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**Original article**

# Classification of Dutch and German avian reoviruses by sequencing the σ C protein

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**Abstract** – We have amplified, cloned and sequenced (part of) the open reading frame of the S1 segment encoding the σ C protein of avian reoviruses isolated from chickens with different disease conditions in Germany and The Netherlands during 1980 up to 2000. These avian reoviruses were analysed phylogenetically and compared with sequences of avian reoviruses in the Genbank database. The avian reoviruses could be grouped in 5 different genotyping clusters and this classification was identical when the sequences were compared of the 5' end, the 3' end or the whole open reading frame of the σ C protein. Therefore sequencing of either part of the gene encoding the σ C protein seems to be reliable for classification. We were unable to identify a correlation between σ C sequences of the avian reoviruses and the disease condition they were isolated from. The sequences found in The Netherlands and in Germany are, like those in Taiwan, more dispersed than the known avian reovirus σ C sequences in the USA and Australia. We did not establish temporal or geographic differences in the avian reoviruses studied.

**avian reovirus / malabsorption syndrome / tenosynovitis / σ C gene / genotype**

## 1. INTRODUCTION

Avian reoviruses (ARV) are important poultry pathogens. However they show a wide heterogeneity in pathogenicity. ARVs have been isolated from chickens without any clinical signs of disease, but they are also associated with disease conditions such as tenosynovitis and malabsorption syndrome (MAS) [3, 4, 10, 16, 17]. Although the relationship between reovirus and tenosynovitis is well-established [16], a causative role is less clear in MAS.

ARVs belong to the genus Orthoreoviruses. They are icosahedral viruses with a dsRNA genome consisting of ten segments, separable into large (L), medium (M), and small (S) classes [13]. The σ C protein is the minor outer-capsid protein and is encoded by the largest open reading frame (ORF) of the S1 segment. Studies with reassortant ARVs showed that the S1

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segment is involved in both viral replication and pathogenesis [1, 8]. The molecular characterisation of ARVs by using PCR and nucleotide sequence analysis of the σC protein has been described [5, 6, 14]. Although only a limited number of sequences of the σ C protein has been reported, these studies indicate the presence of type-specific sequences in the σC protein [5, 6, 14].

The objectives of this study were to assess whether the σ C protein sequence (clustering) of the ARVs and the pathotype are related and whether there are geographic and temporal differences. Also, we compared the grouping in clusters using the sequence data of the whole ORF of the σC protein, the 3' end and the 5' end.

## 2. MATERIALS AND METHODS

### 2.1. Viruses

The viruses used in this study were from different areas in Germany and The Netherlands. They were obtained from Lohmann Animal Health, Cuxhaven, Germany (GE), from the Animal Health Service, Deventer, The Netherlands (NL), or were isolated at our own laboratory (ID-Lelystad Institute for Animal Science and Health, Lelystad, The Netherlands). The viruses were originally isolated from 24 cases of MAS, 11 cases of tenosynovitis or arthritis and from healthy chickens or undefined cases (5 totally). In Table I ARVs are indicated of which the sequence of the S1 segment of the σC gene was submitted by us to Genbank (32 out of 40 strains: 21 from MAS, 7 from tenosynovitis, 4 from healthy or undefined cases). The isolation period was from 1980 up to 2000. All viruses were propagated in embryonated eggs or in primary chicken embryo liver cells (CELC) [8]. Negative controls were non-infected CELC and CELC infected with an avian adenovirus. Two isolates that had shown (by sequencing) to contain more than one type of reovirus (see also 3.3), were cloned by limiting dilution on CELC. Presence of virus was detected by strong cytopathic effect (CPE) in CELC cultures within 24 hours.

### 2.2. Amplification, cloning and sequencing of (part of) the σC gene

RNA was isolated from 200 µL volumes of clarified supernatant from once freeze-thawed CELC using the QIAmp tissue kit (Qiagen, Westburg BV, Leusden, The Netherlands) according to the manufacturer's experienced user protocol for viral nucleic acids. For the RT-PCR, 2 µL RNA was denatured in the presence of 10 pMol forward primer P1 (Tab. II) and 10 pMol reverse primer P4, in a total volume of 9 µL, by heating for 3 min at 100 °C followed by rapid cooling on ice. Subsequently, 11 µL RT-mixture (containing 4 µL 5× first strand buffer (Life Technologies, Breda, The Netherlands), 2 µL 0.1 M DTT, 1 µL Superscript reverse transcriptase I (Life Technologies), 1 µL RNasin (Promega Benelux BV, Leiden, The Netherlands), 1 µL 10mM dNTP's and 2 µL DEPC treated H2O was added. The mixture was incubated 10 min at room temperature followed by incubation for 1 h at 42 °C. Five microliters of the RT reaction was used in a PCR reaction with primers P1 and P4. The PCR mix consisted of 2.5 µL 10 µm forward primer P1, 2.5 µL 10 µm reverse primer P4, 5 µL dNTP's, 5 µL PCR-buffer 10× [with 1.5 mM $MgCl_2$], 0.25 µL Taq DNA polymerase (Perkin Elmer, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and 29.75 µL DEPC treated H2O. The PCR consisted of a denaturation step of 2 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 30 s at 58 °C and 2 min at 72 °C. This was followed by a final extension of 10 min at 72 °C.

The PCR fragments (size 1088 bp) were purified using the QIAEX II protocol (Qiagen, Westburg BV) and cloned in pGEM®-T Easy (Promega Benelux BV). The Flexiprep DNA isolation kit

**Table I.** Viruses used in this study and sequences already submitted to Genbank.

| Isolate | Symptoms[a] | Origin | Year of isolation | Genotype cluster[b] | Genbank accession number |
|---|---|---|---|---|---|
| S1133 | Tenosynovitis | USA | 1973 | 1 | L39002; Shapouri et al., 1995[D] [14] |
| 1733 | Tenosynovitis | USA | 1983 | 1 | AF004857; Vakharia et al., 1997[D] |
| 138 | Tenosynovitis | USA | N | 1 | AF218359; Duncan, 2000[D] |
| 176 | Tenosynovitis | USA | N | 1 | AF218358; Duncan, 2000[D] |
| 750505 | Tenosynovitis | Taiwan | 1986 | 1 | AF204950; Liu et al., 2000[D] |
| 601G | Tenosynovitis | Taiwan | 1992 | 1 | AF297217; Liu et al., 2001[D] |
| 601SI | Tenosynovitis | Taiwan | 1992 | 1 | AF204947; Liu et al., 2000[D] |
| R2/TW | Tenosynovitis | Taiwan | 1992 | 1 | AF297213; Liu et al., 2001[D] |
| GEL01 96T | Tenosynovitis | Germany | 1996 | 4 | AF354221[C] |
| GEL03 97T | Tenosynovitis | Germany | 1997 | 4 | AF354222[C] |
| GEL04 97T | Tenosynovitis | Germany | 1997 | 4 | AF354184[A]; AF354200[B] |
| GEL14 98T | Tenosynovitis | Germany | 1998 | 1 | AF354187[A]; AF354203[B] |
| NLI03 92T | Tenosynovitis | Netherlands | 1992 | 1 | AF354216[B] |
| NLA13 96T | Tenosynovitis | Netherlands | 1996 | 4 | AF354228[C] |
| NLA14 96T | Tenosynovitis | Netherlands | 1996 | 2,4 | AF354211[B]; AF354210[B] |
| 2408 | MAS | USA | 1983 | 1 | AF204945; Liu et al., 2000[D] |
| OS-161 | MAS | Japan | 1970 | 1 | AF204946; Liu et al., 2000[D] |
| 916 | MAS | Taiwan | 1992 | 2 | AF297214; Liu et al., 2001[D] |
| 918 | MAS | Taiwan | 1992 | 4 | AF297215; Liu et al., 2001[D] |
| 1017-1 | MAS | Taiwan | 1992 | 4 | AF297216; Liu et al., 2001[D] |
| GEL05 97M | MAS | Germany | 1997 | 4 | AF354223[C] |
| GEL06 97M | MAS | Germany | 1997 | 1 | AF354224[C] |
| GEL07 97M | MAS | Germany | 1997 | 4 | AF354185[A]; AF354201[B] |
| GEL08 97M | MAS | Germany | 1997 | 4 | AF354186[A]; AF354202[B] |
| GEI09 97M | MAS | Germany | 1997 | 1,4,5 | AF354220[C] (genotype cl 1) |
| GEI10 97M | MAS | Germany | 1997 | 1,4,5 | AF354219[C] (genotype cl 5) |
| GEI11 97M | MAS | Germany | 1997 | 4 | AF354182[A]; AF354198[B] |
| GEL12 98M | MAS | Germany | 1998 | 1 | AF354225[C] |
| GEL13 98M | MAS | Germany | 1998 | 2,3 | AF354226[C]; AF354227[C] |
| GEL15 00M | MAS | Germany | 2000 | 5 | AF 354188[A] |
| NLI01 80M | MAS | Netherlands | 1980 | 4 | AF354195[A]; AF354215[B] |
| NLI02 88M | MAS | Netherlands | 1988 | 4 | AF354229[C] |

**Table I.** (Continued)

| Isolate | Symptoms[a] | Origin | Year of isolation | Genotype cluster[b] | Genbank accession number |
|---|---|---|---|---|---|
| NLA07 96M | MAS | Netherlands | 1996 | 4 | AF354189[A] |
| NLA08 96M | MAS | Netherlands | 1996 | 4 | AF354190[A]; AF354206[B] |
| NLA09 96M | MAS | Netherlands | 1996 | 1 | AF354207[B] |
| NLA10 96M | MAS | Netherlands | 1996 | 4 | AF354191[A]; AF354208[B] |
| NLA11 96M | MAS | Netherlands | 1996 | 4 | AF354209[B] |
| NLI12 96M | MAS | Netherlands | 1996 | 4 | AF354230[C] |
| NLA18 97M | MAS | Netherlands | 1997 | 4 | AF354194[A]; AF354214[B] |
| NLI19 97M | MAS | Netherlands | 1997 | 4 | AF354196[A]; AF354217[B] |
| NLI20 98M | MAS | Netherlands | 1998 | 2 | AF354197[A]; AF354218[B] |
| T6 | Respiratory | Taiwan | 1970 | 1 | AF204948; Liu et al., 2000[D] |
| SOM-4 | Unclear | Australia | N | 5 | L07069; Kool and Holmes, 1993[D] |
| GEL02 96U | Unclear | Germany | 1996 | 1 | AF354183[A]; AF354199[B] |
| NLA16 96U | Unclear | Netherlands | 1996 | 1 | AF354192[A]; AF354212[B] |
| NLA17 96U | Unclear | Netherlands | 1996 | 1 | AF354193[A]; AF354213[B] |
| RAM-1 | Healthy | Australia | 1971 | 5 | L38502; Kool and Holmes, 1995[D] |
| 919 | Healthy | Taiwan | 1992 | 1 | AF204949; Liu et al., 2000[D] |
| NLI21 98H | Healthy | Netherlands | 1998 | 4 | AF354204[B] |

[a] Tenosynovitis means that the particular reovirus was isolated from a case of tenosynovitis. MAS means that the particular reovirus was isolated from a field case of malabsorption syndrome (MAS) or experimental MAS.
[b] Clustering was based on alignment of protein sequences using Omiga™.
[A] Genbank Accession numbers 3' end: AF354182 until AF354197.
[B] Genbank Accession numbers 5' end: AF354198 until AF354218.
[C] Genbank Accession numbers: ORF AF354219 until AF354230.
[D] Genbank Accession numbers submitted to Genbank as indicated, generally unpublished.

(Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was used for DNA isolation followed by sequencing of two clones of each isolate with the ABI PRISM BIGDye kit (Perkin Elmer, Applied Biosystems). Two microliters Flexiprep purified DNA were used for the sequencing PCR for 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Sodium acetate/ ethanol precipitation was used to purify the PCR sequencing reaction products.

After solubilisation in 15 µL Template Suppression Reagent (ABI Prism, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) denaturation for 2 min at 94 °C and cooling on ice, samples were placed and run in the 310 Genetic Analyzer (ABI Prism, Applied Biosystems). Primers P1 and P4 were used for partial sequencing. To sequence the entire $\sigma$C gene the M13 universal forward and reverse primers as well as internal primers were used (Tab. II).

**Table II.** Primers used in this study.

| Code | Sequence | Sense | Location[a] | Specificity |
|---|---|---|---|---|
| P1 | 5'-AGTATTTGTGAGTACGATTG-3' | + | 525-544 | All |
| P2 | 5'-GATACTGTCATTGACTTCGA-3' | + | 666-685 | All |
| P5 | 5'-AACGCGCGCCCTAGA-3' | + | 825-839 | Cluster 1 |
| P6 | 5'-TAGCTGCTACTGTGGC-3' | + | 824-839 | Cluster 4 |
| P10 | 5'-CGCTTCTTCTGTAGGT-3' | + | 825-840 | Cluster 2[b] |
| P11 | 5'-GCAGAGACCGTGCGT-3' | + | 826-840 | Cluster 3 |
| P12 | 5'-TTGGATGATGTAACTGC-3' | + | 835-851 | Cluster 1 |
| P13 | 5'-GTCTCAGTAGCAGAGA-3' | + | 829-843 | Cluster 5 |
| P15 | 5'-CTGCATCTTCTTTGAGC-3' | + | 824-840 | Cluster 2[b] |
| P16 | 5'-GAGTCCTCGATAGGTC-3' | + | 826-841 | Cluster 2[b] |
| P3 | 5'-CCSGTRCGCACGGTCA-3' | – | 1589-1574 | All |
| P4 | 5'-GGCGCCACACCTTAGGT-3' | – | 1613-1597 | All |
| P8 | 5'-AGACATCATGTAGTCAG-3' | – | 1317-1301 | Cluster 1+4 |
| P9 | 5'-AGACATCATGTAATCAG-3' | – | 1317-1301 | Cluster 1+4 |
| P17 | 5'-ACGACATCATATAATCAG-3' | – | 1318-1300 | Cluster 1 |
| P18 | 5'-GGACATCATATAGTCAGT-3' | – | 1317-1300 | Cluster 2[b] |
| P19 | 5'-TCGACATCAAATAATCAGT-3' | – | 1318-1300 | Cluster 3 |
| P20 | 5'-TGCTTAGTTGACATAAGAT-3' | – | 1325-1307 | Cluster 5 |
| M13F | 5'-TGTAAAACGACGGCCAGT-3' | + | | |
| M13R | 5'-CAGGAAACAGCTATGACC-3' | – | | |

[a] Location according to Shapouri et al., 1995 [14].
[b] To sequence the isolates of cluster 2, each isolate needed individual primers.

### 2.3. Alignment and analysis of deduced amino acid sequences

The protein sequences of the $\sigma$C protein were aligned using Omiga$^{TM}$ (Oxford Molecular Ltd., Oxford, United Kingdom). These sequences were compared with known sequences of ARVs S1133 [15], 1733 [12], RAM-1 [6, 7] and SOM4 [5, 6]. Sequences of ARVs that were submitted to GenBank by Duncan in 2000 and by Liu et al. in 2000 and in 2001, were also used for comparison (Tab. I). The alignments were further analysed with the phylogeny program Phylip [2]. A distance matrix was calculated using Protdistance Kimura [2]. A tree was constructed using the UPGMA method. The tree is shown using Treeview [9]. The GenBank accession numbers for the sequences are given in Table I.

## 3. RESULTS

### 3.1. Amplification, cloning and sequencing of (part of) the σC gene

To assess whether ARVs can be grouped by using sequence data of the $\sigma$C protein sequence, 40 ARVs (see Materials and Methods section and Tab. I) were

grown on CELC. Two hundred microliter aliquots of the clarified supernatants of freeze-thawed CELS (4th or 5th passage) were used for RNA isolation. RNA isolated from non-infected CELC and from CELC infected with avian adenovirus were used as controls. Using these RNA samples in the RT-PCR did not result in a specific amplification. The PCR products were cloned in the pGEM®-T Easy vector.

Initially, sequencing was performed only with the RT-PCR primers (P1 and P4, Tab. II). These primers had been chosen from a comparison of the sequences of S1133, RAM-1 and SOM-4. The position of these primers might not be optimal for amplification but showed to be the only conserved sites around the ORF encoding the $\sigma$ C protein. The reverse primer P4 is located at the stop codon region. Therefore the last amino acid of $\sigma$ C could not be determined in this study. The 5' and 3' ends of the $\sigma$ C gene were sequenced with P1 and P4 respectively.

### 3.2. Alignment and analysis of deduced amino acid sequences

Alignment of the amino acid sequences of 3' and 5' ends of the $\sigma$ C gene of 28 ARVs, the published sequences of S1133, RAM-1 and SOM-4, and the sequences determined by Liu et al. and by Duncan yielded 5 clusters of different sequences (for the 3' end see Fig. 1). To confirm the genetic clustering obtained from the sequences of the 3' and 5' ends, the entire $\sigma$ C encoding gene of 12 representative ARVs was sequenced using M13 universal primers and internal primers (Tab. II). Alignment of the full length open reading frames divided the ARVs in exactly the same 5 genotype clusters as obtained with the sequences of the 3' and 5'ends (Fig. 2).

Subsequently, the alignment of the 83 amino-acid long N-terminal sequence fragments obtained using the 5' primer P1, was chosen to allocate the remaining 12 viruses in the genotype clusters. In Table I the ARVs are indicated for which the sequences have been submitted, either by us (32 out of 40 ARVs) or by others.

Most MAS isolates studied group in genotype clusters 1 and 4, few in clusters 2 and 5. Most Dutch and German tenosynovitis isolates studied group in genotype cluster 4 and those from unclear cases in genotype cluster 1.

Table 1 and Figure 1 show the comparison of genotype clustering based on the amino acid sequences of isolates from Germany and The Netherlands with those of the United States, Australia, Taiwan and Japan. The isolates from Germany, The Netherlands and from Taiwan are grouped in several clusters: 5, 3 and 3 respectively, whereas the isolates from the United States and Australia can only be found in one cluster. On the other hand, most isolates from Taiwan are grouped in cluster 1, whereas most isolates from The Netherlands are grouped in cluster 4 and those from Germany both in cluster 1 and 4. The single isolate from Japan was found in cluster 1.

Four isolates, GEI09 97M, GEI10 97M, NLA14 96T and GEL13 98M, showed two distinct amino acid sequences upon sequencing DNA of their cloned plasmids. This suggested the presence of at least two different reoviruses in the primary isolates. To prove this, GEI09 97M and GEI10 97M, which both contained amino acid sequences of cluster 1 and 5, were purified by limiting dilution on CELC. Besides the sequences of the clusters 1 and 5, a third sequence (cluster 4) was obtained by using the limiting dilution method for purification.

## 4. DISCUSSION

Avian reoviruses show a wide heterogeneity in pathogenicity. They are associated with disease conditions like viral arthritis, tenosynovitis and malabsorption syndrome (MAS), but they are also isolated from

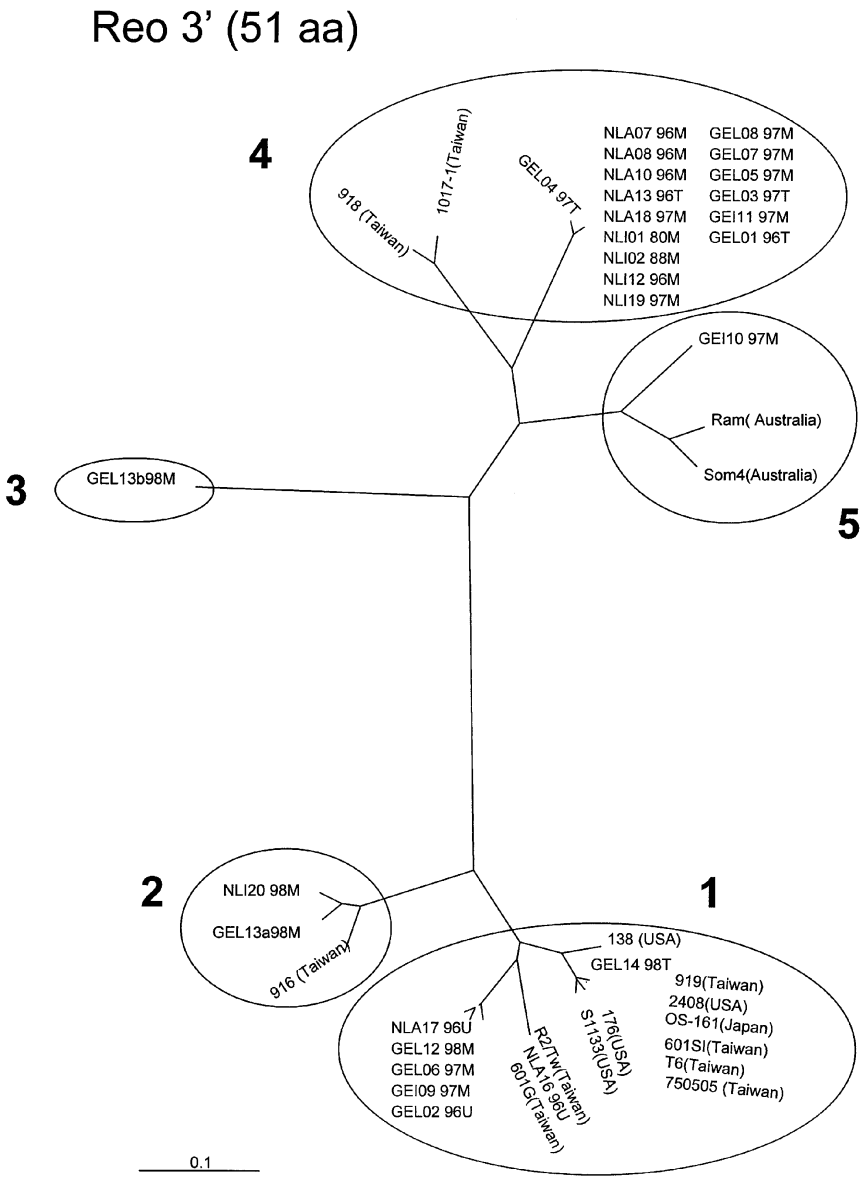


**Figure 1.** Unrooted radial phylogram based on alignments of the 3'end of the σ C gene of AVRs (51 amino acids) using the maximum likelihood method from the PHYLIP program [2]. Distances were calculated using Protdistance Kimura procedure [2]. ARV strains are indicated in Table I.

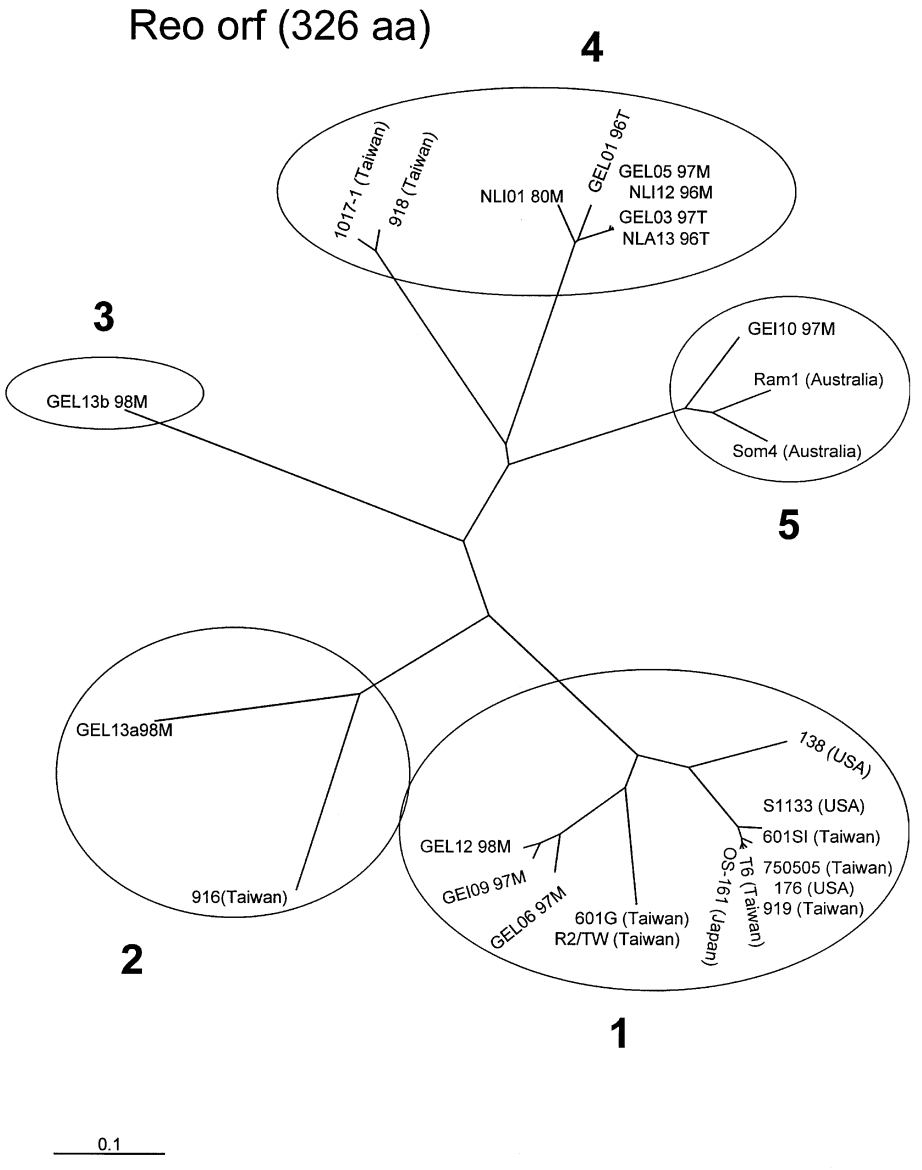


**Figure 2.** Unrooted radial phylogram based on alignments of complete ORFs of the σ C gene of avian reovirus strains (326 amino acids) using the maximum likelihood method from the PHYLIP program [2]. Distances were calculated using Protdistance Kimura procedure [2]. ARV strains are indicated in Table I.

chickens without any clinical signs [3, 4, 10, 16, 17]. Although the relationship between reovirus and tenosynovitis is well-established [16], a causative role is less clear in MAS. The characterisation and classification of ARV isolates might be important in the epidemiology of MAS cases and in the choice of vaccine on farms with problems of MAS and or reoviruses in general. The high number of reoviruses isolated from field cases of MAS in The Netherlands and in Germany prompted us to study the relation between the isolates and the relation between classification and pathogenicity, and temporal and geographic differences in isolates.

Molecular characterisation of ARVs based on nucleotide sequence analysis indicated that USA isolates (S1133, 1733, 2408, and C08) were closely related, but different from Australian isolates (RAM-1 and SOM-4) [6]. We decided to do molecular characterisation of MAS-associated ARVs by using PCR and nucleotide sequence analysis of the σ C protein as described [5, 6, 14]. We have compared these with several Dutch and German ARVs isolated from tenosynovitis cases or from healthy chickens. We were able to classify ARVs in 5 different genotyping clusters (Tab. I, Figs. 1 and 2). The classification in clusters was independent of determination of the 5' end, the 3' end or the whole ORF of the σ C gene. Therefore sequencing of a C representative part of the σ C gene seems to be sufficient. However, we were unable to establish a correlation between σ C sequences of the ARVs and the disease condition they were isolated from.

The sequences found in The Netherlands and in Germany are dispersed like those from Taiwan that have been submitted. In comparison, known ARV σ C sequences in the US (6 sequences submitted at present) and Australia (2 sequences submitted) show a very close relationship per area. Despite the dispersion, most isolates from The Netherlands are grouped in cluster 4 and those from Germany both in cluster 1 and 4, whereas most isolates from Taiwan are grouped in cluster 1. Also, most Dutch and German MAS isolates studied group in genotype clusters 1 and 4, whereas most Dutch and German tenosynovitis isolates studied group in genotype cluster 4. The isolates from unclear cases all group in genotype cluster 1.

Only one avian reovirus in our study had a sequence identical to the well-known reovirus strain S1133 originally isolated from a tenosynovitis case [15]. Vaccination against reovirus infection is mostly performed using strain S1133 [18] or 1733, a strain related to both tenosynovitis and stunting [11]. Yet, most sequences obtained in this study are at far distance from the sequence of both S1133 and 1733 (both cluster 1, Tab. I, Fig. 1). The isolation of ARVs from MAS cases on farms that had unsuccessful vaccination campaigns might have selected for ARVs with genotypes other than the commonly used vaccine strain S1133. Or, the prevalence of ARVs other than S1133 might be the cause for unsuccessful vaccination on those farms. This can be assessed by examining whether vaccination with S1133 provides any (cross) protection against viruses classified in sequence clusters 2–5. In this respect it is interesting to find out whether viruses within certain clusters induce protection against viruses in the same cluster or in other clusters. Three avian reoviruses isolated from MAS cases in Germany had σC sequences that were closely related to the sequences of the Australian isolates RAM-1 and SOM-4 [6]. These sequences have not been published from other locations. Therefore this finding was rather surprising. Since these viruses are not used as a vaccine in Europe, one may wonder about the origin of these viruses. Although the Dutch and German ARVs were isolated from 1980 up to 2000, no differences in time could be established.

This study provides new information at the amino acid level about several Dutch

and German avian reoviruses. The distribution of the isolates in sequence clusters (Tab. I) was based on the sequences found in amplified and cloned viral genomes. Therefore, not all sequences present in the primary virus isolates might have been obtained. Nevertheless, the distribution as presented in Table I is clear.

In conclusion, the classification of ARVs by genotyping could be realised based on the amino acid sequence of part of the σ C gene. However, it was impossible to relate certain reovirus genotypes to specific pathotypes or to establish temporal or geographic differences in isolates.

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