 2014). To obtain the 5' and 3' terminal sequences of the segments, DNA oligonucleotides were ligated to each end of the genomic dsRNA (Lambden et al., 1992). Oligonucleotide primers were designed to amplify and sequence the missing parts of the genome (data not shown).

Complete genome sequences were assembled using the CLC Genomics Workbench software (http://www.clcbio.com). Contigs were aligned with Sanger sequencing reads using

MultAlin online software (http://multalin.toulouse.inra.fr/multalin/) and were edited in GeneDoc software (Nicholas et al., 1997). BLASTn and BLASTx algorithms (https://blast.ncbi.nlm.nih.gov/) were used to identify homologous genes among sequences deposited in GenBank. Codon-based multiple sequence alignments were generated using the Muscle algorithm within the TranslatorX online software (http://translatorx.co.uk). Phylogenetic analysis was performed using the MEGA7 package (Kumar et al., 2016).

3. Results and Discussion

Complete genomic sequences, including the typical 5' and 3' segment termini of the orthoreovirus strains, D2533/4/1-10 (D2533/4) and D2533/6/1-10 (D2533/6), were determined and deposited into the GenBank database under the accession numbers MH520075 to MH520084 and MH520085 to MH520094, respectively. Genomic organization of the studied strains was similar to and corresponded with that of other ARVs. With the exception of S1, which was tricistronic in both strains, all segments were found to encode a single open reading frame (ORF). The homologues of the following protein coding sequences could be identified in both genomes: λA, λB, λC, μA, μB, μNS, σA, σB, σC, σNS, p10 and p17 (Table 1).

Nucleotide (nt) and amino acid (aa) sequences of the coding regions of D2533/4 showed the highest nt/aa identity with classical and novel WRVs of European and Chinese origin (46.7-93.1 % nt/ 41.1-98.2 % aa) (Suppl. material Table 1, 2). The lowest identity values were seen when analysing the σC (nt and aa similarities with novel WRVs, 75.4-76.6 % and 82.9-83.9 %; classical WRVs, 46.7-47.9 % and 41.1-42.6 %, other ARVs 39.9-45.5 % and 26.2-33.2 %). In case of λA, λB, λC, μA, μNS and σNS BLASTn and pairwise distance analyses revealed the highest scores and nt sequence identity values with classical WRVs, while μB, σB, and σC were most similar to novel WRV strains. In the phylogenies performed

with individual genes, D2533/4 clustered with classical (6 genes) and novel (3 genes) WRV strains. Sequence analyses of the σA gene revealed similar scores (ZJ2000M 89%; 03G 88%) and nt identity values with strains belonging to both types of WRVs; accordingly, in the σA phylogeny grouping of the two WRV types was not visible due to several reassortment events that had occurred in the past between the novel and classical WRV strains (Fig. 1). In the σC phylogeny, D2533/4 grouped together with the novel type of WRVs but formed a separate branch in the cluster indicating that this segment was most likely acquired from a divergent WRV strain of a heterologous host species wherein this gene had evolved remarkably. The foreign origin of the S1 segment was also supported by its tricistronic structure as observed in chicken and turkey origin ARVs, as well as in novel WRVs. The selection pressure on coding capacity of the S1 genome segment of avian orthoreoviruses is not well understood. However, losing the FAST protein coding gene along with its apoptotic effect and syncytium forming ability might be beneficial for the virus in the adaptation process to a new host or tissue type and promote its long term survival as it could be observed in less pathogenic classical WRVs (Nibert and Duncan, 2013). Similar reasons might have led to the reduction of the p17 gene (162 aa) which is responsible originally for the regulation of different nuclear and cytoplasmic processes in ARVs and novel WRV strains (Costas et al., 2005; Geng et al., 2009; Huang et al., 2015). The truncated p10 protein (95 aa) of classical WRV strains (including duck and goose origin WRVs detected in France and Hungary, respectively) might have been derived from the original p17 ORF of novel WRVs through the excision of a larger gene fragment during the evolution of WRVs. It is currently unknown whether this event occurred in domestic waterfowl or other hosts; nonetheless, bicistronic σC coding genome segment has not yet been reported from orthoreoviruses of birds other than domestic geese and ducks. The strain 2533/6 shared moderate to low nt and aa sequence identity values with representative members of the seven officially established orthoreovirus species and other

unclassified ARV-like strains (Suppl. material Table 3, Fig. 2). The highest degree of identity was observed with ARV strains (37.3-72.8% nt; 25.2-85.5% aa), TVAV (Tvärminne avian virus; 38.1-68.8% nt; 23.9-80.9% aa) and SSRV (Steller sea lion reovirus; 39.2-69.1% nt; 24.3-76.6% aa). In the genus *Orthoreovirus* specific sequence identity cut-off values have been defined to classify members into species (Attoui et al., 2011). Greater than 75 % nt sequence identity between homologous genes is the cut-off value for most genome segments to classify orthoreovirus strains into the same species, and a nt sequence identity less than 60 % is considered to be the cut-off value to demarcate orthoreoviruses into different virus species. Identity values of 2533/6 and S1133 fell in between these cut-off values or were lower in case of the following homologous genes: λA, λB, µB, σA, σNS and λC, µA, σB, σC, µNS, respectively (Suppl. material Table 3). 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. When comparing 2533/6 and S1133, aa similarity of slightly higher than 85% was found only in case of the λA (85.1%), fell into the demarcation zone in case of λB (75.8%), µB (71.0%), σA (65.4%), and σB (54.2%), and three proteins showed lower values (λC: 55.9%, µA: 60.5%, and σC: 27.1%). µNS and σNS showed 57.0% and 66.0% similarity with the homologue proteins of S1133. In accordance with previous studies (Dandár et al., 2014; Kugler et al., 2016) these data indicated greater genetic distance between putative members of a certain orthoreovirus species than previously assumed implying the need for fine-tuning the sequence identity cut-off values of the currently used classification system (Attoui et al.,

2011). In phylogenies of individual genes, 2533/6 was only distantly related to other known orthoreoviruses of poultry (Fig. 1) and wild birds (e.g., Tvärminne avian virus and Bulbul orthoreovirus), but formed a single monophyletic clade with these strains, just like with NBV and SSRV, suggesting common evolutionary origin of these orthoreoviruses (data not shown).

4. Conclusions

Reassortment of segmented RNA viruses contributes to the emergence of strains with novel genomic constellations (McDonald et al., 2016). In case of influenza viruses, full-genome sequencing and spatial surveillance programs help to discover different reassortants and their evolution is readily traceable as data are rapidly accumulating during disease outbreaks (Dhingra et al., 2018; Ramey et al., 2017). For orthoreoviruses such extensive, world-wide monitoring system and database has not been developed therefore our knowledge about their diversity is still limited, and the exact origin of reassortants and other newly detected viruses in domesticated animals can only be hypothesized. The role of reassortment to generate novel avian orthoreovirus strains is less well understood but recent whole genome sequencing studies indicate that it may be an important mechanism in viral evolution (Farkas et al., 2014, 2016). New constellations of the genomic segments may have an impact on viral phenotype, including those features that are related to pathogenicity and antigenicity. According to our analyses 2533/4 proved to be a triple reassortant strain (Fig. 3), which most probably obtained nine of the genomic segments from classical and novel WRVs; the origin of the S1 segment that codes for a distantly related cell attachment and virus neutralization antigen, σC, remains elusive (Attoui et al., 2011; Kuntz-Simon et al., 2002). Strain 2533/6 might have been acquired from an unknown natural host species; this virus was able to cross the species barrier and successfully replicated in the bursa of Pekin ducklings. High level of nt sequence identity found in the 5' UTR sequences (GCUUUUU/A/C) with other ARVs in

most genomic segments suggests the avian origin of this novel orthoreovirus strain. Detecting novel variants of WRVs in Europe is fascinating and fittingly complements our current understanding of the diversity of orthoreoviruses in waterfowl. It will be intriguing to explore whether these strains are naturally attenuated in domestic poultry and whether they may serve as candidates for development of new vaccines.

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Declarations of interest: none.

7. References

Attoui, H., Mertens, P.P.C., Becnel, J., Belaganahalli, S., Bergoin, M., Brussaard, C.P., Chappell, J.D., Ciarlet, M., del Vas, M., Dermody, T.S., Dormitzer, P.R., Duncan, R., Fang, Q., Graham, R., Guglielmi, K.M., Harding, R.M., Hillman, B., Makkay, A., Marzachì, C., Matthijnssens, J., Milne, R.G., Mohd Jaafar, F., Mori, H., Noordeloos, A.A., Omura, T., Patton, J.T., Rao, S., Maan, M., Stoltz, D., Suzuki, N., Upadhyaya, N.M., Wei, C., Zhou, H., 2011. Family *Reoviridae*, in: King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.) Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, pp. 541–554.

Bányai, K., Borzák, R., Ihász, K., Fehér, E., Dán, Á., Jakab, F., Papp, T., Hetzel, U., Marschang, R.E., Farkas, S.L., 2014. Whole-genome sequencing of a green bush viper reovirus reveals a shared evolutionary history between reptilian and unusual mammalian orthoreoviruses. Arch. Virol. 159:153-158. https://doi.org/10.1007/s00705-013-1796-2

Chen, Z., Zhu, Y., Li, C., Liu, G., 2012. Outbreak-associated novel duck Reovirus, China. Emerg. Infect. Dis. 18:1209-1211. https://doi.org 10.3201/eid1807.120190

Costas, C., Martínez-Costas, J., Bodelón, G., Benavente, J. 2005. The Second Open Reading Frame of the Avian Reovirus S1 Gene Encodes a Transcription-Dependent and CRM1-Independent Nucleocytoplasmic Shuttling Protein. J. Virol. 79:2141-2150. https://doi.org/10.1128/JVI.79.4.2141-2150.2005

Dandár, E., Huhtamo, E., Farkas, S.L., Oldal, M., Jakab, F., Vapalahti, O., Bányai, K., 2014. Complete genome analysis identifies Tvärminne avian virus as a candidate new species within the genus *Orthoreovirus*. J. Gen. Virol. 95:898-904. https://doi.org/10.1099/vir.0.060699-0

Dhingra, M.S., Artois, J., Dellicour, S., Lemey, P., Dauphin, G., Von Dobschuetz, S., Van Boeckel, T.P., Castellan, D.M., Morzaria, S., Gilbert, M., 2018. Geographical and historical

patterns in the emergences of novel highly pathogenic avian influenza (HPAI) H5 and H7 viruses in poultry. Front Vet.Sci. 5:84. https://doi.org/10.3389/fvets.2018.00084.

Farkas, S.L., Dandár, E., Marton, S., Fehér, E., Oldal, M., Jakab, F., Mató, T., Palya, V., Bányai, K., 2014. Detection of shared genes among Asian and European waterfowl reoviruses in the whole genome constellations. Infect. Genet. Evol. 28:55-57. https://doi.org/10.1016/j.meegid.2014.08.029

Farkas, S.L., Marton, S., Dandár, E., Kugler, R., Gál, B., Jakab, F., Bálint, Á., Kecskeméti, S., Bányai, K., 2016. Lineage diversification, homo- and heterologous reassortment and recombination shape the evolution of chicken orthoreoviruses. Sci. Rep. 6:36960. https://doi.org/10.1038/srep36960

Geng, H., Zhang, Y., Liu-Partanen, Y., Vanhanseng Guo, D., Wang, Y., Liu, M., Tong, G., 2009. Apoptosis induced by duck reovirus p10.8 protein in primary duck embryonated fibroblast and Vero E6 cells. Avian Dis. 53:434-440. https://doi.org/10.1637/8514-110408-Reg.1

Huang, W.R., Chiu, H.C., Liao, T.L., Chuang, K.P., Shih, W.L., Liu, H.J., 2015. Avian Reovirus Protein p17 Functions as a Nucleoporin Tpr Suppressor Leading to Activation of p53, p21 and PTEN and Inactivation of PI3K/AKT/mTOR and ERK Signaling Pathways. PLoS One. 10(8):e0133699. https://doi.org/10.1371/journal.pone.0133699

Jansen van Vuren, P., Wiley, M., Palacios, G., Storm, N., McCulloch, S., Markotter, W., Birkhead, M., Kemp, A., Paweska, J.T., 2016. Isolation of a novel fusogenic orthoreovirus from Eucampsipoda africana bat flies in South Africa. Viruses 8:65. doi: 10.3390/v8030065.

Kugler, R., Marschang, R.E., Ihász, K., Lengyel, G., Jakab, F., Bányai, K., Farkas, S.L., 2016. Whole genome characterization of a chelonian orthoreovirus strain identifies significant

genetic diversity and may classify reptile orthoreoviruses into distinct species. Virus Res. 215:94-98. https://doi.org/10.1016/j.virusres.2016.02.005

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33:1870-1874. https://doi.org/10.1093/molbev/msw054

Kuntz-Simon, G., Blanchard, P., Cherbonnel, M., Jestin, A., Jestin, V., 2002. Baculovirus-expressed muscovy duck reovirus sigmaC protein induces serum neutralizing antibodies and protection against challenge. Vaccine 20:3113-3122.

Lambden, P.R., Cooke, S.J., Caul, E.O., Clarke, I.N., 1992. Cloning of noncultivatable human rotavirus by single primer amplification. J. Virol. 66:1817-1822.

Ma, G., Wang, D., Shi, J., Jiang, T., Yuan, Y., Zhang, D., 2012. Complete genomic sequence of a reovirus isolate from Pekin ducklings in China. J. Virol. 86:13137. https://doi.org/10.1128/JVI.02512-12

Markussen, T., Løvoll, M., Tengs, T., Dahle, M.K., Wiik-Nielsen, C.R., Grove, S., Lauksund, R.S., Finstad, Ø.W., Robertsen, B., Rimstad, E., 2013. Sequence Analysis of the Genome of Piscine Orthoreovirus (PRV) Associated with Heart and Skeletal Muscle Inflammation (HSMI) in Atlantic Salmon (*Salmo salar*). PLoS ONE. 8(7):e70075. https://doi.org/10.1371/journal.pone.0070075

McDonald, S.M., Nelson, M.I., Turner, P.E., Patton, J.T., 2016. Reassortment in segmented RNA viruses: mechanisms and outcomes. Nat. Rev. Microbiol. 14:448-460. https://doi.org/10.1038/nrmicro.2016.46

Nibert, M.L., Duncan, R., 2013. Bioinformatics of recent aqua- and orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications. PLoS ONE. 8(7):e68607. https://doi.org/10.1371/journal.pone.0068607

Nicholas, K.B., Nicholas, H.B. Jr, Deerfield, D.W. II, 1997. GeneDoc: analysis and visualization of genetic variation. Embnet News 4:14.

Palya, V., Glávits, R., Dobos-Kovács, M., Ivanics, É., Nagy, É., Bányai, K., Reuter, G., Szűcs, G., Dán, Á., Benkő, M., 2003. Reovirus identified as cause of disease in young geese. Avian Pathol. 32:129-138. https://doi.org/10.1080/030794502100007187

Ramey, A.M., Hill, N.J., Cline, T., Plancarte, M., De La Cruz, S., Casazza, M.L., Ackerman, J.T., Fleskes, J.P., Vickers, T.W., Reeves, A.B., Gulland, F., Fontaine, C., Prosser, D.J., Runstadler, J.A., Boyce, W.M., 2017. Surveillance for highly pathogenic influenza A viruses in California during 2014-2015 provides insights into viral evolutionary pathways and the spatiotemporal extent of viruses in the Pacific Americas Flyway. Emerg. Microbes Infect. 6(9):e80. https://doi.org/10.1038/emi.2017.66.

Rosseel, T., Scheuch, M., Höper, D., De Regge, N., Caij, A.B., Vandenbussche, F., Van Borm, S., 2012. DNase SISPA-next generation sequencing confirms Schmallenberg virus in Belgian field samples and identifies genetic variation in Europe. PLoS ONE. 7(7):e41967. https://doi.org/10.1371/journal.pone.0041967

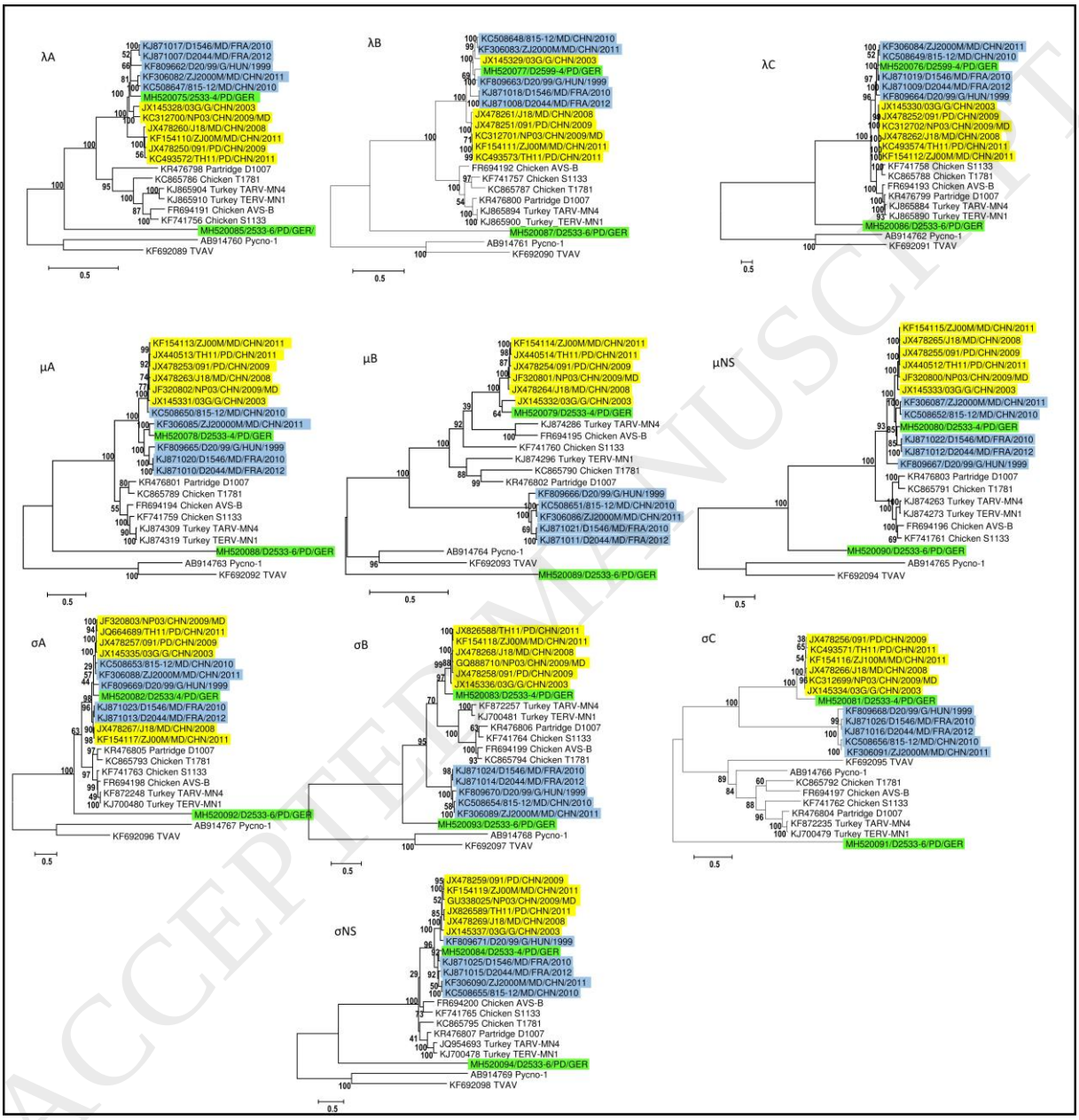
Yun, T., Ye, W., Ni, Z., Chen, L., Yu, B., Hua, J., Zhang, Y., Zhang, C., 2012. Complete genomic sequence of goose-origin reovirus from China. J. Virol. 86:10257. https://doi.org/10.1128/JVI.01692-12

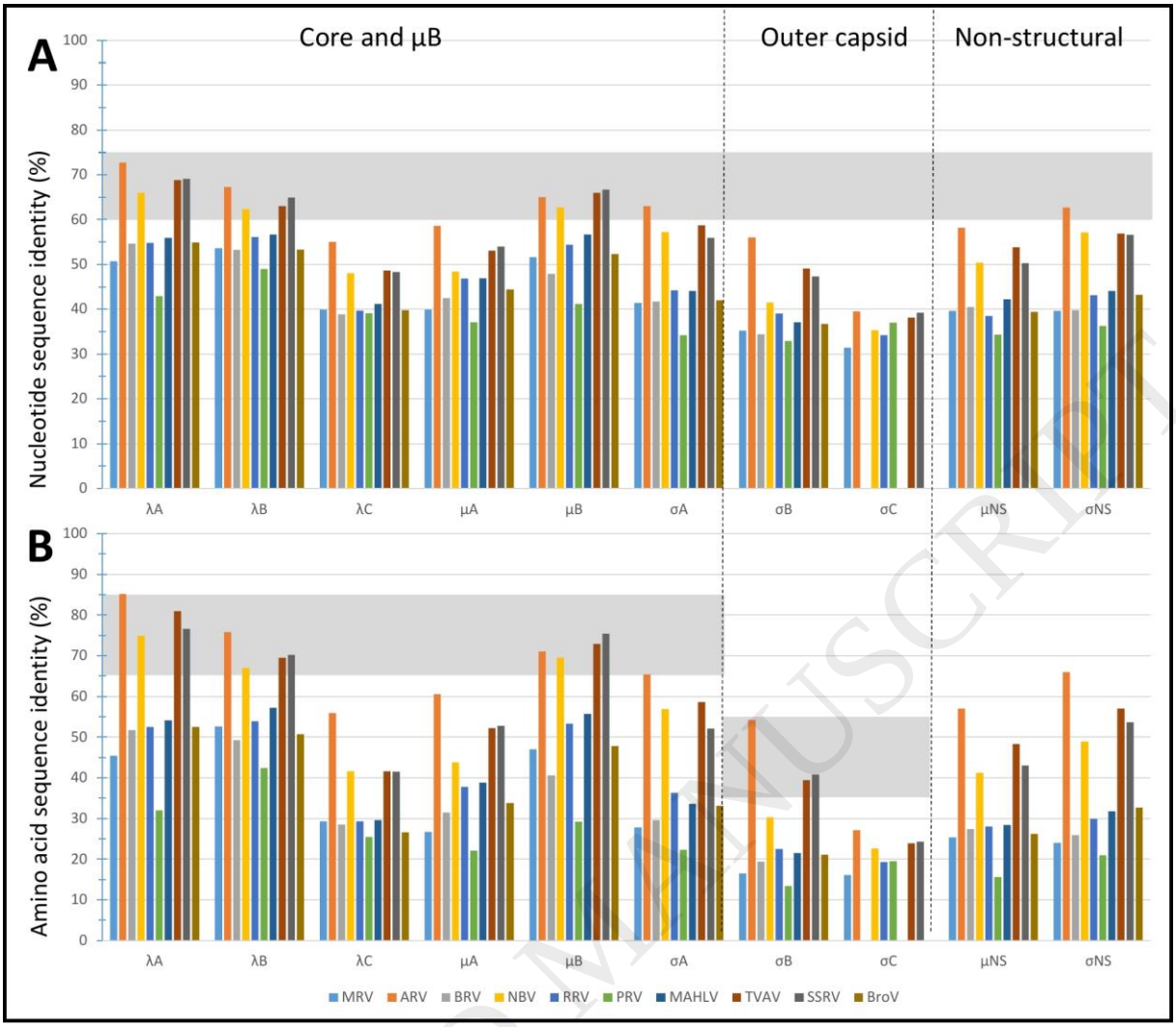
Figure caption

Figure 1. Unrooted phylogenetic trees showing the clustering of avian and waterfowl origin reoviruses based on the nucleotide sequences of the corresponding genome segments of different viruses. Phylogenetic calculations were carried out using the maximum-likelihood method applying the best-fit models calculated for each gene. Classical and novel waterfowl origin strains, and 2533/4 and 2533/6 are indicated with blue, yellow and green rectangles, respectively, in the phylogenetic trees. The scale bar is proportional to the genetic distance.

Figure 2. Comparative diagram based on the percentile nucleotide (panel A) and amino acid (panel B) sequence identities of different genome segments between the strain 2533/6/1-10 and the representative strains of the seven 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, and three unclassified orthoreovirus strains, Broome virus (BRV), Steller sea lion reovirus (SSRV), and Tvärminne avian virus (TVAV), respectively. The bars are ordered according to the virus list at the bottom. In panel A the grey area indicates the species demarcation cut-off values (60–75%). In panel 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.

Figure 3. Simplified schematic illustration of the putative genomic compositions of strain 2533/4/1-10 and the possible parent viruses based on nucleotide-distance comparison and phylogenetic analysis.





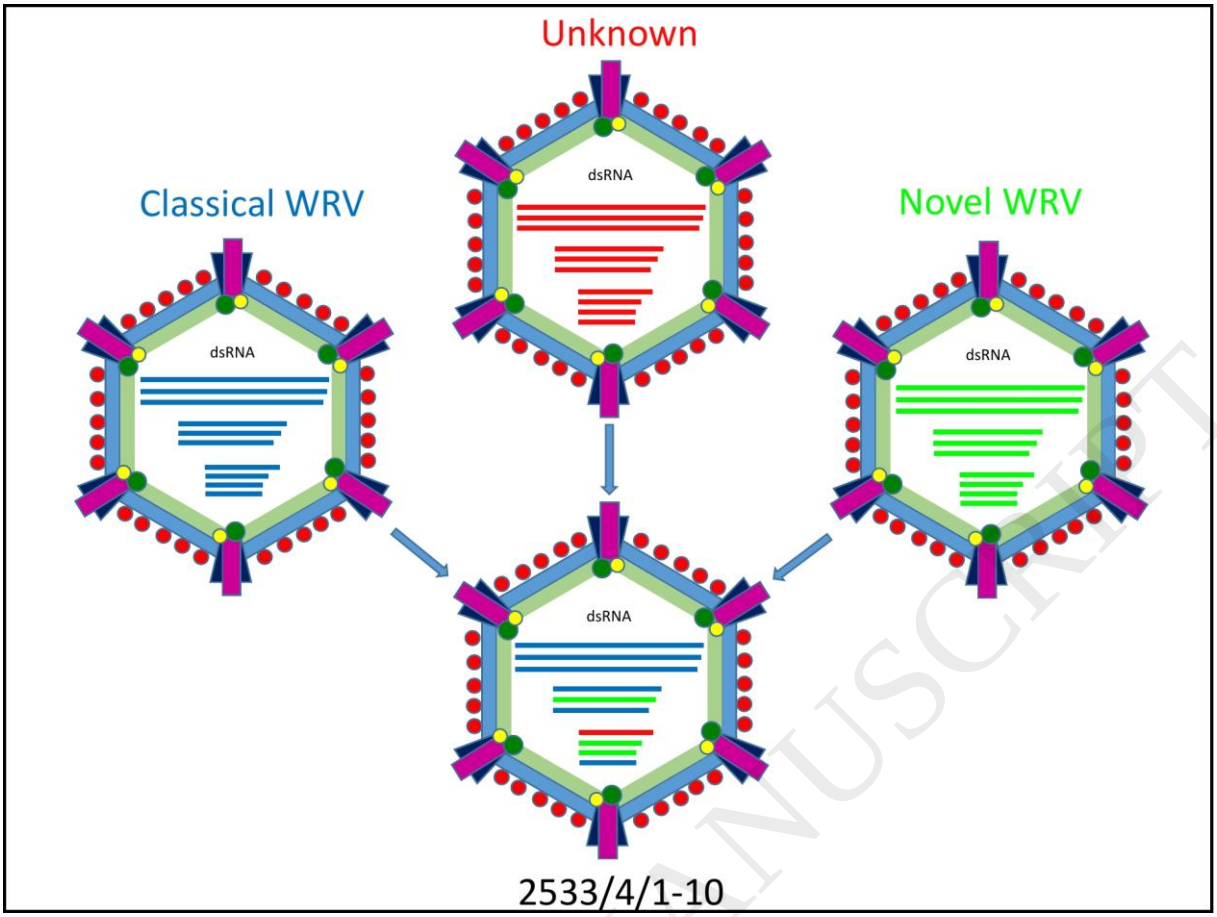


Table 1. General features of the duck orthoreovirus strain D2533/4/1-10 and D2533/6/1-10

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
D2533/4/1-10	L1	3958	20 - 3882 - 56	GCUUUU/UUCAUC	λA (Core shell)	1293	D2044 (KJ871007): 90%
	L2	3907	12 - 3858 - 37	GCUUUU/UUCAUC	λC (Core turret)	1285	D2044 (KJ871009): 90%
	L3	3829	13 - 3780 - 36	GCUUUU/UUCAUC	λB (Core RdRp)	1259	D20/99 (KF809663): 93%
	M1	2284	13 - 2199 - 72	GCUUUU/UUCAUC	μA (Core NTPase)	732	ZJ2000M (KF306085): 90%
	M2	2158	29 - 2031 - 98	GCUUUU/UUCAUC	μB (Outer shell)	676	J18 (JX478264):90%
	M3	1997	25 - 1908 - 64	GCUUUU/UUCAUC	μNS (NS factory)	635	D2044 (KJ871012): 90%
	S1	1568	19 - 294 -32 489 969	GCUUUU/UUCAUC	p10 (FAST) p17 (NS other) σC (Outer fiber)	97 162 322	ZJ00M (KF154116): 77%
	S2	1324	15 - 1251 - 58	GCUUUU/UUCAUC	σA (Core clamp)	416	ZJ2000M (KF306088): 89%
	S3	1202	30 - 1104 - 68	GCUUUU/UUCAUC	σB (Outer clamp)	367	03G (JX145336): 85%
	S4	1191	23 - 1104 - 64	GCUUUU/UUCAUC	σNS (NS RNAb)	367	D1546 (KJ871025): 95%
D2533/6/1-10	L1	3998	20 - 3921 - 57	GCUUUU/UUCAUC	λA (Core shell)	1306	601G (AY641736): 74%
	L2	3896	12 - 3852 - 32	GCUUUU/UUCAUC	λC (Core turret)	1283	ZJ2000M (KF306084): 64%
	L3	3825	13 - 3780 - 32	GCUUUU/UUCAUC	λB (Core RdRp)	1259	GuanxiR2 (KF741727): 69 %
	M1	2279	12 - 2196 - 71	GCUUUU/UUCAUC	μA (Core NTPase)	731	16821-M-06 (KX398305): 66%
	M2	2150	30 - 2022 - 98	GCUUUU/UUCAUC	μB (Outer shell)	673	Pycno-1 (AB914764): 68%

	M3	1990	21 - 1908 - 61	GCUUUU/UUCAUC	µNS (NS factory)	635	924-Bi-05 (KX398267): 68%
	S1	1573	22 - 282 - 34 369 1014	GCUUUU/UUCAUC	p10 (NS FAST) p17 (NS other) σC (Outer fiber)	93 122 337	HN5d (KT861593): 73%
	S2	1325	15 - 1251 - 59	GCUUUU/UUCAUC	σA (Core clamp)	416	S12 (EF076764): 67%
	S3	1201	30 - 1104 - 67	GCUUUU/UUCAUC	σB (Outer clamp)	367	091 (JX478258): 66%
	S4	1190	23 - 1104 - 63	GCUUUU/UUCAUC	σNS (NS RNAb)	367	16821-M-06 (KX398311): 66%