SEQUENCE AND PHYLOGENETIC ANALYSIS OF THE S-CLASS GENOME SEGMENTS OF A DUCK ORTHOREOVIRUS

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Summary. – In spite of common properties duck orthoreoviruses (DRVs) are antigenically different from other avian orthoreoviruses (ARVs). We analyzed the S-class genome segments of the DRV S12 and compared them with S-class genome segments of other orthoreoviruses compared to those of ARV S1133. The full-length S-class σA, σB, σNS, and σC genes were determined and compared with other ARVs to study the degree of genetic divergence and evolution of DRVs. The alignment of the DRV S12 σA, σB, σNS, and σC genes with DRV 89026 showed 90.0%, 93.6%, 88.0%, and 93.1% nucleotide identity and 97.1%, 94.3%, 95.8%, and 93.7% amino acid identity, respectively. The alignment of the DRV S12 σA, σB, σNS, and σC genes with other ARVs revealed 76.0–77.1%, 52.5–55.1%, 78.4–79.6%, and 2.7–9.9% nucleotide identity and 89.5–91.2%, 61.4–62.0%, 91.6–92.7% and 22.6–26.7% amino acid identity, respectively. Phylogenetic analyses demonstrated that DRVs were quite different from other ARVs and provided the evidence for the diversity among avian orthoreoviruses.

Key words: duck orthoreovirus; σA, σB, σNS, and σC genes; phylogenetic analysis

Introduction

All orthoreoviruses consist of 10 dsRNA genome segments packaged into a non-enveloped icosahedral double-capsid shell 70–80 nm in diameter (Schnitzer *et al*., 1982; Joklik, 1983). The genomic segments can be separated into three size classes: large (segments L1-L3), medium (segments M1-M3), and small (segments S1-S4) (Spandidos and Graham, 1976; Gouvea and Schitzer, 1982; Heffels-Redmann *et al*., 1992; Wu *et al*., 1994; Kuntz-Simon *et al*., 2002a). The species *Avian orthoreovirus* along with the species *Mammalian* (MRV), *Baboon* (BRV), *Nelson Bay* (NBV) and *Reptilian orthoreovirus* comprise the genus *Orthoreovirus* within the family *Reoviridae*. DRVs have been classified as duck isolates belonging to the species *Avian orthoreovirus* (Faquet *et al*., 2005).

DRV is an important poultry pathogen involved in the several diseases including viral arthritis/tenosynovitis, growth retardation, pericarditis, myocarditis, enteritis, hepatitis, bursa and thymic atrophy, osteoporosis, respiratory syndromes, and sudden death (Gaudry *et al*., 1972; Malkinson *et al*., 1981; Rosenberger and Olson, 1991; McNulty, 1993; Hollmen *et al*., 2002; Kuntz-Simon *et al*., 2002b). DRV could cause high morbidity and up to 50% mortality in young ducks (Malkinson *et al*., 1981; Heffels-Redmann *et al*., 1992) and dead birds show macroscopically fibrinous pericarditis, friable liver and marbled spleen (Gaudry *et al*., 1972; Menendez *et al*., 1975; Malkinson *et al*., 1981; Marius-Jestin *et al*., 1988; Palya *et al*., 2003). The

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Abbreviations: DRV(s) = duck orthoreovirus(es); MRV = Mammalian orthoreovirus; ARV(s) = Avian orthoreovirus(es); NBV = Nelson Bay orthoreovirus; BRV = Baboon orthoreovirus; TRV = Turkey orthoreovirus; GRV = Goose orthoreovirus

DRV strains are antigenically different from other ARV strains (Heffels-Redmann *et al*., 1992; Hollmen *et al*., 2002; Kuntz-Simon *et al*., 2002a,b) and as a result, the vaccination of ducks with ARV vaccine yields poor results (Marius, 1983).

Recently, S1 and S3 gene segments of turkey isolate of Avian orthoreovirus (TRV) (Kapczynski *et al*., 2002; Sellers *et al*., 2004; Day *et al*., 2007), σ1/σC and σA genes of goose isolate of Avian orthoreovirus (GRV) (Banyai *et al*., 2005; Zhang *et al*., 2006a), and M and S-class gene segments of 17 other ARVs were described. However, S-class gene segments were reported only for DRV strain S14 M (Zhang *et al*., 2007) and two French strains DRV 89026 and DRV 89330 (Kuntz-Simon *et al*., 2002a).

The aim of this study was to present the new sequence information for the S-class (σA, σB, σNS, and σC genes) genome segments of DRV strain S12, to examine the sequence diversity among DRVs and other orthoreoviruses and to extend our general knowledge about this genus. Based on the paired identities between homologous proteins of DRV isolates and other orthoreoviruses, we suggested that the species *Avian orthoreovirus* comprise highly divergent viruses.

Materials and Methods

Viruses. DRV S12 was isolated from the liver of dead Muscovy duck with enteritis, hepatitis, and movement difficulties. This strain was maintained by cultivation in the Muscovy duck embryonic fibroblast (DEF) monolayers or chorioallantoic chamber of Muscovy duck eggs (Zhang *et al*., 2006a). ARV S1133 was obtained from the Institute of Veterinary Drug Control, China.

SDS-PAGE. Viral RNA was purified from the infected DEF cells or the allantoic fluid of infected eggs using Trizol Reagent (BRL-Life Technologies). The samples of viral dsRNA were boiled for 2 mins prior to loading on 10% polyacrylamide gel. After the SDS-PAGE the gels were stained with silver staining kit (Invitrogen).

RT-PCR. The primers used for amplification of the S-class genome segments are listed in Table 1. Purified dsRNA was used to generate cDNA clones by RT-PCR. The sequences and locations of primers are shown in Table 1. In the RT-PCR test, 1 μg of purified dsRNA was denatured in boiling water for 10 mins, chilled on ice for 5 mins, and then used as a template. The one-tube RT-PCR was performed according to the procedures provided by the TaKaRa PCR Thermal Cycler (TaKaRa, Japan). Reverse transcription step was carried out at 42°C for 30 mins. PCR reactions were subjected to 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, extension for 2 mins at 72°C, and one final extension cycle at 72°C for 7 mins.

cDNA cloning and sequencing. Purified PCR products were cloned into plasmid pMD18-T vector (Invitrogen) that was used for transformation of *Escherichia coli* BL21 (DE3) according to the manufacturer's recommendation. Recombinant plasmid DNA was purified by using a Qiagen plasmid Miniprep kit (Valencia). The universal forward and reverse primers M13 were used to complete sequencing with Automated Laser Fluorescence DNA Sequencer (ABI).

Sequence analysis. Nucleotide and deduced aa sequences of the DRV S-class genome segments were analyzed using the DNASTAR software. This software was also used for alignment and comparison with the available nt and aa sequences with σB, σA, σNS, and σC genes of other reoviruses genes from GenBank (including ARV, DRV, GRV, TRV, BRV, NBV, and MRV1-3). A phylogenetic tree was constructed by using the parsimony program PAUP (Swofford, 1991). The accession numbers for each orthoreovirus were:

ARV S1133: σC(L39002), σA(AF104311), σB(U20642), σNS(U95952);
ARV 138: σC(AF218359), σA(059717), σB(AF059721), σNS(AF059725);
ARV 176: σC(AF218358), σA(AF059716), σB(AF059720), σNS(AF059724);
ARV 916: σC(AF297214), σA(AF294764), σB(AY008383), σNS(AF294774);
ARV 918: σC(AF297215), σA(AF294766), σB(AF301373), σNS(AF294775);
ARV R2/TW: σC(AF297213), σA(AF294765), σB(AF301472), σNS(AF294778);
ARV RAM-1: σC(L38502);
TRV TX98: σ2(AY444911), NC98 σ2(AF465799);
GRV D20/99: σC(AJ717737);
NBV: σC(AF218360), σ1(AF059718), σNS(AF059726), σ2(AF059722);

Table 1. Primers used for PCR amplification of DRV S12 S-class genome segments

Primer sequence (5'-3')	Target gene	Position (nt)	Amplicon size (bp)
pA: GCTTTTTCTCCCACGATGGCGCGTG	σA	1–25	1302
pAR: GATGAATACACCCACGCGCTACCGGAG		1298–1324	
pB: CTCGGGAATCGCACGTTCGGCGT	σB	8–30	1100
pBR: CGTGCTTCTTGACCCGGTCCACG		1135–1157	
pNS: GCTTTTTGAGTCCTTGTGCAG	σNS	1–21	1151
pNSR: GATGAGTAAGAGTCCAAGTCG		1171–1191	
pC: GCCTGGAACGAATACCTTCACCATG	σC	255–279	820
pCR: GCCGAGCTCCCTCAAATGGTCGCAATGGA		1072–1100	

BRV: σ1(AF059719), σ2(AF059723), σNS(AF059727);
MRV1 T1L: σ1(NC004267), σ2(NC004268), σNS(NC004266), σ3(NC004265);
MRV2 T2J: σ1(NC004264), σ2(NC004263), σNS(NC004269), σ3(NC004273);
MRV3 T3D: σ1(NC004277), σ2(NC004279), σNS(NC004283), σ3(NC004276);
DRV 89026: σA(AJ278102), σB(AJ006476), σNS(AJ133122), σC(AJ310525);
DRV 89330: σB(AJ243881);
DRV S12: σA(EF076764), σB(DQ643971), σNS(DQ325536), σC(DQ643970).

Results

SDS-PAGE analysis

The migration pattern of DRV S12 and ARV S1133 genomic dsRNA were analyzed by SDS-PAGE (Fig. 1). The electrophoresis revealed that DRV S12 contained a genome composed of 10 segments of dsRNA that were separated into three size classes with three large (L), three medium (M), and four small (S) segments. DRV S12 and ARV S1133 L and M gene segments migrated to similar positions in the gel, while S-class segments differed evidently in their electrophoretic migration. The S1 gene segment of DRV S12 migrated more closely to the other S-class genome segments, while the S1 segment of ARV S1133 migrated closer to the M-class genome segments (Fig. 1).

Sequence analysis of σC, σB, σNS, and σA gene segments

Examination of the aa sequence derived from the σC gene segment of DRV S12 isolate revealed the presence of a heptapeptide repeat and a leucine zipper pattern (Fig. 2), as

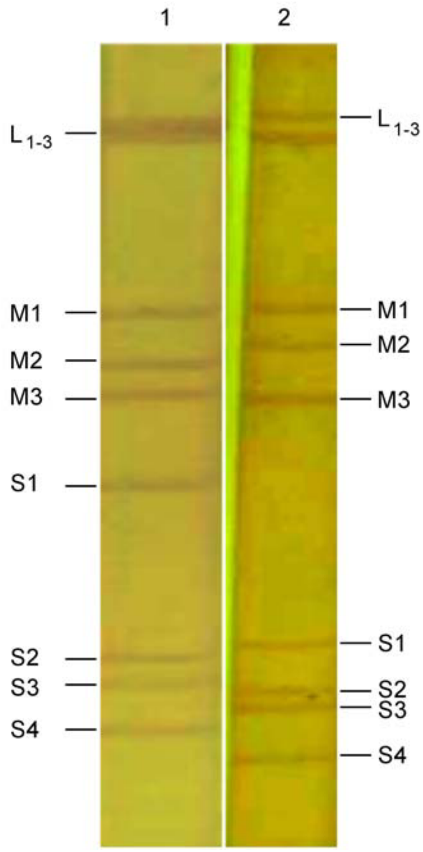

Fig. 1

SDS-PAGE of purified dsRNA of ARV S1133 (lane 1) and DRV S12 (lane 2)

The designations of the segments are on both sides. Large (L1-3), middle (M1-3), and small (S1-4) gene segments.

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>                                                                                 a..d...a..d...a..d...a..d...
S12     MSETPAP------------------------PGYSVPSCSPGSRDLTRSDVLALILSFNPVSRSDVEDLSRRLSSLQSSYDALFEEVLRLRVRMDMIYN   75
89026   MSGTPAP------------------------PGYSVPSCSPGSRGLTRSDVLALILSFNPVSRSDVEDLSRRLSSLQSSYDALFEEVLRLRVRMDMMYN   75
GRV     MSGTLAP------------------------PGCSVPFCSPGSRGLTRSDVLALILSFNPVSRSDVEDLSRRLSLLQSSYDALFEGVLRLRVRVDMIYN   75
S1133   MAGLNPSQRREVVSLILSLTSNVNISHGDLTPIYERLTNLEASTELLHRSISDISTTVSNIS-ANLQDMTHTLDDVTANLDGLRTTVTALQDSVSILST   98

S12     SLSK-----------------------------------------SLSDLGDRVAKLERETSHIKTVRAPLHVTDGELAILAKPSFCTLDPVLSGTSLNAVF  136
89026   SFSD-----------------------------------------SLSDLGDRVAKLEREASYIKTVRAPLHVTDGELALLAKPSFCTLDPVLSGTSLNAVF  136
GRV     SFSE-----------------------------------------SLSGLGDRVTKLERGVSLIKTVRAPLHVTDGELTLLAKPSFCTLDPVLSGTSLNAVF  136
S1133   NVTDLTNRSSAHAAILSSLQTTVDGNSTAISNLKSDISSNGLAITDLQDRVKSLESTASHGLSFSPPLSVADGVVSLDMDPYFCSQRVSLTSYSAEAQL  197

S12     ATGSWLAKIEAGASIASFILNLRFHHFGQRTTLFLSSPNVVTVFPGSSVYLVLDTSRLIDPTYDTSPLTPSPAFAANMFMVDMVFDDASTKESFSLSTT  235
89026   ATGSWLAKIEAGVSVASFVLNMRFHHFGQRTTLFLSSPNVVTVFPGSSVYLVLDTSRLIDPTYDTALLTPSPAFAANMFMVDMVFDDASTKESFSLSTT  235
GRV     ATGSWLAKIEAGVSIASFVLNLRFHHFGQRTTLFLSSPNVVTVFPGSSVYLVLDASRLIDPTYDTSLLTPSPAFAANMFMVDMVFDEASTKESFSLSTT  235
S1133   MQFRWMARGTNGSS-DTIDMTVNAHCHGRRTDYMMSSTGNLTV-TSNVVLLTFDLSDITHIPSDLARLVPSAGFQAASFPVDVSFTRDSATHAYQ--AY  292
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Fig. 2

Deduced aa sequences of the σC proteins for DRV S12 (S12), DRV 89026 (89026), GRV D20/99 (GRV), and ARV S1133 (S 1133)

The numbers of aa positions are on the right. Solid line above the first row indicated the leucine zipper patterns. Amino acids marked (a) or (d) indicated the first and fourth position of the heptapeptide pattern. The solid-line box indicated the putative zinc finger domain.

Table 2. Identities of homologous σA, σB, σNS, and σC genes of some orthoreoviruses in %

	S12	89026	GRV	S1133	R2-TW	916	918	NBV	BRV	T1L
S12	97,94,96,94	–,–,–,91	89,61,92,23	91,62,92,23	91,62,92,26	90,62,92,25	60,32,50,19	29,18,27,–	28,–,23,14	
89026	90,94,88,93		–,–,–,91	88,61,90,23	90,61,89,22	89,61,89,26	89,61,89,24	60,32,50,20	28,16,27,–	28,–,23,16
GRV	–,–,–,90	–,–,–,91		–,–,–,2	–,–,–,21	–,–,–,27	–,–,–,26	–,–,–,8	–,–,–,–	–,–,–,14
S1133	76,60,79,5	76,64,77,3	–,–,–,3		96,97,94,75	96,96,94,58	98,96,94,75	60,35,50,22	30,19,28,–	29,–,23,11
R2-TW	76,64,79,3	76,64,79,3	–,–,–,3	88,90,80,77		99,98,98,58	99,98,98,49	60,35,50,22	29,18,28,–	29,–,23,10
916	77,63,79,3	76,64,78,3	–,–,–,4	87,91,80,62	94,97,97,63		97,98,98,51	60,35,50,26	29,18,28,–	29,–,23,14
918	77,60,78,10	76,64,78,3	–,–,–,3	97,91,81,60	90,97,97,61	89,97,96,62		60,35,49,22	29,19,28,–	28,–,23,8
NBV	60,18,50,5	58,19,51,4	–,–,–,4	62,19,57,6	62,19,57,6	62,19,56,7	62,19,57,12		32,15,27,–	30,–,23,8
BRV	3,6,–,5	3,6,3,–	–,–,–,–	4,7,4,–	3,6,9,–	5,6,19,–	4,7,9,–			28,–,20,–
T1L	12,–,6,2	3,–,3,2	–,–,–,2	2,–,6,2	2,–,2,5	3,–,2,2	9,–,2,4	7,–,8,2	8,–,3,–	

(–) = sequences not available. Bottom left and top right numbers indicate nt and deduced aa identities, respectively. Each pairwise comparison includes values for σA, σB, σNS, and σC genes.

described in detail for DRV and ARV (Liu *et al*., 1997; Kuntz-Simon *et al*., 2002a; Zhang *et al*., 2006b). The leucine zipper pattern is indicated between aa 49 and 66. The heptapeptide pattern was found in the putative α-helical coiled-coil motifs predicted by the MultiCoil program. Alignment of aa sequences of σC proteins revealed insertion of two large gaps at the N-terminal part of the DRV S12, DRV 89026 and GRV. Sequence analysis revealed that DRV S12 σC gene shared 93.1% and 89.9% identity to DRV 89026 and GRV D20/99 respectively, at the nucleotide level and with 93.7% and 91.0% identity respectively, at the aa level (Table 2). The degree of similarity to ARVs, NBV, and MRV1 T1L were only 21.0–26.7%, 17.8%, and 14–16% at aa level, respectively.

The predicted aa sequence of DRV S12 σB gene comprising 367 aa showed the Mr of 40.4 K, and an isolectric point of 6.67 with a –2.22 charge at pH 7. The size of other ARVs σB was determined previously (Wichramasinghe *et al*., 1993; Le Gall-Reculé *et al*., 1999). The possible secondary structure of DRV S12 σB protein had the following features: 19% of the aa were in the form of α-helices, 29% were in the form of ß-sheets, 33% were within turns, and 19% were in random coils. The N-terminal half of the molecule contained 63% of the predicted helices. Within the S12 σB amino acid sequence, a C-X_2-C-X_{17}-H-X_2-C zinc-binding motif (between aa 55–75) and a motif rich in basic amino acids (^{287}KKVSHYR^{293}) were identified (Fig. 3). The alignments of σB deduced aa sequences of DRV S12, DRV 89026, ARV S1133, and TRV NC98 revealed the most of the conserved aa present at the N-terminus (aa 1–113).

The nucleotide sequence comparison revealed that DRV S12 σB gene showed 93.6% identity to DRV 89026; 58.8–63.8% identity to ARVs; 18.2% similarity to the NBV, and only 4–6% to MRV1 T1L and BRV homologous gene (Table 2). The aa sequence comparison demonstrated that DRV S12 σB shared approximately 60.9–62.5% identity to ARVs, 32.3% to NBV, 17.7% to BRV, and 13.9–15.6% to MRV1 T1L (Table 2).

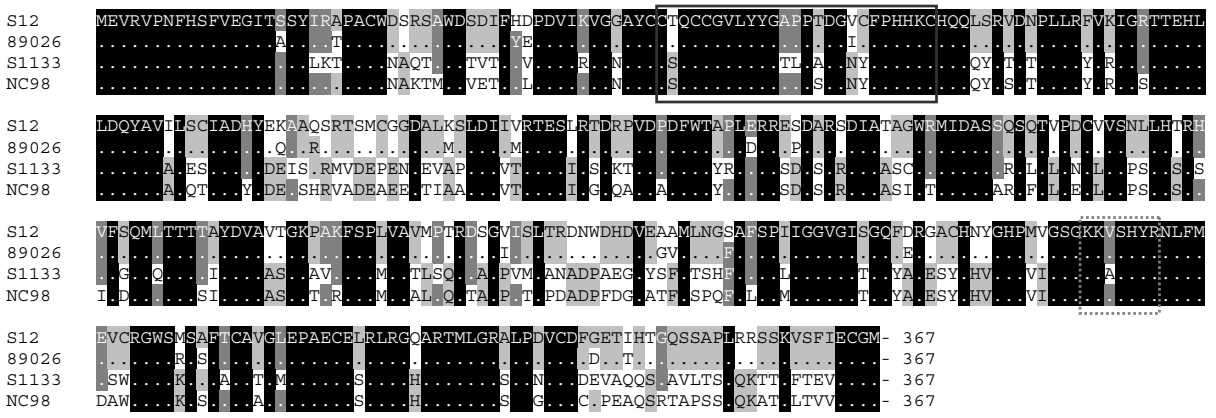

Fig. 3

Deduced aa sequences of the σB proteins for DRV S12 (S12), DRV 89026 (89026), ARV S1133 (S 1133), and TRV NC98 (NC98)

The numbers of aa positions are on the right. The solid-line box indicated the putative zinc finger domain and the dotted-line box indicated the basic aa motif.

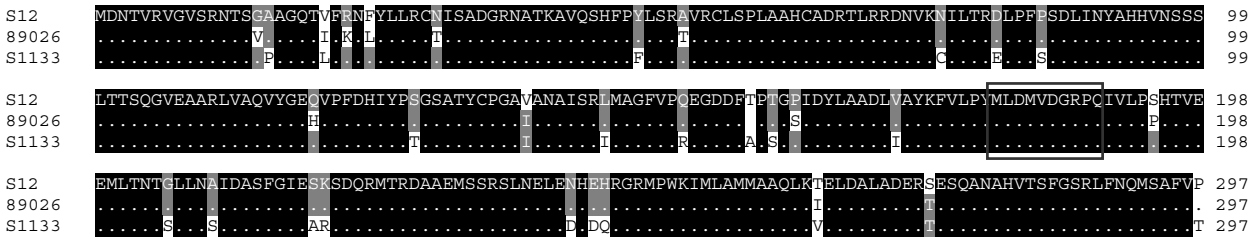

Fig. 4

Deduced aa sequences of the σNS proteins for DRV S12 (S12), DRV 89026 (89026), and ARV S1133 (S 1133)

The numbers of aa positions are on the right. The solid-line box indicated the sequence aa 180–188. Small upper lines indicated the potential glycosylation sites.

DRV S12 σNS gene contains ORF of 368 codons starting with an ATG (at nt 24 to 26) and terminating at nt 1125 to 1127 with a TAG codon and a 3'-untranslated region of 64 nt. Compared to other ARVs, there is one nt deletion at the position 1155. The Mr of DRV S12 σNS protein was estimated to 40.4 K that is consistent with the reported Mr for S1133 σNS of 40.6 K (Chiu and Lee, 1997). The possible secondary structure of the σNS protein determined by the Garnier method had the following features: 36% of residues were in the form of α-helices, 26% in the form of ß-sheets, 17% within turns, and 22% in random coils. The α-helices were not uniformly distributed (data not shown) and about 60% of them were in the aa 197–313 region, as previously reported for σNS of MRV and ARVs (Chiu and Lee, 1997; Wiener and Joklik, 1987). The sequence of the σNS protein of DRV S12 was shown as a whole in Fig. 4. Boxed amino acids residues ^{180}MLDMVDGRP^{188} are required for ssRNA binding. The protein contains four potential glycosylation sites (at aa 12–14, 31–33, 39–41, and 96–98) (Fig. 4). The strains DRV S12 and ARV S1133 σNS proteins had the same aa sequence ^{180}MLDMVDGRP^{188} representing the epitope required for ssRNA binding (Yin *et al*., 1998; Huang *et al*., 2005). The DRV S12 σNS protein contained 38 acidic, 37 basic, 25 aromatic, and 131 hydrophobic amino acids. This protein is relatively rich in methionine (16 residues) and proline (17 residues), but relatively poor in cysteine (3 residues).

The σNS gene of DRV S12 showed 87.9% identity to DRV 89026, and 78.4–79.6% identity to ARVs at the nt level. The sequence identities at the predicted aa level were 95.9% to DRV 89026 and 91.6–92.7% to ARVs. The aa sequence of DRV S12 σNS showed 49.8% identity to the NBV counterpart. Sequence comparison of σNS protein with MRV1 T1L and BRV homologues revealed extensive sequence divergence, especially at the aa level 23.2–23.4% and 27.4%, respectively (Table 2).

The σA gene contains a long ORF and its start codon has a strong context (ACGATGG) for initiation with adenine at position –3 and guanine at position +4 (Kozak, 1986). Thus, it is sufficient to encode a protein of 415 aa with Mr about 46.1 K and an isolectric point of 8.3. The sequence of the DRV S12 σA is shown its entirety in Fig. 5 and only the

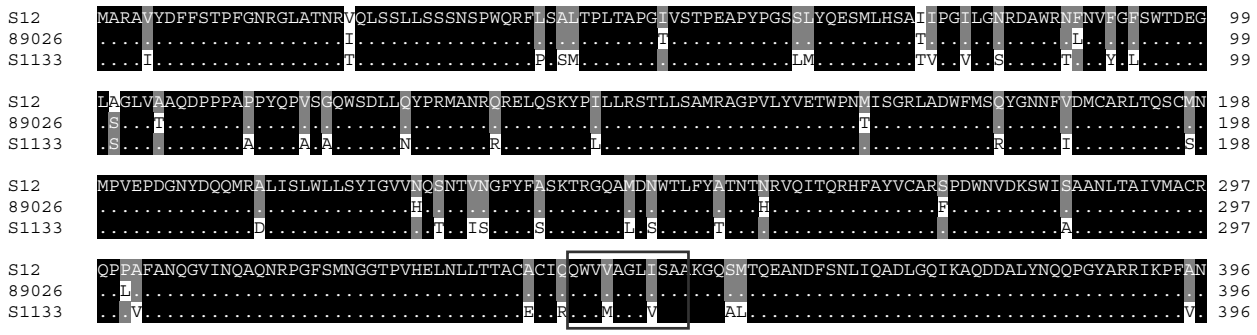

Fig. 5

Deduced aa sequences of the σA proteins for DRV S12 (S12), DRV 89026 (89026), and ARV S1133 (S 1133)

The numbers of aa positions are on the right. The box indicated the sequence aa 340–350.

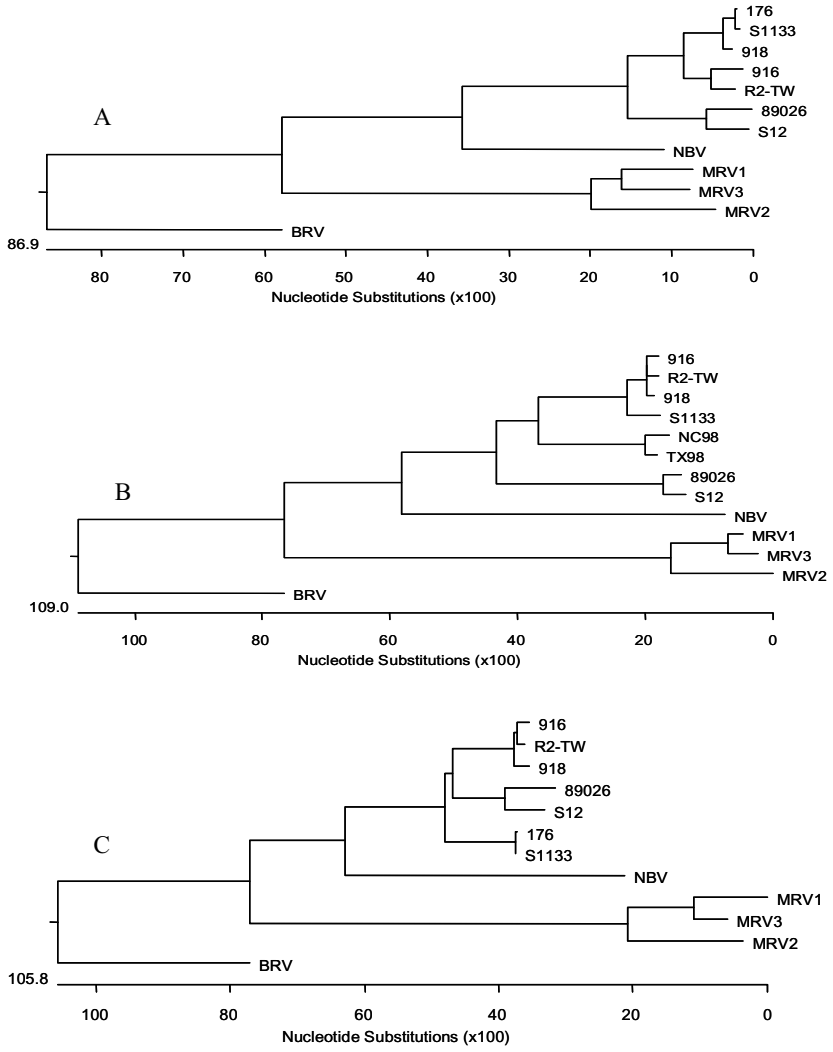

Fig. 6

Phylogenetic trees of orthoreoviruses based on the nucleotide sequences of their σA (A), σB (B), and σNS (C) genes

The codes for analyzed viruses are listed in *Materials and Methods*.

mismatches are indicated. The σA protein possible secondary structure determined by the Garnier method had the following features: 14% of aa were in the form of α-helices, 34% in the form of ß-sheets, 24% within turns, and 28% in random coils. The regions containing α-helix were not uniformly distributed in the molecule. About 60% of these regions were located close to the C-terminus (Nibert *et al*., 1990) and in the small region at the extreme C-terminus with a high α-helix score (Yin *et al*., 2000). Although σA protein has three glycosylation sites predicted, it has been shown that it is not a glycoprotein (Varela *et al*., 1996). Three-fourth of the protein consisted mainly of ß-turns and ß-strands that were similar to the three isolates of MRV (T1L, T2J, and T3D) (Dermody *et al*., 1991) and other ARVs σA that possessed dsRNA binding activity (Yin *et al*., 2000; Martinez-Costas *et al*., 2000). The sequence analysis showed that DRV S12 σA protein at the epitope II region (^{340}QWVVAGLISAA^{350}) (Huang *et al*., 2005) contains two aa substitutions in position 343 (from methionine to valine) and position 347 (from valine to threonine) in comparison with σA protein of ARV S1133 (Fig. 5).

The nucleotide sequence comparison revealed that DRV S12 σA gene showed 90.0 % identity to DRV 89026, 76.0–77.1% identity to ARVs respectively, 60.1% identity to the NBV, only 5–12% identity to MRV1 T1L, and 3% to BRV homologous gene (Table 2). The aa sequence comparison

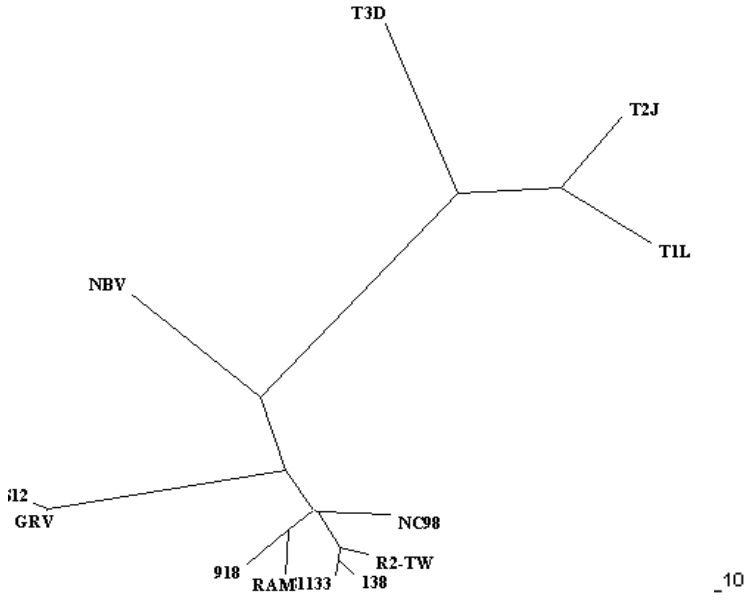

Fig. 7

Phylogenetic tree of orthoreoviruses based on the nucleotide sequence of their σC genes

The codes for analyzed viruses are listed in *Materials and Methods*.

demonstrated that DRV S12 σA protein shared approximately 97.1% identity to DRV 89026, 89.5–91.2% to ARVs, 32.3% identity to NBV, 17.7% to BRV, and 13.9–15.6% to MRV1 T1L homologous protein (Table 2).

Phylogenetic analysis

The evolutionary relationship between DRVs and the other orthoreoviruses was determined by phylogenetic analysis of σA, σB, σNS, and σC genes (Fig. 6 and Fig. 7). The established σA, σB, and σNS trees confirmed the classification of DRVs into the defined species *Avian orthoreovirus* (Faquet *et al.,* 2005). The DRVs exhibited the highest degree of variability in their σC proteins with only 23% to 27% amino acid identities, which lent support to the previous observation that the DRVs were unique among the ARVs (Kuntz-Simon *et al*., 2002).

Discussion

The migration patterns of genomic dsRNA segments of DRV S12 in SDS-PAGE showed that 10 dsRNA segments were separated into three size classes: large (L1-3), medium (M1-3) and small (S1-4). This separation was consistent with the other members of the genus *Orthoreovirus* (Hrdy *et al*., 1979; Wu *et al*., 1994). The S-class segments of the strains ARV S1133 and DRV S12 reflected evidently the difference in the electrophoretic migration of segments. Indeed, there was no DRV S12 segment found at the ARV S1133 S1 segment position, what was consistent with previous reports for other DRV and GRV strains (Kuntz-Simon *et al*., 2002a; Palya *et al*., 2003), but different from report of Heffels-Redmann *et al*. (1992). The differences could be due to the different strain of DRV used and different SDS-PAGE experimental conditions.

Amino acid sequence analysis showed that the DRV S12 σB had a CCHC zinc finger motif in the N-terminal region that was in agreement with the recent investigations of DRVs, TRVs, and MRVs (Mabrouk and Lemay, 1994; Schiff *et al*., 1998; Le Gall-Recule *et al*., 1999; Kapczynski *et al*., 2002). There were two repeated basic amino acid motifs conserved in all three serotype MRV1-3 that were assumed to be involved in the binding to nucleic acids (Berg, 1986; Mabrouk and Lemay, 1994). DRV S12 σB protein has only one basic stretch (^{287}KKVSHYR^{293}) as well as the published ARV S1133 sequence (^{287}KKASHYR^{293}), suggesting that the mentioned basic stretch might be required for dsRNA-binding activity (Wang *et al*., 1996).

By comparing DRVs and ARVs σC amino acid sequences, the DRVs σC had a structure related to that of ARVs σC (Shapouri *et al*., 1995) and MRV σ1(Nibert *et al*., 1990; Fraser *et al*., 1990). Kuntz-Simon *et al*. (2002b) have demonstrated that baculovirus-expressed σC could induce serum-

neutralizing antibodies as described previously for σC of ARVs (Schnitzer *et al*., 1982; Wichramasinghe *et al*., 1993; Theophilos *et al*., 1995).

The identity of σB and σC protein for DRV S12 and ARVs were only 60.9–62.5% and 21.5–25.7%, respectively at the aa level, suggesting that DRV is a distinct virus, but related to ARVs. This result agreed with previous serological data showing that DRV was antigenically different from ARVs (Heffels-Redmann *et al*., 1992; Kuntz-Simon *et al*., 2002a,b; Hollmen *et al*., 2002). Based on the group and type-specific epitopes, the genes σB and σC might have been subjected to an initial increased rate of evolution under strong immunological selection. Comparison of the DRV S12 σB and σC protein sequences with the σ3 and σ1 of the MRV T1L revealed 14–15% identity that were of the same order of magnitude as those between ARVs and MRVs (11–14%).

Sequence of the σNS of DRV S12 showed the ^{180}MLDMVDGRP^{188} sequence, which is identical to the ARVs epitope B suggesting that DRV and ARVs might share the same mechanism in ssRNA binding activity (Yin *et al*., 1998; Huang *et al*., 2005). The secondary structure of S12 σA was very similar to that of ARVs σA and MRVs σ2 indicating that the σA gene might have similar function in mentioned strains. The region related to the epitope II on the S12 σA showed sequence variation only at the positions 343 and 347 suggesting that these 2 aa substitutions did not affect the reactivity of epitope II and therefore were not critical for antibody binding.

The 10% and 12% diversity of σA and σNS genes between the Chinese strain DRV S12 and French strain DRV 89026 suggested that they have been evolving separately in different continents and consequently, they represented distinct lineages.

The phylogenetic tree of σA, σB, and σNS proteins had similar topology, suggesting that DRVs evolved independently as indicated by Kuntz-Simon *et al*. (2002a). Phylogenetic analysis of σC gene revealed that the DRV, GRV, ARV, NBV, and MRV belonged to different groups with a distinct ancestral origin. We may speculate that DRVs, GRVs, TRVs, and ARVs of chicken origin could have evolved uniquely in fowls. The high sequence homology between DRVs and GRVs (Palya *et al*., 2003; Banyai *et al*., 2005; Zhang *et al*., 2006b) suggested that they could be the same species of virus, as indicated by Zhang *et al*. (2006a).

The cross-species transmissions between mammalian species occur in nature, so it may still be possible that the reassortment occurs between DRV and other ARVs, despite there is no evidence for naturally occurring reassortment. Further molecular studies dealing with orthoreovirus strains isolated from different avian species could clarify more deeply the relationships between different isolates within the species *Avian orthoreovirus.*

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References

Bányai K, Palya V, Benkő M, Bene J, Havasi V, Melegh B, Szűcs G (2005): The goose reovirus genome segment encoding the minor outer capsid protein, σ1/σC, is bicistronic and shares structural similarities with its counterpart in Muscovy duck reovirus. *Virus Genes* **31**, 285–291.

Berg JM (1986): Potential metal-binding domains in nucleic acid binding proteins. *Science* **232**, 485–487.

Chiu CJ, Lee LH (1997): Cloning and nucleotide sequencing of the S4 genome segment of avian reovirus S1133. *Arch. Virol*. **142**, 2515–2520.

Day JM, Pantin-Jackwood MJ, Spackman E (2007): Sequence and phylogenetic analysis of the S1 genome segment of turkey-origin reoviruses. *Virus Genes* (in press).

Dermody TS, Schiff LA, Nibert ML, Coombs KM, Fields BN (1991): The S2 gene nucleotide sequences of prototypes strains of the three reovirus serotypes: characterization of reovirus core protein sigma 2. *J. Virol.* **65**, 5721–5731.

Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (Ed.) (2005): *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*. Elsevier-Academic Press, Amsterdam.

Gaudry D, Charles J, Tektoff J (1972): A new disease expressing itself by a viral pericarditis in Barbary ducks. *CR Acad. Sci*. **274**, 2916–2919.

Gouvea V, Schnitzer TJ (1982): polymorphism of the migration of double-stranded RNA genome segments of avian reoviruses. *J. Virol*. **43**, 465–471.

Heffels-Redmann U, Müller H, Kaleta EF (1992): Sreuctural and biological characteristics of reoviruses isolated from Muscovy ducks (Cairina moschata). *Avian Pathol*. **21**, 481–491.

Hollmén T, Franson JC, Kilpi M, Docherty DE, Hansen WR, Hario M (2002): Isolation and characterization of a reovirus from common eiders (Somateria mollissima) from Finland. *Avian Dis*. **46**, 478–484.

Hrdy DB, Rosen L, Fields BN (1979): Polymorphism of the migration of double-stranded RNA segments of reovirus isolates from humans, cattle, and mice. *J. Virol*. **31**, 104–111.

Huang PH, Li YJ, Su YP, Lee LH, Liu HJ (2005): Epitope mapping and functional analysis of sigma A and sigma NS proteins of avian reovirus. *Virology* **332**, 584–595.

Joklik WK (1983): The reovirus particle. In Joklik WK (Eds): *The Reoviridae*. Plenum Press, New York, pp. 9–78.

Kapczynski DR, Sellers HS, Simmons V, Schultz-Cherry S (2002): Sequence analysis of the S3 gene from a turkey reovirus. *Virus Genes* **25**, 95–100.

Kozak M (1986): Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosome. *Nucleic Acids Res*. **9**, 5233–5252.

Kuntz-Simon G, Le Gall-Reculé G, De Boisséson C, Jestin V (2002a): Muscovy duck reovirus σC protein is atypically encoded by the smallest genome segment. *J. Gen. Virol*. **83**, 1189–1200.

Kuntz-Simon G, Blanchard P, Cherbonnel M, Jestin A, Jestin V (2002b): Baculovirus-expressed muscovy duck

reovirus σC protein induces serum neutralizing antibodies and protection against challenge. *Vaccine* **20**, 3113–3122.

Le Gall-Reculé G, Cherbonnel M, Claire A, Blanchard P, Jestin A, Jestin V (1999): Molecular characterization and expression of the S3 gene of muscovy duck reovirus strain 89026. *J. Gen. Virol*. **80**, 195–203.

Liu HJ, Giambrone JJ (1997): Amplification, cloning and sequencing of the σC-encoded gene of avian reovirus. *J. Virol. Methods* **63**, 203–208.

Mabrouk T, Leman G (1994): Mutations in a CCHC zinc-binding motif of the reovirus sigma 3 protein decrease its intracellular stability. *J. Virol*. **68**, 5787–5790.

Malkinson M, Perk K, Weisman Y (1981): Reovirus infection of young muscovy ducks (Cairina moschata). *Avian Pathol*. **10**, 433–440.

Marius V (1983): Prévention de la réovirose du canard de Barbarie a íaide ďun vaccine a virus inactive mis au point au laboratoire natonal de pathologie aviaire de Ploufrgan. *Áviculteur* **437**, 65–67.

Marius-Jestin V, Lagadic M, Le Menec Y, Bennejean G (1988): Histological data associated with muscovy duck reovirus infection. *Vet. Rec*. **123**, 32–33.

Matinez-Costas J, Gonzalez-lepez C, Vakharia VN, Benavente J (2000): possible involvement of the double-stranded RNA-binding core protein σA in the resistance of avian reovirus to interferon. *J. Virol*. **74**, 1124–1131.

McNulty MS (1993): Reovirus. In McFerran JB, McNulty MS (Eds): *Virus Infections of Birds*. Elsevier Science Publishers, Netherlands, pp. 181–193.

Menendez NA, Calnek BW, Cowen BS (1975): Localization of avian reoviruses (FDO isolate) in tissues of mature chickens. *Avian Dis*. **19**, 112–117.

Nibert ML, Dermody TS, Fields BN (1990): Structure of the reovirus cell-attachment protein: A model for domain organization of σ1. *J. Virol*. **64**, 2976–2989.

Palya V, Glávits R, Dobos-Kovács M, Ivanics BE, Nagy E, Bányai K, Teuter G, Szűcs G, Dán Á, Benkő M (2003): Reovirus identified as cause of disease in young geese. *Avian Pathol.* **32**, 129138,

Rosenberger JK, Olson NO (1991): Reovirus infections. In Canlnek BW, Barnes HJ, Beard CW, Reid WM, Yoder Jr HW (Eds): *Diseases of Poultry*. Iowa State University Press, Ames Iowa, pp. 639–647.

Schiff LA, Nibert M, Co MS, Brown EG, Fields BN (1988): Distinct binding sites for zinc and double-stranded RNA in the reovirus outer capsid protein σ3. *Mol. Cell. Biol*. **8**, 273–283.

Schnitzer TJ, Ramos T, Gouvea V (1982): Avian reovirus polypeptides: analysis of intracellular virus-specified products, virions, top component, and cores. *J. Virol*. **43**, 1006–1014.

Sellers HS, Linnemann EG, Pereira L, Kapczynski DR (2004) Phylogenetic analysis of the sigma 2 protein gene of turkey reoviruses. *Avian Dis*. **48**, 651–657.

Spandidos DA, Gramham AF (1976): Physical and chemical characterization of an avian reovirus. *J. Virol*. **19**, 968–976.

Swofford DL (1991): *Phylogenetic Analysis Using Parimony*. Computer Program Distributed by Illinois National History Survey. Champagne, IL, USA.

Theophilos MB, Huang JA, Holmes IH (1995): Avian reovirus σC protein contains a putative fusion sequence and induces fusion when expressed in mammalian cells. *Virology* **208**, 678–684.

Varela R, Martinez-Costas J, Mallo M, Benavente J (1996): Intracellular posttranslational modifications of S1133 avian reovirus proteins. *J. Virol*. **70**, 2974–2981.

Wang Q, Bergeron J, Mabrouk T, Lemay G (1996): Site-directed mutagenesis of the double-stranded RNA binding domain of bacterially-expressed sigma 3 reovirus protein. *Virus Res*. **41**, 141–151.

Wiener JR, Joklik WK (1987): Comparison of the reovirus serotype 1, 2, and 3 S3 genome segments encoding the non-structural protein σNS. *Virology* **161**, 332–339.

Wu YW, Shien JH, Lee LH, Shieh HK (1994): Analysis of the double-stranded RNA genome segments among avian reovirus field isolates. *J. Virol. Methods* **48**, 119–122.

Wickramasinghe R, Meanger J, Enriquez CE, Wilcox GE, (1993): Avian reovirus proteins associated with neutralization of virus infectivity. *Virology* **194**, 688–696.

Yin HS, Lee LH (1998): Identification and characterization of RNA-binding activities of avian reovirus no-structural protein sigma NS. *J. Gen. Virol*. **79**, 1411–1413.

Yin HS, Shieh HK, Lee LH (2000): Synthesis in Escherichia coli of avian reovirus core protein σA and its dsRNA-binding activity. *Virology* **266**, 33–41.

Zhang Y, Liu M, Ouyang S, Hu Q, Guo DC, Han Z (2006a): Detection and Identification of Avian, Duck, and Goose Reoviruses by RT-PCR: Goose and Duck Reoviruses Aggregated the Same Specified Genogroup in Orthoreovirus Genus II. *Arch Virol*. **151**, 1525–1538.

Zhang Y, Liu M, Hu Q, Ouyang S (2006b): Characterization of the σC-encoding gene from Muscovy duck reovirus. *Virus Genes* **36**, 165–170.

Zhang Y, Guo DC, Geng HW, Liu M, Hu Q, Wang J, Tong G, Kong XG, Liu C (2007): Characterization of M-class genome segments of muscovy duck reovirus S14. *Virus Res*. **125**, 42–53.