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The nucleopolyhedroviruses of *Rachiplusia ou* and *Anagrapha falcifera* are isolates of the same virus

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The 7·8 kb *Eco*RI-G fragment of *Rachiplusia ou* multicapsid nucleopolyhedrovirus (RoMNPV), containing the polyhedrin gene, was cloned and sequenced. The sequence of the fragment was 92·3% identical to the sequence of the corresponding region in the *Autographa californica* (Ac)MNPV genome. A comparison of the *Eco*RI-G sequence with other MNPV sequences revealed that RoMNPV was most closely related to AcMNPV. However, the predicted amino acid sequence of RoMNPV polyhedrin shared more sequence identity with the polyhedrin of *Orygia pseudotsugata* MNPV. In addition, the RoMNPV sequence was almost completely identical (99·9%) to a previously published 6·3 kb sequence of *Anagrapha falcifera* MNPV (AfMNPV). The *Eco*RI and *Hin*dIII restriction fragment profiles of RoMNPV and AfMNPV also were nearly identical, with an additional *Eco*RI band detected in RoMNPV DNA. Bioassays of these viruses with three different hosts (the European corn borer, *Ostrinia nubilalis* Hübner, the corn earworm, *Helicoverpa zea* Boddie, and the tobacco budworm, *Heliothis virescens* Fabricius) failed to detect any differences in the biological activities of RoMNPV and AfMNPV. These results indicate that RoMNPV and AfMNPV are different isolates of the same virus. The taxonomic relationship of Ro/AfMNPV and AcMNPV is discussed.

Introduction

Baculoviruses are invertebrate-specific pathogens of the family *Baculoviridae* that have been isolated primarily from species of the insect order Lepidoptera. Members of this family in the genus *Nucleopolyhedrovirus* have been developed as foreign gene expression vectors (Jarvis, 1997) and biopesticides (Black *et al.*, 1997; Van Beek & Hughes, 1998). The type species of this genus, the *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), is one of a group of genetically similar viruses that includes NPVs isolated from *Galleria mellonella* L., *Trichoplusia ni* Hübner and *Rachiplusia ou* Guenée (Jewell & Miller, 1980; Smith & Summers, 1979, 1980).

Rachiplusia ou (Ro)MNPV was first isolated in 1960 during an epizootic in the mint looper, *Rachiplusia ou*, in Indiana (Paschke & Hamm, 1961; Paschke & Sweet, 1966). Restriction enzyme digest and nucleic acid hybridization studies show that RoMNPV is closely related to AcMNPV (Jewell & Miller, 1980; Smith & Summers, 1980, 1982). Homologous recombination between the genomes of these two viruses has been observed during co-transfection and co-infection of cell lines and insects, further underscoring the degree of nucleotide sequence identity between these viruses (Croizier *et al.*, 1988; Summers *et al.*, 1980). Although a restriction map of RoMNPV has been assembled (Smith & Summers, 1980; Summers *et al.*, 1980), there are no published reports of RoMNPV gene sequences.

We are developing recombinant clones of RoMNPV for control of the European corn borer, *Ostrinia nubilalis*, a major agricultural pest. As a preliminary step towards this goal, we cloned and sequenced an RoMNPV restriction fragment containing the polyhedrin (*polh*) gene. Here we report the analysis of this sequence and consider implications for the classification of this virus as a species separate and distinct from AcMNPV.

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The GenBank accession number of the sequence reported in this paper is AF068270.

Methods

■ **Viruses, cells and insects.** AcMNPV strain C6 (Possee, 1986), RoMNPV strain R1 (Smith & Summers, 1980) and *Anagrapha falcifera* (Af)MNPV (Chen *et al.*, 1996) were propagated in *Spodoptera frugiperda* cell lines (Vaughn *et al.*, 1977) and titred by plaque assay. Sf21 cells were grown in Ex-Cell 405 medium (JRH Biosciences) supplemented with 3% foetal bovine serum (Intergen) and antibiotics (1 U/ml penicillin, 1 µg/ml streptomycin; Sigma). Sf9 cells were grown in TNM-FH medium (JRH Biosciences) which was also supplemented with 3% foetal bovine serum, antibiotics and 0·1% Pluronic F-68 (JRH Biosciences). Eggs of *Ostrinia*

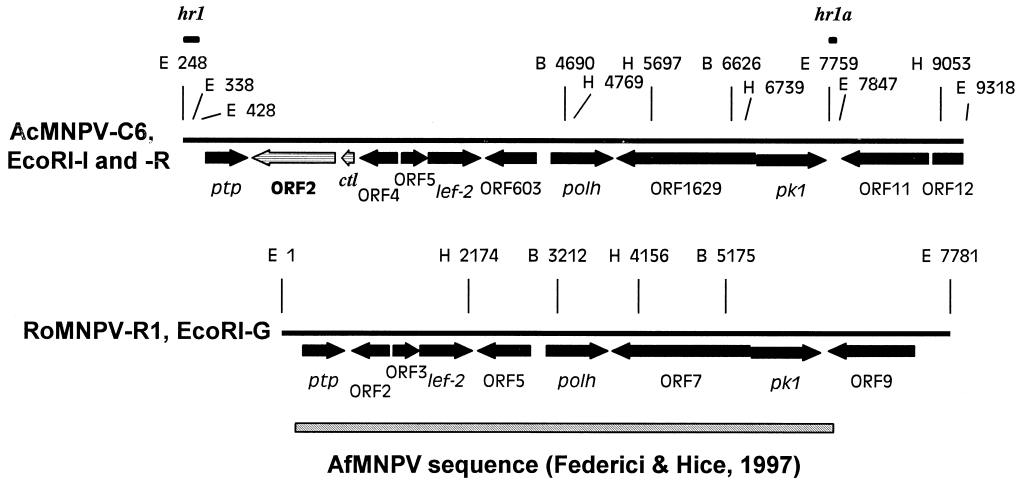

Fig. 1. Physical map of restriction sites and genes on the RoMNPV-R1 *Eco*RI-G fragment and the corresponding region in AcMNPV-C6. Restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII) along with their nucleotide positions in the *Eco*RI-G and the AcMNPV-C6 genomic sequences. The positions of homologous regions (*hr*s) on the AcMNPV sequence are also indicated. ORFs in *Eco*RI-G are numbered in accordance with the standard established for AcMNPV (Ayres *et al*., 1994), with *ptp* designated as ORF1. Arrowheads indicate ORF orientation, and the AcMNPV ORF2 and *ctl* genes, which are absent from *Eco*RI-G, are represented with hatched arrows. The position of the AfMNPV sequence reported by Federici & Hice (1997) is indicated with a bar below the *Eco*RI-G map.

nubilalis as well as diet for these insects were obtained from the USDA/ARS Corn Insects and Crop Genetics Research Unit in Ames, IA, USA. Eggs of *Heliothis virescens* and *Helicoverpa zea* were obtained from the USDA/ARS Southern Insect Management Research Unit in Stoneville, MS, USA and diet for these species was obtained from BioServ (Frenchtown, NJ, USA) and Southland Products (Lake Village, AR, USA), respectively.

■ **Viral DNA isolation and restriction digest.** Sf9 cells were infected with NPVs at an m.o.i. of 1. Budded virus (BV) was harvested at 5 days post-infection. BV was precipitated by overnight incubation on ice with an equal volume of 20% polyethylene glycol–1 M NaCl. After pelleting by centrifugation, the BV was resuspended in 10 mM Tris–HCl–1 mM EDTA pH 8·0 and incubated for 3 h at 37 °C with 1% SDS and 1 mg/ml proteinase K. Viral DNA was purified by phenol–chloroform extraction and ethanol precipitation. Five μg of viral DNAs was digested with restriction enzymes for 3 h, and restriction fragments were separated by electrophoresis on a 0·8% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination.

■ **DNA sequencing.** The plasmid pUC19M (Clontech) is a variant of pUC19 in which the *Eco*RI site has been substituted with an *Eco*RV site. An *Eco*RI site was inserted into pUC19M by digesting with *Sal*I, filling in the termini with Klenow fragment, and attaching *Eco*RI adaptors (Promega) to the blunt ends. This plasmid, called pUC19M-RI, was used to clone the RoMNPV-R1 *Eco*RI-G fragment, as well as overlapping *Kpn*I and *Bam*HI subfragments of *Eco*RI-G. Nested unidirectional deletions of the subfragments were created by the method of Henikoff (1984) and sequenced using M13 forward and reverse primers by automated dideoxy terminator sequencing (Sanger *et al*., 1977) at the Iowa State University DNA Sequencing and Synthesis Facility. Compilation of overlapping sequences and analysis of the final assembled sequence of *Eco*RI-G were carried out with the programs of the Genetics Computer Group Wisconsin package (version 9.0; Devereux *et al*., 1984) and the Baylor College of Medicine Search Launcher web page (http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher/launcher.html). The GenBank accession number of the RoMNPV *Eco*RI-G sequence is AF068270.

■ **Insect bioassays.** Viral occlusions were prepared from cadavers of virus-killed *H. virescens* by a standard method (O'Reilly *et al*., 1992). Lethal concentration bioassays were conducted using the droplet feeding method of Hughes & Wood (1981) with five different concentrations of occlusions and 35 larvae per dose. Dose–mortality relationships were analysed by probit analysis using the POLO program (Russell *et al*., 1977). Statistical analysis of LC_{50}s was carried out by the lethal dose ratio comparison method of Robertson & Preisler (1992). All bioassays were repeated at least three times.

Results

Sequence of the RoMNPV *polh* locus

The *Eco*RI-G fragment of RoMNPV contains the *polh* gene for this virus (Summers *et al*., 1980). This fragment was cloned and sequenced. Alignment of the 7781 nucleotide (nt) *Eco*RI-G sequence with the AcMNPV-C6 genomic sequence (Ayres *et al*., 1994) using the GCG BESTFIT program revealed 93·2% sequence identity with 32 gaps to nt 248–9318 of the AcMNPV sequence. With the exception of ORF 2, *ctl* and ORF 12, RoMNPV had the same array of genes found in AcMNPV in this region (Fig. 1). Consequently, we have adapted the same ORF numbering scheme used by Ayres *et al*. (1994) for AcMNPV, with ORF 1 corresponding to *ptp*. Approximately 1275 nt that in AcMNPV contains the ORF 2 and *ctl* ORFs were absent from the corresponding region in RoMNPV, and in their place was a 51 nt sequence with no significant similarity to other baculovirus sequences. The RoMNPV sequence which aligns with AcMNPV ORF 12 has a 5 nt insertion at codon position 16 which causes a frameshift and results in termination of the ORF at position 25. The *Eco*RI-G fragment aligns with the regions in AcMNPV containing most of the 'homologous region' elements *hr1* and all of *hr1a*, but much of the AcMNPV

Table 1. Comparison of deduced amino acid sequences for genes in RoMNPV-R1 *Eco*RI-G

RoMNPV gene	Percentage sequence identity with gene from other NPVs			
	AfMNPV	AcMNPV	BmNPV	OpMNPV
ptp	100%	98·8%	95·8%	60·5%
ORF 2 (AcMNPV ORF 4)	99·3%	96%	94%	66%
ORF 3 (AcMNPV ORF 5)	100%	98·2%	90·8%	57·4%
lef-2	99·5%	98·6%	93·8%	54·4%
ORF 5 (AcMNPV ORF 603)	100%	87·1%	–	–
polh	100%	90·2%	93·1%	96·3%
ORF 7 (AcMNPV ORF 1629)	100%	93·2%	88·2%	31·7%
pk1	100%	97·4%	97·0%	67·4%
ORF 9 (AcMNPV ORF 11)	–	95·3%	89·0%	44·4%

hr sequence in this region was not present in the RoMNPV sequence.

We also found that the *Eco*RI-G sequence was almost completely identical (99·9% identity with two gaps) to a 6289 nt sequence of the same region of AfMNPV (Federici & Hice, 1997). The AfMNPV sequence begins with an *Eco*RI site which is 152 nt downstream of the first *Eco*RI site in *Eco*RI-G and ends at nt 6437 of *Eco*RI-G (Fig. 1). The nucleotide sequence divergence between these viruses in this region consists of five 1 and 2 nt mismatches that occur within *ptp*, ORF2, *lef2*, and *polh*, and two 1 nt deletions that are present in intergenic regions of RoMNPV. The predicted amino acid sequences of RoMNPV *ptp*, ORF 3, ORF 5, *polh*, ORF 7 and *pk1* are 100% conserved with the corresponding AfMNPV ORFs (Table 1), while RoMNPV ORF 2 has a serine-to-cysteine substitution at codon position 116 and *lef2* has an arginine-to-alanine substitution at position 63 when compared to the AfMNPV sequences. The predicted amino acid sequence of RoMNPV ORF 9 lies outside the AfMNPV sequence.

Comparison of the ORFs in *Eco*RI-G with homologous ORFs from AcMNPV, *Orygia pseudotsugata* (Op)MNPV and *Bombyx mori* (Bm)NPV revealed that RoMNPV appears to be most closely related to AcMNPV (Table 1). As described for AfMNPV (Federici & Hice, 1997), the RoMNPV polyhedrin amino acid sequence shares greater sequence identity with OpMNPV than with AcMNPV, suggesting that RoMNPV acquired its *polh* gene by recombination with a more distantly related baculovirus. A sequence identity search revealed that the polyhedrin of *Epiphyas postvittana* MNPV (Hyink *et al.*, 1998) possessed the highest degree of sequence identity (97·1%) with RoMNPV polyhedrin.

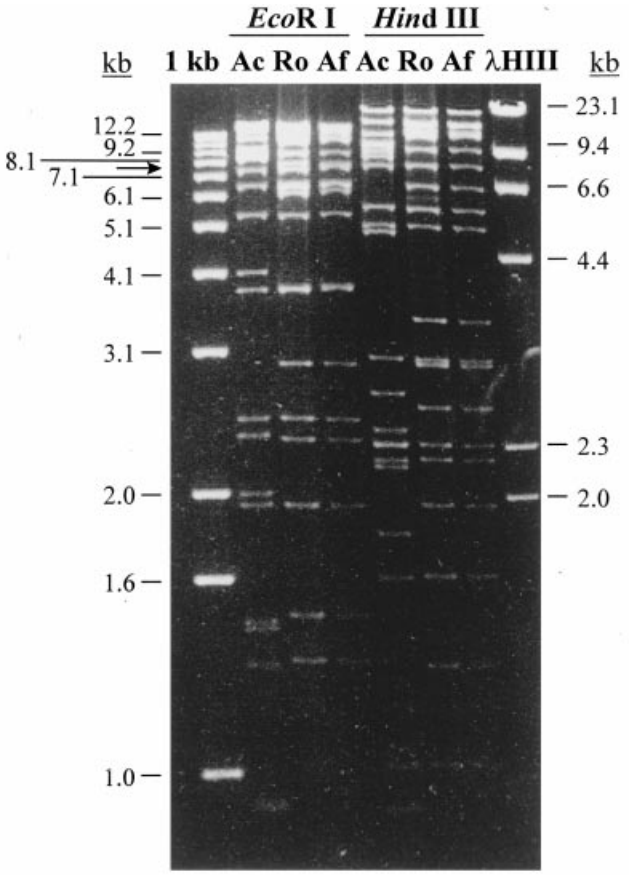

Fig. 2. *Eco*RI and *Hin*dIII digests of AcMNPV-C6 (Ac), RoMNPV-R1 (Ro) and AfMNPV (Af) viral DNA. Size standards (indicated in kb) are the 1 kb ladder (1 kb, Gibco BRL) and *Hin*dIII fragments of phage λ (λHIII, Promega). The arrow indicates an additional *Eco*RI band in RoMNPV DNA.

Restriction digest and bioassay analysis

Restriction digests of RoMNPV and AfMNPV DNA yielded nearly identical fragment patterns, confirming that these viruses are the same (Fig. 2). An additional band migrating between the 7 and 8 kb markers was present in the RoMNPV *Eco*RI fragment pattern (Fig. 2).

Table 2. Dose–mortality response of neonate larvae infected with RoMNPV, AfMNPV and AcMNPV

Host/virus	$LC_{50} \times 10^5$ (95% CL)*	Slope (±SE)	Heterogeneity
Ostrinia nubilalis			
RoMNPV-R1	$36{\cdot}9^a$ (11·96–72·6)	1·0 (±0·197)	0·67
AfMNPV	$31{\cdot}4^a$ (0·39–74·49)†	1·18 (±0·232)	1·61
AcMNPV-C6	$1647{\cdot}8^b$ (511·6–3644·9)	0·82 (±0·195)	0·86
Helicoverpa zea			
RoMNPV-R1	$1{\cdot}1^a$ (0·44–2·04)	1·84 (±0·277)	1·32
AfMNPV	$0{\cdot}8^a$ (0·59–1·14)	2·12 (±0·328)	0·42
AcMNPV-C6	$2{\cdot}6^b$ (0·69–5·58)	1·30 (±0·239)	1·03
Heliothis virescens			
RoMNPV-R1	$0{\cdot}7^a$ (0·39–1·13)	1·72 (±0·299)	0·76
AfMNPV	$0{\cdot}8^a$ (0·39–1·14)	1·71 (±0·296)	0·27
AcMNPV-C6	$1{\cdot}1^a$ (0·60–1·63)	1·66 (±0·270)	0·80

* Polyhedra/ml. For each host, values with different letters are significantly different at $P < 0{\cdot}05$. LC_{50}s and 95% confidence limits (95% CL) were determined by probit analysis. Statistical analysis of LC_{50}s was carried out by the lethal dose ratio comparison method of Robertson & Preisler (1992).
† 90% CL.

Lethal concentration (LC) bioassays were carried out to compare the biological activities of RoMNPV and AfMNPV. In our LC bioassays, RoMNPV and AfMNPV were equally virulent against larvae of *Ostrinia nubilalis*, *Helicoverpa zea* and *Heliothis virescens* (Table 2). Both viruses had significantly lower LC_{50}s than AcMNPV against *O. nubilalis* and *H. zea*, which is consistent with previous studies showing that RoMNPV is more virulent against *O. nubilalis* than AcMNPV (Lewis & Johnson, 1982) and that AfMNPV is more effective against *H. zea* than AcMNPV (Hostetter & Puttler, 1991).

Discussion

In the process of assembling a transfer vector to make recombinant RoMNPV, we discovered that RoMNPV and the more recently described AfMNPV are different isolates of the same virus. AfMNPV infects a wide variety of agriculturally significant Lepidoptera (Hostetter & Puttler, 1991), and much work evaluating its potential as a biological control agent has been published. The differences between RoMNPV and AfMNPV are so minor that there is little reason to distinguish between the two viruses. In this case, AfMNPV should be referred to as RoMNPV, since the latter was originally isolated in 1960 from *Rachiplusia ou*.

It is possible that the original isolate of RoMNPV contained a virus that is different and distinct from AfMNPV and that we have selectively amplified an AfMNPV contaminant in our stock of RoMNPV-RI. However, Smith & Summers (1980) worked with two separate stocks of RoMNPV and found that all the clones derived from these stocks yielded identical *Eco*RI fragment patterns. This fragment pattern matched that obtained by Jewell & Miller (1980), who worked with an RoMNPV stock that was a few passages removed from the original isolate of Paschke & Sweet (1966) and that had undergone three more passages before analysis of viral DNA. The *Eco*RI fragment patterns of our stock of RoMNPV-R1 exactly matched the fragment patterns obtained by these other two groups. It seems unlikely, then, that our RoMNPV is a selectively amplified AfMNPV contaminant.

There has been some controversy over whether RoMNPV/AfMNPV should be classified as a variant of AcMNPV or as a separate species (Smith & Summers, 1980; Volkman *et al.*, 1995; Federici & Hice, 1997). Federici & Hice (1997) proposed that Ro/AfMNPV should be classified as a variant of AcMNPV. However, there are differences between these viruses that argue against this classification.

(1) Restriction fragment differences. It has been stated that the differences between AcMNPV and RoMNPV/AfMNPV are of the same magnitude as those existing among other viruses regarded as AcMNPV variants, such as *Trichoplusia ni* (Tn) and *Galleria mellonella* (Gm) MNPVs (Federici & Hice, 1997). However, this is not the case. Smith & Summers (1979) found that TnMNPV and GmMNPVs had over 90% of restriction fragments with identical or highly similar mobilities to AcMNPV restriction fragments. This degree of relatedness was similar to that found for other AcMNPV variants (Smith & Summers, 1979), and also to *Spodoptera exempta* MNPV (Brown *et al.*, 1984). RoMNPV, in contrast, only had 35 of 60 fragments (58·3%) co-migrate with AcMNPV fragments. Smith & Summers (1980) concluded that while TnMNPV and GmMNPV should be considered variants of AcMNPV, RoMNPV is more distantly related.

(2) Sequence divergence. Although the *Eco*RI-G nucleotide sequence identity with AcMNPV was 93·2%, 32 gaps were required to produce this alignment. For a DNA virus, this indicates that a small but significant degree of sequence divergence has taken place. Although RoMNPV and AcMNPV can undergo recombination with each other, recombination has also been observed between AcMNPV and *Cydia pomonella* granulovirus, which share little nucleotide sequence identity (Crook *et al*., 1993). Hence, it is uncertain to what extent the occurrence of recombination can serve as a criterion in the classification of baculovirus species.

(3) Missing *hr* elements and ORFS. Baculovirus genomes have 'homologous regions' (*hr*s) that function as viral transcriptional enhancers and DNA replication origins (Possee & Rohrmann, 1997). In AcMNPV, each *hr* consists of two to eight repeats of an imperfect palindromic sequence with an *Eco*RI site at the centre of the palindrome. The Ro/AfMNPV genome has five regions with sequence similarity to AcMNPV *hr4L*, but none of these map to the *Eco*RI-G fragment (Chen *et al*., 1996). Although we found single copies of the *hr1* and *hr1a* palindromes in the RoMNPV *Eco*RI-G sequence, much of the rest of the *hr* sequences were missing.

In addition, an intact AcMNPV ORF 12 homologue was not found in RoMNPV. Although the function of ORF 12 is unknown, its absence from the genomes of OpMNPV and BmNPV (Ahrens *et al*., 1997; Possee & Rohrmann, 1997) suggests that it does not play an essential role in the nucleopolyhedrovirus life-cycle.

A large segment containing AcMNPV ORF 2 and *ctl* is also missing from the *Eco*RI-G region, another feature shared by OpMNPV and BmNPV. Federici & Hice (1997) did not detect hybridization of a probe containing ORF 2 and *ctl* sequences to AfMNPV DNA under low-stringency conditions, indicating that Ro/AfMNPV does not contain these genes or that they are present but with highly diverged sequences. Multiple ORF 2- and *ctl*-like sequences are present in the genomes of OpMNPV, BmNPV and *Lymantria dispar* MNPV (Ahrens *et al*., 1997; Possee & Rohrmann, 1997; Kuzio *et al*., 1999).

(4) Host-range differences. Federici & Hice (1997) state that variability in biological activities is common among virus variants and is not used as a criterion in species demarcation. However, there is nothing in the polythetic species concept, endorsed by the International Committee on the Taxonomy of Viruses (ICTV) for the definition of a virus species (Mayo & Pringle, 1998), that precludes the use of variability in biological activities against different hosts as one of the polythetic criteria. Ro/AfMNPV and AcMNPV infect many of the same species, but there are multiple differences in their host ranges. One of the distinguishing characteristics of AfMNPV is its greater virulence against *Helicoverpa zea* (Hostetter & Puttler, 1991) when compared to AcMNPV. Differences in the susceptibilities of another eight host species to AfMNPV and AcMNPV were also observed by Hostetter & Puttler (1991). In addition, Ro/AfMNPV infects species not susceptible to AcMNPV, such as the tobacco hornworm (*Manduca sexta* L.) and the navel orangeworm (*Amyelois transitella* Walker) (Hostetter & Puttler, 1991; Vail *et al*., 1993).

In conclusion, it appears that the group of NPVs closely related to AcMNPV can be divided into two separate and distinct lineages. One lineage contains AcMNPV, TnMNPV, GmMNPV and *Spodoptera exempta* MNPV. The other lineage contains RoMNPV. The taxonomic classification of these baculovirus lineages (either as a single species or as separate species) needs to be clarified. This will require the establishment of polythetic criteria for demarcation of baculovirus species (Van Regenmortel *et al*., 1997), as published recently for reoviruses of the genus *Coltivirus* (Attoui *et al*., 1998).

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