© INSTITUT PASTEUR/ELSEVIER
Paris 1991

Res. Virol.
1991, 142, 489-494

Restriction map of the *Casphalia extranea* densovirus genome

G. Fédière [(1)], S. Herder [(1)], K.N. Kouassi [(1)], X. Léry [(1)],
D. Dauthuille [(1)] and M. Bergoin [(2)]

[(1)] *Laboratoire d'Entomovirologie ORSTOM, BPV 51 Abidjan (Côte-d'Ivoire), and*
[(2)] *Station de Recherches de Pathologie comparée INRA-CNRS, 30380 St. Christol-lez-Alès (France)*

SUMMARY

A physical map of the *Casphalia extranea* densovirus genome (*Ce*DNV) was constructed. The size of the intact viral genome was estimated to be 4.9 kilobases or 1.6 MDa (single strand). The double-stranded *Ce*DNV genomic DNA was cleaved with 26 restriction endonucleases and 20 restriction sites were mapped on the genome. The *Ce*DNV DNA restriction map was compared to those of other densoviruses. Southern blotting hybridization experiments failed to reveal any homology between the genomes of *Ce*DNV and *Junoniacoenia* densovirus (*Jc*DNV).

Key-words: DNA, Densovirus, *Parvoviridae,* Restriction map; *Casphalia extranea.*

INTRODUCTION

Densoviruses (DNV) originally discovered in heavily infected larvae of the greater wax moth *Galleria mellonella* (Meynadier *et al.*, 1964) have been since isolated from several species of insects, mainly Lepidoptera (see review by Kawase, 1985). The virions of DNV are small icosahedral non-enveloped particles, 20 to 23 nm in diameter and their capsid contains 4 polypeptides (Tijssen *et al.*, 1976; Kelly *et al.*, 1980; Bando *et al.*, 1985). Their genome is a single-stranded (ss) linear DNA molecule 5-6 kb in length (Barwise and Walker, 1970; Kelly and Bud, 1978; Nakagaki and Kawase, 1980; Jousset *et al.*, 1986; Bando *et al.*, 1987). Owing to their properties, DNV have been classified within the family *Parvoviridae* as the genus *Densovirus,* along with the two genera *Parvovirus* and *Dependovirus* of vertebrate parvoviruses (Matthews, 1982; Siegl *et al.*, 1985). They are characterized by their autonomous replication (unlike the dependoviruses) and by the separate encapsidation in equimolar ratios of strands of each polarity (Barwise and Walker, 1970; Kurstak *et al.*, 1971; Kelly *et al.*, 1977). As a consequence of this property, DNA extraction from DNV virions in high salt buffer resulted in the formation of double-stranded (ds) DNA molecules (Truffaut *et al.*, 1967; Barwise and Walker, 1970; Kelly *et al.*, 1977).

Submitted December 15, 1990, accepted November 12, 1991.

Corresponding author: Prof. Max Bergoin, Station de Recherches de Pathologie comparée, INRA-CNRS, 30380 St. Christol-lez-Alès (France).

ORSTOM Fonds Documentaire
2 9 JUIL. 1992
N° : 36.384 ex 1
Cote : B P32

A small DNA virus has been recently isolated from *Casphalia extranea* (Lepidoptera, *Limacodidae*), one of the major oil palm pests in West Africa (Fédière, 1983). Laboratory and field tests with this virus have shown its high pathogenicity and ability to control natural populations of its host (Fédière *et al.*, 1986). Preliminary results indicated that this virus shared most of the properties of densoviruses, including a capsid built up of 4 polypeptides and a DNA of about 5.7 kb (Fédière, 1983). Recently, we succeeded in multiplying the virus in an established cell line (Fédière *et al.*, 1990). In order to further investigate the basic properties of the *C. extranea* densovirus (*Ce*DNV) genome, we have established the restriction map of its dsDNA form.

MATERIALS AND METHODS

Virus purification

Virus was purifed from infected larvae collected in the fields (Fédière, 1983). Larvae were first homogeneized in Tris (0.05 M)-SDS (0.06 %) buffer pH 7.8. After filtration through cheese cloth and clarification (9,000 *g*, 5 min), the virus was concentrated by high speed centrifugation (Ti-55 Beckman rotor, 40,000 rpm, 2 h). The viral pellets, resuspended in Tris (0.05 M pH 7.8) buffer were dispersed by ultrasonication and then clarified (9,000 *g*, 5 min). The resulting supernatant, containing virus particles, was layered onto a 15-45 % (w/w) saccharose gradient prepared in Tris buffer and centrifuged (SW 28, Beckman rotor, 27,000 rmp, 2 h). The virus band was removed with a peristaltic pump connected to an UV spectrophotometer (Beckman UV-5230). Generally, 2 or 3 gradients were necessary to obtain a satisfactory purification of the virus.

DNA extraction

The virus suspension in TE buffer (0.01 M Tris, 0.01 M EDTA, pH 8.0) was treated with SDS (5 mg/ml final concentration) and incubated at 60°C for 15 min. The DNA solution was deproteinized by mixing with an equal volume of a phenol/chloroform solution and gentle shaking at room temperature. The mixture was centrifuged for 5 min at 5,000 *g* and the same extraction procedure was applied to the upper aqueous phase using phenol only, then chloroform only, and finally, ether.

The deproteinized DNA solution was then dialysed against TEN buffer (0.01 M Tris pH 8.0; 0.001 M EDTA; 0.1 M NaCl) for 72 h, with a change of buffer every 12 h and finally ethanol precipitated overnight at −20°C.

The DNA precipitate was resuspended in TE buffer and the purity and concentration of the solution checked by UV spectrophotometry.

Digestion

The DNA was digested with the following endonucleases: *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*II, *Hae*III, *Cfo*I, *Hin*cII, *Hin*dIII, *Hpa*I, *Hpa*II, *Kpn*I, *Nhe*I, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sma*I, *Stu*I, *Taq*I, *Xba*I, *Xho*I, under conditions specified by the supplier.

The digestion volume was approximately 50 µl and 1 to 2 µl of enzyme were added in order to have at least one unit per µg of DNA. The digested fragments were analysed by electrophoresis on horizontal agarose mini-gels (Helling *et al.*, 1974) of appropriate concentrations (1.2 to 2 %) in TAE buffer (40 mM Tris pH 8.0; 40 mM sodium acetate; 1 mM EDTA). To separate restriction fragments too small to be detected by agarose gel electrophoresis, polyacrylamide slab gels (7.8 and 9 %) were also used. After migration, gels were stained with ethidium bromide (1 mg/ml) and photographed under UV light. The size of restriction fragments was determined by linear regression according to the method of Southern (1979) using double *Eco*RI-*Hin*dIII

*Ad*DNV	=	*Acheta domestica* DNV.
*Bm*DNV	=	*Bombyx mori* DNV.
*Ce*DNV	=	*Casphalia extranea* DNV.
ds	=	double-stranded.
EDTA	=	ethylene diamine tetraacetate.
*Gm*DNV	=	*Galleria mellonella* DNV.
ITR	=	inverted terminal repeat.
*Jc*DNV	=	*Junonia coenia* DNV.
SDS	=	sodium dodecyl sulphate.
ss	=	single-stranded.
TAE buffer	=	Tris-acetate-EDTA buffer.
TE buffer	=	Tris-EDTA buffer.
TEN buffer	=	Tris-EDTA-NaCl buffer.

digestion fragments of bacteriophage lambda DNA as standards. This method permits the calculation of a correction coefficient (L) to avoid error in measurement due to the inflexion in the upper part of the calibration curve.

Southern blot hybridization

DNA restriction fragments were transferred onto nylon membranes (Boerhinger Mannheim) and then hybridized with a total genomic probe of either *Ce*DNV or *Jc*DNV DNA, both labelled with digoxigenin-11-dUTP according to the supplier's protocol (Boerhinger Mannheim).

In order to reuse a membrane for a second hybridization, it was first decolorized and treated following the recomandations of the supplier.

RESULTS

The DNA of *Ce*DNV was submitted to a range of restriction enzymes. The following endonucleases: *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Hae*II, *Hha*I, *Cfo*I, *Hpa*I, *Kpn*I, *Nhe*I, *Not*I, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, *Sma*I and *Stu*I were found to have no cleavage site in the viral DNA.

The enzymes *Eco*RV, *Xba*I and *Xho*I cleaved the *Ce*DNV DNA once. The other enzymes tested had more than one restriction site: *Eco*RI and *Hinc*II (3 sites), *Dra*I and *Hin*dIII (4 sites), *Hae*III and *Taq*I (6 sites). Digestion with *Hpa*II appeared to be poorly reproducible by generating an inconstant number of fragments. The sizes of the restriction fragments of the *Ce*DNV ge-

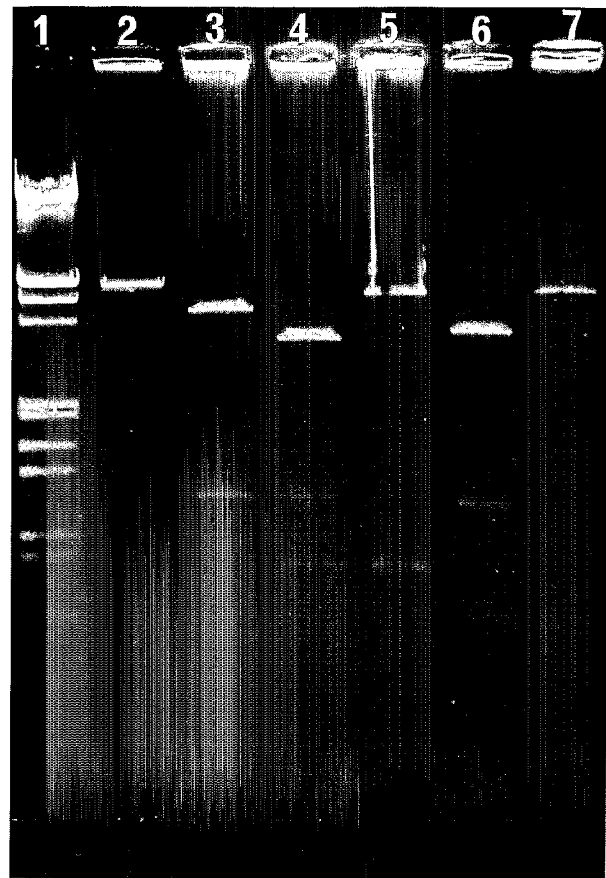

Fig. 1. Electrophoretic profile of restricted genomic DNA from *Ce*DNV.

Lane 1: *Eco*RI-*Hin*dIII lambda DNA digest; lane 2: undigested DNA; lane 3: *Eco*RV digest; lane 4: *Eco*RV+*Xba*I digest; lane 5: *Xba*I digest; lane 6: *Eco*RV+*Xbo*I digest; lane 7: *Xho*I digest.

Table I. Number and size (in kb) of the restriction fragments from the genomic DNA of *Ce*DNV.

	Fragments						
Enzymes	A	B	C	D	E	F	G
*Dra*I	2.72	1.18	0.75	0.14	0.11	—	—
*Hinc*II	1.68	1.43	1.13	0.66	—	—	—
*Eco*RI	3.49	0.58	0.51	0.32	—	—	—
*Eco*RV	3.83	1.07	—	—	—	—	—
*Hae*III	1.87	1.09	0.65	0.56	0.20	0.25	0.22
*Hin*dIII	2.39	0.89	0.82	0.42	0.38	—	—
*Taq*I	1.25	1.08	0.86	0.73	0.64	0.22	0.12
*Xba*I	4.22	0.68	—	—	—	—	—
*Xho*I	4.40	0.50	—	—	—	—	—

The sizes of the individual fragments generated by each enzyme were adjusted according to the value of 4.9 kb estimated for the undigested genomic DNA.

nome are shown in table I. Due to the limitations of the method used, it was not possible to detect DNA fragments with a size smaller than 80 bp. The molecular weight of undigested dsDNA estimated by electrophoresis on agarose gel was 3.2 MDa (4.9 kbp) or 1.6 MDa for the ss molecule.

Complete mapping of the restriction sites on the genome was achieved by using classical techniques of total or partial digestion with each of the different enzymes or simultaneous digestion with pairs of restriction enzymes (see fig. 1). The restriction map of the *Ce*DNV genome was obtained by ordering the 29 restriction fragments and positioning the 20 restriction sites (see fig. 2). The unique *Eco*RV restriction site served for ordering the fragments on the genome. It was arbitrarily decided that the large fragment (A) represented the right hand side of the molecule. The recognition sites of the other enzymes were positioned relative to this.

The restriction sites for enzymes *Hpa*II, *Dra*I and *Hinc*II were not mapped on the genome, and

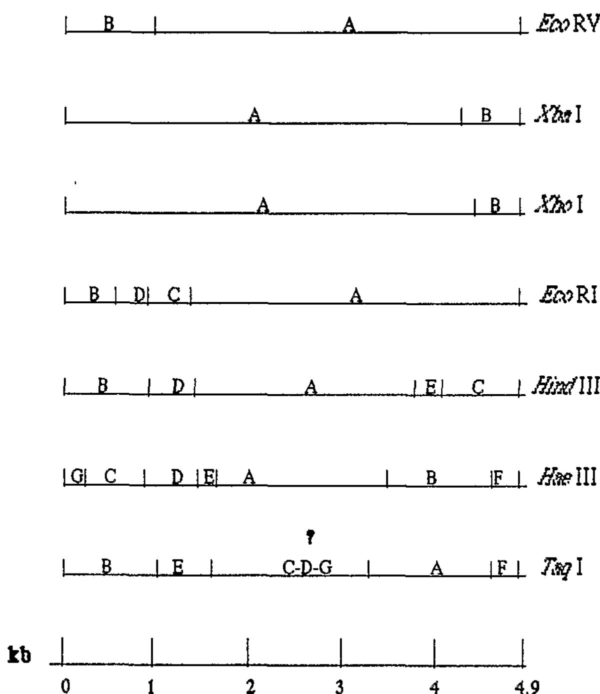

Fig. 2. Restriction map of the *Ce*DNV genomic DNA as determined by the following endonucleases: *Eco*RV, *Xba*I, *Xho*I, *Eco*RI, *Hind*III, *Hae*III and *Taq*I.

the restriction fragments C, D and G of *Taq*I enzyme (see fig. 2), in the central part of the molecule, could not be ordered accurately because none of the enzymes used cleaved the DNA molecule in this region.

We looked for homologies between *Ce*DNV DNA and *Jc*DNV DNA. For this purpose, restriction fragments of both genomes, were electrophoresed in 1 % agarose gel, transferred onto a nylon membrane and hybridized first with a total genomic *Ce*DNV probe, then rehybridized with a total genomic *Jc*DNV probe (see "Materials and Methods"). As shown in figure 3, both *Ce*DNV and *Jc*DNV DNA probes hybridized only with the restriction fragments of their homologous DNA. We concluded from these results that the two genomes have no significant sequence homology.

DISCUSSION

A restriction map of the *Ce*DNV DNA was derived from 7 restriction enzymes and 20 sites have been positioned on the genome. In order to know if the proposed map orientation conforms to the convention adopted for other parvoviruses (Armentrout *et al.,* 1978), *i.e.* the 3'-OH terminus of the "minus" (viral) strand as the left end of the molecule; the 3' and 5' extremities have yet to be defined. Cleavage of the *Ce*DNV genome by *Hae*III generated 2 small fragments of similar size which mapped at both ends of the genome (see fig. 2). Symmetrical cleavages were also found in other densovirus genomes (Bando *et al.,* 1987; Jousset *et al.,* 1990), which very likely indicates the presence of inverted repeats at both ends of the genome.

With a size of 4.9 kb, the *Ce*DNV genome is closer to the 5.0-kb DNA of *Bombyx mori* DNV (*Bm*DNV; Bando *et al.,* 1990) or to the 5.2-kb genome of *Acheta domestica* DNV (*Ad*DNV, Jousset *et al.,* 1986) than to the 5.9-kb DNA of *Jc*DNV and *Gm*DNV (Jousset *et al.,* 1990). However, the restriction map of the *Ce*DNV genome shows little similarity with that of *Bm*DNV (Bando *et al.,* 1987), *Ad*DNV (Jousset, personnal communication), *Jc*DNV or

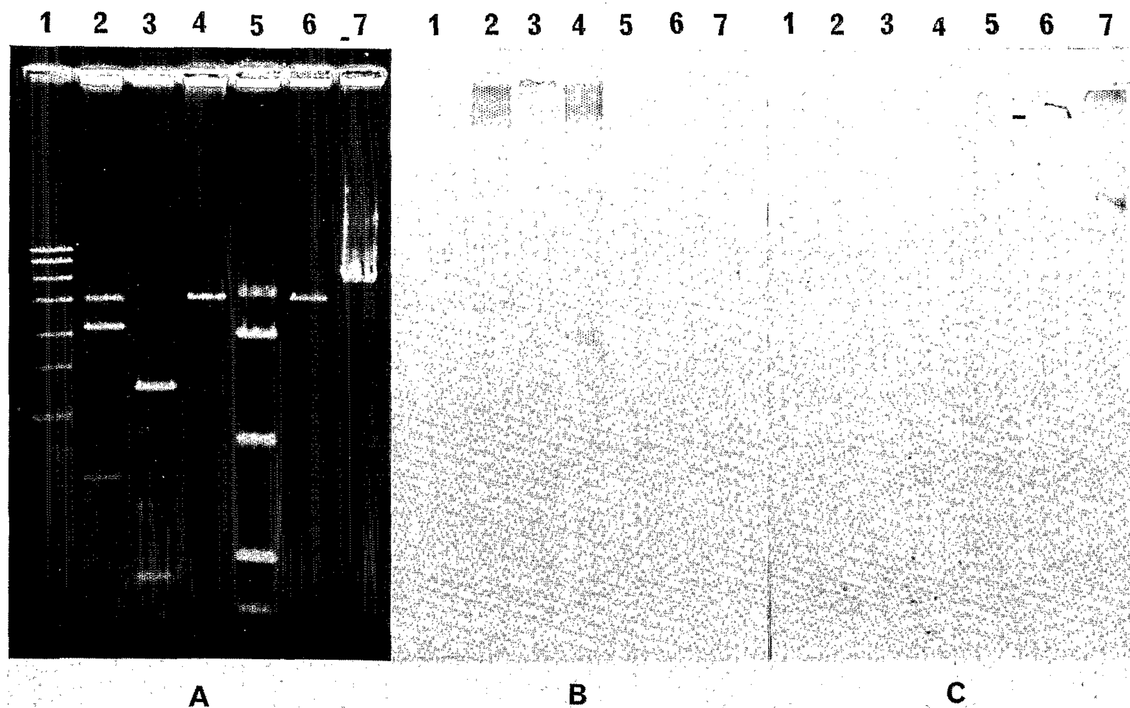

Fig. 3. Southern blotting hybridization between *Ce*DNV and *Jc*DNV genomes.

A) Electrophoretic profiles of restricted and uncleaved *Ce*DNV and *Jc*DNV genomic DNA. Lane 1: *Eco*RI fragments of phage SPP1 DNA used as molecular weight marker (Boehringer Mannheim); lane 2: *Eco*RV digest of *Ce*DNV DNA; lane 3: *Hin*dIII digest of *Ce*DNV DNA; lane 4: undigested *Ce*DNV DNA; lane 5: *Eco*RI digest of *Jc*DNV DNA; lane 6: *Hin*dIII digest of *Jc*DNV DNA; lane 7: undigested *Jc*DNV DNA.

B) Southern blot of DNA fragment profiles shown in A revealed by a digoxigenin-labelled *Ce*DNV DNA probe.

C) The nylon membrane shown in B was dehybrydized and rehybridized using a digoxigenin-labelled *Jc*DNV DNA probe.

*Gm*DNV (Jousset *et al.*, 1990). Furthermore, Southern hybridization failed to reveal any homology between *Ce*DNV and *Jc*DNV DNA. This lack of homology may reflect the lack of serological relationship observed between *Ce*DNV, *Jc*DNV and *Gm*DNV (Fédière, 1983).

On the basis of the length of their inverted terminal repeats (ITR), we recently proposed to separate the densoviruses into 2 categories: those with a genomic size averaging 6 kb possessing long ITR and those with a smaller size (about 5 kb) with shorter ITR (Jourdan *et al.*, 1990). Additional experiments will be necessary to further define these differences and their significance and to precisely define the relationship of *Ce*DNV to other densoviruses.

Carte physique de restriction du génome du densovirus de *Casphalia extranea*

Nous avons dressé la carte physique de restriction de la forme bicaténaire de l'ADN du densovirus de *Casphalia extranea* Walker (*Ce*DNV; Lepidoptère, *Limacodidae*). La taille du génome a été estimée à 4,9 kilobases, soit 1,6 MDa (forme monocaténaire). L'ADN génomique a été digéré par 26 endonucléases, et 20 sites de restriction ont été cartographiés par 7 enzymes. Cette carte a été comparée à celles d'autres densovirus. Aucune homologie de séquence n'a été détectée entre l'ADN génomique du *Ce*DNV et celui du densovirus de *Junonia coenia.*

Mots-clés: ADN, Densovirus, *Parvoviridae,* Carte de restriction; *Casphalia extranea.*

References

Armentrout, R., Bates, R., Berns, K., Carter, B., Chow, M., Dressler, K., Fife, W., Hauswirth, W., Hayward, G., Lavelle, G., Rhode, S., Strauss, S., Tattersall, P. & Ward, D. (1978), A standardized nomenclature for restriction endonuclease fragments, *in* "Replication of parvoviruses" (D. Ward & P. Tattersall) (pp. 523-526). Cold Spring Harbor Laboratory, New York.

Bando, H., Kusuda, J. & Kawase, S. (1987), Molecular cloning and characterization of *Bombyx* densovirus genomic DNA. *Arch. Virol.,* 93, 139-146.

Bando, H., Kusuda, J., Gojobori, T. & Kawase, S. (1985), (Genome structure and origin of Bombyx densonucleosis virus DNA). *Jap. J. Genetics,* 60, 584.

Bando, H., Choi, H., Ito, Y. & Kawase, S. (1990), Terminal structure of a densovirus implies a haipin transfer replication which is similar to the model for AAV. *Virology,* 179, 57-63.

Barwise, A.H. & Walker, I.O. (1970), Studies of the DNA of a virus from *Galleria mellonell* L. *FEBS Letters,* 6, 13-16.

Fédière, G. (1983), Recherches sur des viroses épizootiques de lépidoptères *Limacodidae* ravageurs de palmacées. Thèse, Univ., Montpellier II, 103 pp.

Fédière, G., Monsarrat, P., Mariau, D. & Bergoin, M., A Densovirus of *Casphaliaextranea* (Lepidoptera, *Limacodidae*): characterization and use for biological control, *in* "Fundamental and applied aspects of invertebrate pathology" (R.A. Samson, J.M. Vlak & D. Peters) p. 705. Proc 4th int. Colloq. Invertebr. Pathol., Veldhoven (The Netherlands), August 1986.

Fédière, G., Léry, X., Quiot, J.M. & Monsarrat, P. (1990), Replication of the Densovirus of *Casphalia extranea* (Lepidoptera, Limacodidae) on an established cell line. *J. Invertebr. Path.,* 56, 132-134.

Helling, R.B., Goodman, H.M. & Boyer, H.W. (1974), Analysis of R.*Eco*RI fragments of DNA from lambdoid bacteriophages and other viruses by agarose gel electrophoresis. *J. Virol.,* 14, 1235-1244.

Jourdan, M. (1990), Clonage et séquençage du génome infectieux d'un parvovirus d'insecte, le densovirus du lépidoptère *Junonia coenia*. Th. Doct., Univ. Montpellier 2, Décembre 1990, 172 p.

Jousset, F.X., Compagnon, B. & Bergoin, M. (1986), Comparison of the restriction map and infectivity of the genome of three Densoviruses, *in* "Fundamental and applied aspects of invertebrate pathology" (R.A. Samson, J.M. Vlak & D. Peters) p. 121. Proc. 4th int. Colloq. Invertebr. Pathol., Veldhoven (The Netherlands), August 1986.

Jousset, F.X., Jourdan, M., Compagnon, B., Mialhe, E., Veyrunes, J.-C. & Bergoin, M. (1990), Restriction maps and sequence homologies of two densovirus genomes. *J. gen. Virol.,* 71, 2463-2466.

Kawase, S. (1985), Pathology associated with densoviruses, *in* "Viral insecticides for biological control" (K. Maramorosch, K.E. Sherman) (pp. 197-231). Academic Press, New York, London.

Kelly, D.C., Moore, N.F., Spilling, C.R., Barwise, A.H. & Walker, O. (1980), Densonucleosis virus structural proteins. *J. gen. Virol.,* 36, 224-235.

Kelly, D.C. & Bud, H.M. (1978), Densonucleosis virus DNA: analysis of fine structure by electron microscopy and agarose gel electrophoresis. *J. gen. Virol.,* 40, 33-43.

Kelly, D.C., Barwise, A.H. & Walker, I.O. (1977), DNA contained by two densonucleosis viruses. *J. Virol.,* 21, 396-407.

Kurstak, E., Vernoux, J.P., Niveleau, A. & Onji, P.A. (1971), Visualisation du DNA du virus de la densonucléose (VDN) à chaînes monocaténaires complémentaires de polarités inverses, plus et moins. *C.R. Acad. Sci.* (Paris) *(Sér. D),* 272, 762-765.

Matthews, R.E.F. (1982), Classification and nomenclature of viruses. *Intervirology,* 17, 1-199.

Meynadier, G., Vago, C., Plantevin, G. & Atger, P. (1964), Virose d'un type inhabituel chez le Lépidoptère *Galleria mellonella* L. *Rev. Zool. agric. appl.,* 63, 207-208.

Nakagaki, M. & Kawase, S. (1980), DNA of a new parvolike virus isolated from the silkworm *Bombyx mori. J. Invertebr. Path.,* 35, 124-133.

Siegl, G., Bates, R.C., Berns, K.I., Carter, B.J., Kelly, D.C., Kurstak, E. & Tattersall, P. (1985), Characteristics and taxonomy of *Parvoviridae. Intervirology,* 23, 61-73.

Southern, E.M. (1979), Measurement of DNA length by gel electrophoresis. *Analyt. Biochem.,* 100, 319-323.

Tijssen, P., Van der Hurk, J. & Kurstak, E. (1976), Biochemical, biophysical, and biological properties of densonucleosis virus. — I. Structural proteins. *J. Virol.,* 17, 686-691.

Truffaut, N., Berger, G., Niveleau, A., May, P., Bergoin, M. & Vago, C. (1967), Recherches sur l'acide nucléique du virus de la densonucléose du lépidoptère *Galleria mellonella* L. *Arch. ges. Virusforsch.,* 21, 469-474.