

Characterization of a new densovirus infecting the German cockroach, *Blattella germanica*

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A new DNA virus (*Parvoviridae: Densovirinae, Densovirus*) was isolated and purified from descendants of field-collected German cockroaches, *Blattella germanica*. Viral DNA and cockroach tissues infected with *B. germanica* densovirus (*BgDNV*) were examined by electron microscopy. Virus particles, about 20 nm in diameter, were observed both in the nucleus and in the cytoplasm of infected cells. Virus DNA proved to be a linear molecule of about 1.2 µm in length. *BgDNV* isolated from infected cockroaches infected successfully and could be maintained in BGE-2, a *B. germanica* cell line. The complete *BgDNV* genome was sequenced and analysed. Five open reading frames (ORFs) were detected in the 5335 nt sequence: two ORFs that were on one DNA strand encoded structural capsid proteins (69.7 and 24.8 kDa) and three ORFs that were on the other strand encoded non-structural proteins (60.2, 30.3 and 25.9 kDa). Three putative promoters and polyadenylation signals were identified. Structural analysis of the inverted terminal repeats revealed the presence of extended palindromes. The genome structure of *BgDNV* was compared with that of other members of the family *Parvoviridae*; the predicted amino acid sequences were aligned and subjected to phylogenetic analyses.

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INTRODUCTION

The family *Parvoviridae* includes autonomously replicating DNA viruses belonging to two subfamilies: *Parvovirinae*, members of which infect vertebrates, and *Densovirinae*, which infect invertebrates (Fédière, 2000). Three genera are recognized within the *Densovirinae*: *Densovirus*, *Iteravirus* and *Brevidensovirus*. Densoviruses infect arthropods, mostly insects, in which densovirus infection has been detected in species of six insect orders (Lepidoptera, Diptera, Orthoptera, Dictyoptera, Odonata and Hemiptera) and in decapod crustaceans (Tijssen & Bergoin, 1995; Shike *et al.*, 2000; van Munster *et al.*, 2003; Wang *et al.*, 2005). Their name, denso-nucleosis virus or densovirus (DNV), refers to histopathological and ultrastructural characteristics of infected cells, the nuclei of which become hypertrophied and contain dense, dark, Feulgen stain-positive virion masses (Vago *et al.*, 1966). In most cases, DNV infection causes death of the host (Tanada & Kaya, 1993).

Icosahedral, non-enveloped DNV particles are 18–26 nm in diameter and harbour a single-stranded, linear DNA of about 4000–6000 nt. Some DNVs (e.g. *AeDNV*, isolated from the mosquito *Aedes aegypti*) contain predominantly an mRNA-complementary DNA strand in their particles, whereas others (e.g. *JcDNV*, isolated from the butterfly *Junonia coenia*) produce particles with different strands in similar amounts (Bergoin & Tijssen, 2000). When both plus and minus strands are packed in particles, total DNA isolation at high ionic strength results in annealing of the complementary strands, yielding double-stranded DNA (dsDNA).

The viral coding sequences are flanked by inverted terminal repeats (ITRs) of about 500 nt that assume hairpin configurations at the ends of DNV DNA and may form secondary-structural elements. The nucleotide sequences of the two ITRs may be identical (e.g. *JcDNV*; Dumas *et al.*, 1992), as in vertebrate adeno-associated viruses, or different (e.g. *AeDNV*; Afanasiev *et al.*, 1991). ITRs play an important role in autonomous replication and packaging of virus DNA (Bergoin & Tijssen, 2000).

The GenBank/EMBL/DDBJ accession number for the *BgDNV* genome sequence described in this paper is AY189948.

Supplementary material is available in JGV Online.

The DNV genomes sequenced so far each contain several open reading frames (ORFs). ORFs occur in one or in both DNA strands, depending on the virus type, and encode structural proteins or viral capsid polypeptides (VP) and non-structural proteins (NS) (Bergson & Tijssen, 2000).

DNVs tend to be highly host-specific, they infect most tissues of their hosts, they do not appear capable of infecting vertebrates and they resist extreme environmental conditions, thus making them effective biological-control agents against populations of agricultural and medically important pests (Corsini *et al.*, 1996; Afanasiev & Carlson, 2000; Carlson *et al.*, 2000). In addition, DNVs provide convenient transduction vectors for delivery of foreign genes that can be used to genetically manipulate insect populations (Afanasiev & Carlson, 2000; Carlson *et al.*, 2000; Bossin *et al.*, 2003). A large portion of the virus genome can be substituted with foreign genes, as long as the terminal sequences, which are required for replication and packaging, remain intact.

Here, we report on the isolation of a new DNV (*Blattella germanica* DNV or BgDNV) from the cockroach *Blattella germanica* and describe its ultrastructure, pathology, histopathology and infectivity for cockroaches and *B. germanica* cell lines. We have cloned and sequenced the viral DNA and compared its genome structure and predicted amino acid sequences with those of other parvoviruses. We conclude that this virus is a new member of the genus *Densovirus*.

METHODS

Total DNA was isolated from *B. germanica* individuals of a cockroach colony (P6) that originated from a natural population in a pig farm in North Carolina, USA. Isolation of total and plasmid DNA, endonuclease digestion, gel electrophoresis and fragment elution from agarose gel were carried out according to published protocols (Sambrook *et al.*, 1989; Mukha *et al.*, 1995).

Cell culture and preparation of infectious inoculum. *B. germanica* cell lines BGE-1 and BGE-2, isolated previously from embryonic tissues (Kurtti & Brooks, 1977), were used to propagate BgDNV. Cells were grown in antibiotic-free L-15B medium supplemented with fetal bovine serum (5%), tryptose phosphate broth (5%) and bovine lipoprotein concentrate (0.1%), pH 7.0 (Munderloh & Kurtti, 1989). Cultures were incubated at 25 °C and fed weekly by replacing spent medium with fresh medium. Subcultures were made every 3–4 weeks with an initial seeding density of approximately 5×10^5 cells ml⁻¹. Cultures were inoculated with virus prepared from insects infected with the P6 virus strain. Individual infected insects were bisected; one part of the body was kept in the freezer, whilst the other was used for total DNA extraction and analysis by agarose-gel electrophoresis (see below). Frozen tissues from the second half were homogenized in cell-free cell-line medium, passed through a 0.22 µm filter and the filtrate, which presumably contained virus particles, was inoculated into flasks containing *B. germanica* cells.

Electron microscopy. DNA preparations were examined by transmission electron microscopy (TEM; magnification $\times 30\,000$). A mixture of virus and plasmid DNA (2:1) was prepared as described in the section on DNA (plasmid) preparation for TEM by Dykstra & Reuss (2003).

For TEM of cells, *B. germanica* tissues were fixed with 4F:1G fixative, rinsed in phosphate buffer, osmicated, dehydrated in an ethanolic series culminating in acetone, infiltrated with Spurr's resin and polymerized at 70 °C. Ultrathin sections were stained with methanolic uranyl acetate and Reynolds' lead citrate (Dykstra & Reuss, 2003).

PCR. PCR amplifications were carried out by using *Taq* DNA polymerase (Promega) and a PTC-100 Thermal Cycler (MJ Research Inc.). Two primers (5'-CAGGATTGCCATAATAGAAG and 5'-CATCATCTTGGTTAGACTGTC) were used for PCR amplification of an approximately 500 bp fragment, spanning the middle region of the virus genome. Each reaction contained 0.1 µg DNA template, 1.5 mM MgCl₂, 1 mM each dNTP and 0.2 pmol each primer. The PCR regimen was as follows: initial template denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 1 min, and a final 7 min elongation step at 72 °C.

Virus-genome cloning. Virus dsDNA was digested with *Pst*I to two fragments and resolved in 0.7% agarose gel (Sambrook *et al.*, 1989). The tailing mixture (200 µl), containing 0.3 µg virus DNA fragment, 100 mM potassium cacodylate (pH 7.2), 2 mM CoCl₂, 0.2 mM dithiothreitol (DTT), 0.1 mM dGTP and 30 U terminal deoxynucleotidyl transferase (Gibco-BRL), was incubated at 37 °C for 1 h. Plasmid DNA (pUC19) was digested with *Pst*I and resolved in 0.7% agarose gel. The linearized DNA was tailed with poly(dC). The reaction mixture (50 µl), containing 0.1 µg plasmid DNA, 100 mM potassium cacodylate (pH 7.2), 2 mM CoCl₂, 0.2 mM DTT, 0.02 mM dCTP and 15 U terminal deoxynucleotidyl transferase, was incubated at 37 °C for 30 min. Virus and plasmid DNAs were precipitated with ethanol, washed with 70% ethanol and dissolved in 50 µl water.

The annealing mixture (10 µl) contained 0.25 µg virus DNA, 0.05 µg plasmid DNA, 10 mM Tris/HCl (pH 8.0), 0.1 M NaCl and 1 mM EDTA. The mixture was incubated at 65 °C for 5 min and at 57 °C for 1 h. After incubation, 5 µl of the mixture was used to transform competent *Escherichia coli* XL2-Blue MRF' cells (Stratagene).

Sequencing and sequence analysis. The plasmids containing fragments of the virus genome (see Results) were sequenced according to Sanger *et al.* (1977) with a dGTP BigDye Terminator kit (Applied Biosystems) on an ABI PRISM 377 sequencer.

Promoters, poly(A) tracts, ORFs and their protein products were predicted and multiple and pairwise comparisons were done with the BCM Search Launcher software package (Smith *et al.*, 1996) (<http://searchlauncher.bcm.tmc.edu>). Secondary structure of ITRs was predicted by using the FOLD program (Zuker & Stiegler, 1981). A thermal-denaturation profile of BgDNV dsDNA was computed according to Poland's algorithm (Poland, 1974; Steger, 1994) with a program available at <http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html>.

Phylogenetic reconstruction. Multiple alignments were performed with CLUSTAL W 1.75 (Thompson *et al.*, 1994). The sequences used in the alignments correspond to the NS-1 amino acid fragment located between two highly conserved replication-initiation and helicase motifs (van Munster *et al.*, 2003) of the following viruses: *Diatraea saccharalis* DNV (DsDNV), JcDNV, *Galleria mellonella* DNV (GmDNV), *Aedes albopictus* DNV (AaDNV), *Bombyx mori* DNV (BmDNV-5), infectious hypodermal and hematopoietic necrosis virus (IHHNV), *Casphalia extranea* DNV (CeDNV), *Periplaneta fuliginosa* DNV (PfDNV) and BgDNV.

Phylogenetic trees were constructed by using the cluster algorithm TreeTop (http://www.genebee.msu.su/services/phtree_reduced.html). In the clustering algorithm, the distance between groups of sequences is used for setting of the branching order. This distance is defined as the arithmetic mean of pairwise distances between elements of the two

groups. The root is determined as a point on one of the branches such that the distances from it to all hanging nodes (corresponding to sequences) are equal. Confidence levels were estimated by using bootstrap-resampling procedures (1000 trials).

RESULTS AND DISCUSSION

Virus detection, purification, pathology and histopathology

Electrophoresis of cockroach total DNA in 0.7% agarose gel revealed an additional DNA band of approximately 5 kb (Fig. 1, lane 2) in some *B. germanica* individuals. The cockroach colony (P6) was maintained in the laboratory for 5 years and it originated from cockroaches captured in an infested pig farm in North Carolina, USA. Most of the individuals possessing the ~5 kb band also displayed several symptoms of pathology, including lethargy, flaccidity, poorly coordinated movements and partial or complete paralysis of the hind legs. Similar symptoms have been reported for DNV infection of other insects, including the cockroach *Periplaneta fuliginosa* and the cricket *Acheta domestica* (Meynadier *et al.*, 1977; Suto *et al.*, 1979; Tanada & Kaya, 1993; Hu *et al.*, 1994).

Electron microscopy of ultrathin tissue sections of infected individuals revealed that this virus exhibits polytropic pathology, infecting cells of the digestive system and fat body. Cells of different tissues were similar in cytopathological features, all having unusually structured chromatin with most of the nucleoplasm occupied by virus particles. Particles of approximately 20 nm in diameter were observed both within the nucleus and in the cytoplasm of infected cells. In the nucleus, virus particles were closely packed in an electron-dense virogenic stroma (Fig. 2a), which occasionally

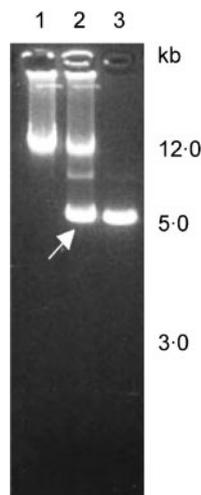


Fig. 1. Agarose-gel electrophoresis (0.7%) of total DNA isolated from uninfected (lane 1) and infected (lane 2) *B. germanica* individuals and of purified virus DNA (lane 3). Virus DNA is indicated by an arrow.

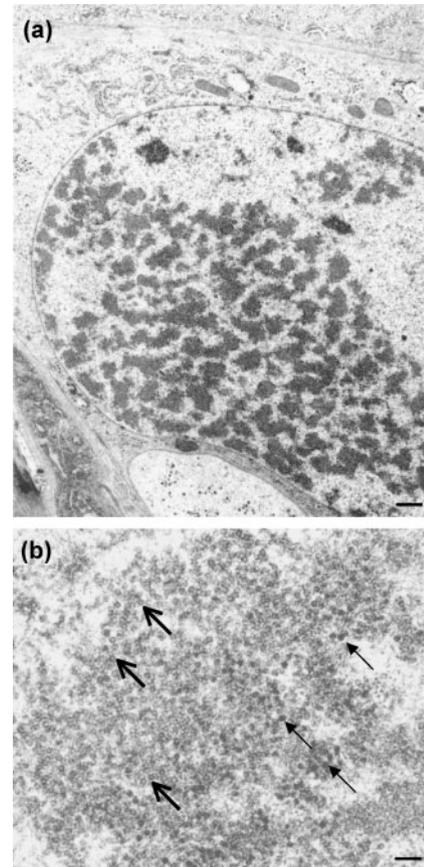


Fig. 2. Electron-microscopic image of *BgDNV*-infected cells of the *B. germanica* digestive tract. (a) Virogenic stroma and (b) virus particles having an electron-transparent core and an electron-dense wall (thick arrows) or seen as electron-dense spheres (thin arrows). Bars, 1 μm (a); 100 μm (b).

formed paracrystalline arrays. Virus particles could be divided into two types based on ultrastructural detail: those having an electron-transparent core surrounded by an electron-dense wall and others exhibiting electron-dense spheres (Fig. 2b). All of these features are characteristic of DNVs (Garzon & Kurstak, 1976; Chao *et al.*, 1984, 1985).

The ~5 kb fraction was isolated from agarose gels and purified (Fig. 1, lane 3). TEM indicated that the purified fraction contained linear DNA of approximately 1.2 μm in length (Fig. 3). The DNA proved to be double-stranded; its restriction map was constructed with *Bg*III, *Eco*RI and *Pst*I (Fig. 4).

Infection of *B. germanica* cell lines

Maintenance of the virus, analysis of its life cycle and studies of integration into the host genome are best done with host-cell lines. We infected, by using virus particles, two virus-free *B. germanica* cell lines, BGE-1 and BGE-2 (Kurtti & Brooks, 1977). Line BGE-1 was isolated from embryos in the germ-band stage and BGE-2 from embryos undergoing dorsal

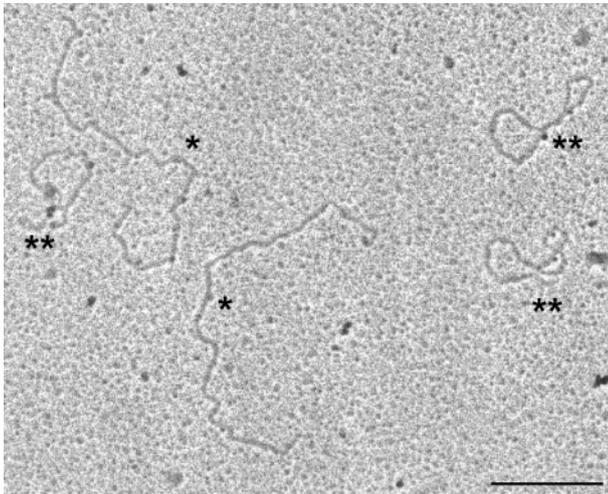


Fig. 3. Electron-microscopic image of (*) *BgDNV* DNA and (**) marker circular DNA of pUC19. Bar, 300 nm.

closure. Originally isolated in a culture medium patterned after cockroach haemolymph (Landureau & Jollès, 1969), these cell lines were adapted to L-15B, a medium designed specifically for tick-cell culture (Munderloh & Kurtti, 1989), but which supports cells from a wide range of different arthropod species (Kurtti *et al.*, 2005).

The filtrate prepared from infected P6 cockroaches was infectious for cultured *B. germanica* BGE-2 cells, but not for BGE-1 cells. The amount of virus replication in these two different cell lines is shown in Fig. 5. The quantity of *BgDNV* DNA (relative to the genomic DNA) increased dramatically during the first 20 days after infection of the BGE-2 cells. However, no virus DNA was found in total DNA extracts of infected BGE-1 cells. The results with BGE-2 show conclusively that *BgDNV* alone is responsible for the infection. The two lines derived from two different embryonic stages are made up of distinctly different cell types. The tetraploid BGE-1 cells are mainly spindle-shaped or multipolar, whereas the diploid BGE-2 cells are round, smaller and contain many cytoplasmic inclusions. We are presently investigating the details of *BgDNV* cell and tissue specificity.

Virus DNA cloning

Full-length DNV genomes are difficult to clone in a circular plasmid vector, because of instability of their extended

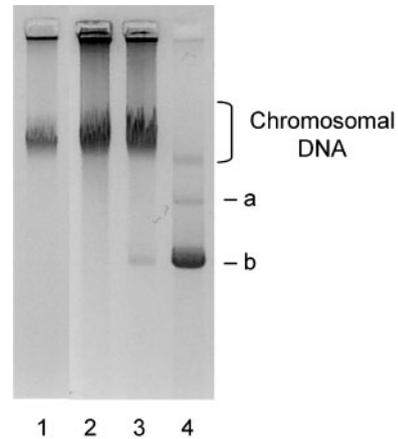


Fig. 5. Dynamics of virus replication in the BGE-1 and BGE-2 cell lines. Agarose-gel electrophoresis (0.7%) of total DNA isolated from infected BGE-1 cells (lane 1) and uninfected (lane 2) and infected (lanes 3 and 4) BGE-2 cells. Lane 3 shows total DNA isolated from infected cells 10 days after virus infection and lanes 1 and 4 show total DNA isolated from infected cells 20 days after virus infection. Multimeric (a) and monomeric (b) virus forms are shown, confirmed by Southern analysis (data not shown).

terminal palindromic regions when both (left and right) are present in the plasmid together (Deiss *et al.*, 1990). An *EcoRI*–*EcoRI* fragment (Fig. 4a) spanning the middle part of the virus genome was cloned by standard methods (Sambrook *et al.*, 1989) in pUC19 linearized by the restriction enzyme *EcoRI*. To clone virus fragments containing the ITRs, we used the following approach.

Virus dsDNA, isolated from agarose gel, was digested with *PstI*. The fragments containing the ITR regions (Fig. 4b, c) were cloned separately in the pUC19 vector. The fragments (Fig. 4b, c) and pUC19 DNA, linearized by the restriction enzyme *PstI*, were 3'-tailed with poly(dG) and poly(dC), respectively, annealed and then used to transform *E. coli*. The cloned fragments, each containing one end of the virus genome, remained stable in the recombinant plasmids maintained in *E. coli* XL2-Blue MRF' cells.

The resulting plasmids, containing fragments a, b and c (Fig. 4a–c), were used to sequence the *B. germanica* virus genome.

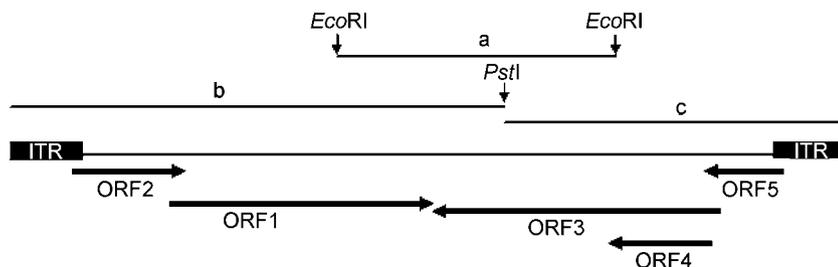


Fig. 4. ORF arrangement and *BgDNV* DNA cloning. Restriction map of the virus DNA and virus fragments cloned in the pUC19 plasmid. (a) *EcoRI*–*EcoRI*; (b) *PstI*–left ITR; (c) *PstI*–right ITR. Arrows indicate the direction of ORF transcription.

Nucleotide sequence of *Bg*DNV genome and structure of its ITRs

The *Bg*DNV genome is 5335 nt (GenBank accession no. AY189948). Pairwise comparisons with other DNV sequences available from GenBank/EMBL revealed only low similarity (48–51 %) with the *Bg*DNV genome (data not shown). Nevertheless, several motifs proved to be evolutionarily conserved (100 % identity) among DNVs isolated from insects of two distant orders, Lepidoptera (moths and butterflies) and Blattodea (cockroaches) (Fig. 6). The motifs are in the region of nt 2923–3221 of the *Bg*DNV genome (Fig. 6) and correspond to the 3' end of ORF3 (Fig. 4). Therefore, motifs 1 and 2 may be used to construct universal degenerate primers suitable for seeking new insect DNVs.

The right and left ends of the *Bg*DNV genome are, respectively, 216 and 217 nt and form ITRs (Fig. 7). Analysis with the BLAST program (Altschul *et al.*, 1990) did not reveal any appreciable similarity between these and other virus sequences available from GenBank/EMBL (data not shown). Imperfect palindromes of 192 nt that are contained in the *Bg*DNV ITRs may form secondary structures. By using the FOLD program (Zuker & Stiegler, 1981), we identified the most probable secondary structure of *Bg*DNV ITR (see Supplementary Fig. S1, available in JGV Online). Parvovirus ITRs may form three types of secondary structures (T-, Y- or I-shaped hairpins), which are important for replication (Siegl & Tratschin, 1987; Bergoin & Tijssen, 2000). In contrast to adeno-associated viruses (Srivastava *et al.*, 1983) and *Jc*DNV (Dumas *et al.*, 1992), *Bg*DNV ITRs produce I-shaped, but not T- or Y-shaped, hairpins. A similar ITR secondary structure is characteristic of *B19 virus*, a human parvovirus (Shade *et al.*, 1986), *Bombyx mori* (moth) DNV 1 (*Bm*DNV-1) (Bando *et al.*, 1990) and *P. fuliginosa* DNV (*Pf*DNV) (Yamagishi *et al.*, 1999). Thus, the secondary structure of ITRs differs between two evolutionarily related DNVs, *Gm*DNV and *Jc*DNV (approx. 90 % genomic DNA similarity). The functional significance of the difference in ITR secondary structure among DNVs is still obscure.

Possibly, changes resulting in a new ITR conformation take place in early divergence, suggesting saltatory evolution of the taxon (Altukhov, 2003).

The G + C content of *Bg*DNV ITRs (59.0 mol%) is higher than that of the entire virus genome (39.6 mol%), which may contribute to the stability of the ITR secondary structure. A thermal-denaturation profile was constructed for the first 950 nt (Fig. 7, on the left) of *Bg*DNV DNA according to Poland's algorithm (Poland, 1974; Steger, 1994). The central region of the virus genome proved to have a denaturation temperature of approximately 80 °C, whilst two peaks (approx. 95 °C) were observed for ITRs (see Supplementary Fig. S2, available in JGV Online). Hence, the palindromes possibly play an important role in replicative dsDNA stabilization (Siegl & Tratschin, 1987). Whilst the G + C content of the virus generally reflects the G + C content of its host cells (Bergoin & Tijssen, 2000), the ITR G + C content is usually high in members of the family *Parvoviridae*, being 60 % in *Pf*DNV (Yamagishi *et al.*, 1999), 50 % in *Bm*DNV (Bando *et al.*, 1990) and 46–60 % in autonomously replicating parvoviruses (Bloom *et al.*, 1988). An exception is *Ae*DNV, with ITRs having only 27 mol% G + C (Afanasiev *et al.*, 1991).

Genome organization

ORFs and deduced amino acid sequences. The *Bg*DNV genome contains five ORFs, two (1 and 2) on one strand and three (3–5) on the other (Fig. 4). The ORF arrangement is typical for the genus *Densovirus* (Bergoin & Tijssen, 2000).

ORF1 (1887 nt; nt 922–2808) encodes a predicted protein (PP) of 628 amino acid residues, corresponding to a molecular mass of 69.7 kDa. ORF2 (690 nt; nt 243–932) encodes a PP of 229 residues (24.8 kDa). ORF3 (1594 nt; nt 440–2811) encodes a PP of 530 residues (60.2 kDa). ORF4 (790 nt; nt 4397–3608) encodes a PP of 262 residues (30.3 kDa). ORF5 (652 nt; nt 5055–4404) encodes a PP of 216 residues (25.9 kDa). The amino acid sequences and

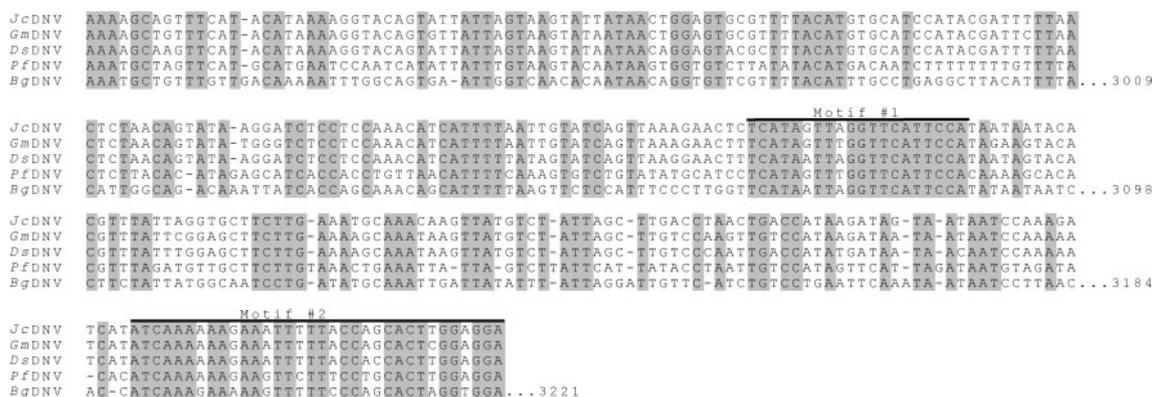


Fig. 6. Comparison of a fragment of *Bg*DNV ORF3 with corresponding sequences of other insect DNVs. Identical nucleotides are shaded and conserved motifs 1 and 2 are indicated.

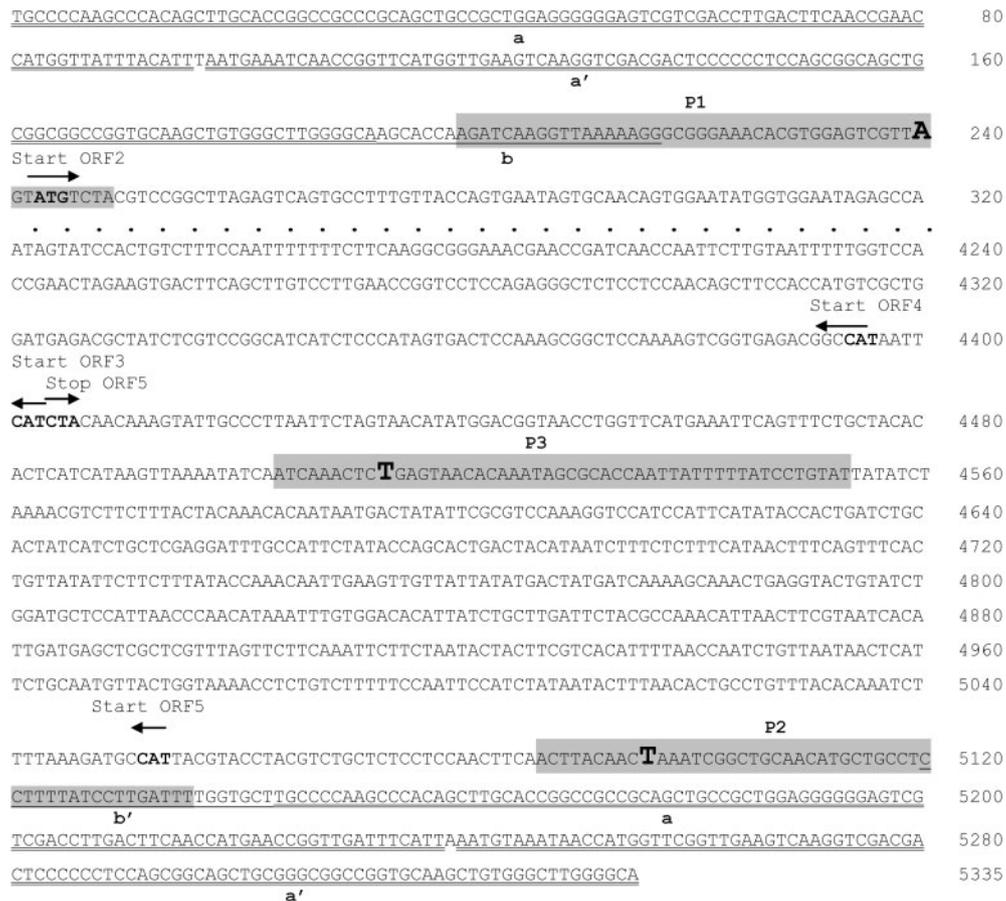


Fig. 7. Nucleotide sequence of ITRs in the *BgDENV* genome. Imperfect palindromic sequences *a* and *a'* (double-underlined) and *b* and *b'* (underlined) are shown. Translation-initiation codons are in bold. Putative promoters (P1, P2 and P3) are shaded. Transcription-initiation start points are shown as large, bold letters. Arrows indicate the direction of transcription.

putative functions of the deduced protein products are listed in Supplementary Table S1, available in JGV Online.

The predicted *BgDENV* protein sequences were compared with protein sequences of other DNVs with an adapted version of the BLAST program (Altschul *et al.*, 1997). The total similarity was very low. However, several relatively short motifs showed substantial homology, being evolutionarily conserved among various insect DNVs, including *BgDENV*, *PfDENV*, *JcDENV*, *GmDENV*, *DsDENV* and *Myzus persicae* DNV (*MpDENV*) (see Supplementary Table S1, available in JGV Online). The putative functions of the PPs corresponding to the ORFs are as following: ORF1 and ORF2, capsid proteins; ORF3, non-structural protein (NS-1), which is important for virus replication (Bergoin & Tijssen, 2000); ORF4 and ORF5, non-structural proteins with unknown function.

Promoters. We used two algorithms for predicting putative transcription-regulatory sequences: Bucher's algorithm (Bucher & Trifonov, 1986; Bucher, 1990) and NNPP, the Neural Network Promoter Prediction program (Reese, 2001; http://www.fruitfly.org/seq_tools/promoter.html). A

putative promoter, P1, with a score of 1.0, was revealed at nt 200–249, with a transcription-initiation start point at nt 240 (Fig. 7). P1 probably controls transcription of ORF1 and ORF2. Both algorithms revealed a second putative promoter sequence, P2, on the second strand between nt 5136 and 5086, with a transcription-initiation start point at nt 5095 (Fig. 7). The transcription of ORF3–ORF5 is probably controlled by P2. A third promoter, P3, with a score of 0.88 in the NNPP program, is on the same strand as P2 between nt 4553 and 4504, with a transcription-initiation start point at nt 4513; P3 might control the transcription of ORFs 3 and 4.

Polyadenylation signals. Polyadenylation signals were found in positions 937–942 and 2818–2823 on the DNA strand containing ORF1 and ORF2. The complementary strand contains an adenylation signal at position 2810–2815.

Like other DNVs, *BgDENV* contains an ambisense-coding genome, with the structural and non-structural proteins being encoded by several genes in opposite directions (Fig. 4). Nevertheless, the genome structure of *BgDENV* has

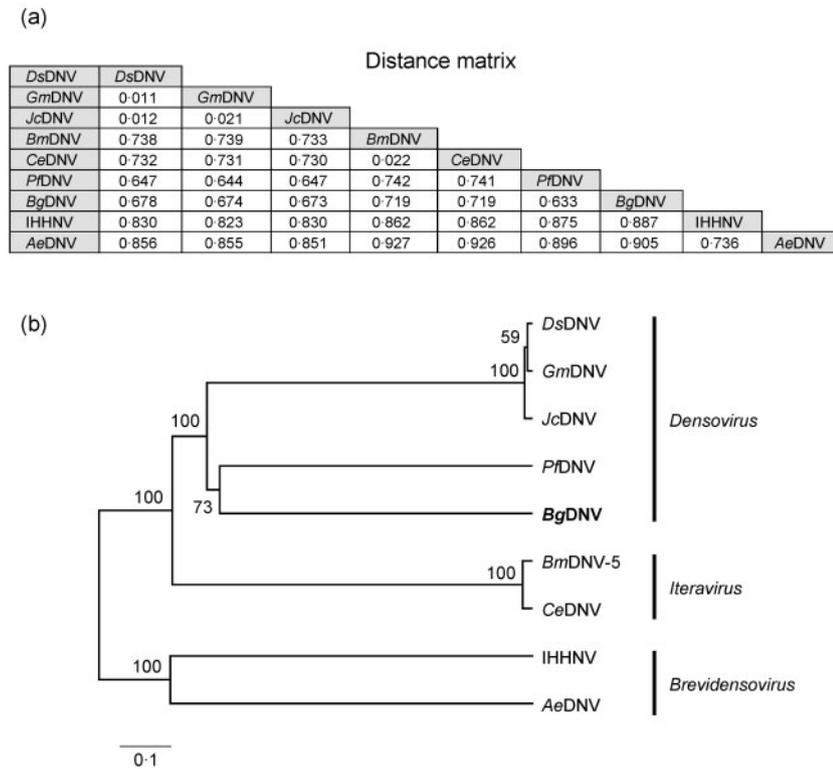


Fig. 8. Phylogenetic analysis of *BgDNV*. The predicted amino acid sequences corresponding to the highly conserved fragments of NS-1 of the insect parvoviruses were aligned and used to produce (a) a distance matrix and (b) a phylogenetic tree. Bootstrap values are indicated as numbers at each branch (1000 replications). Bar, 10% distance.

several unique features compared with that of other insect DNVs, especially concerning ORFs encoding capsid proteins. The two *BgDNV* ORFs encoding structural proteins each contain a polyadenylation signal, whereas synthesis of *JcDNV*, *GmDNV* and *Mythimna loreyi* DNV (*MIDNV*) capsid proteins may be initiated from several codons of one ORF (Dumas *et al.*, 1992; Gross *et al.*, 1990; Tijssen *et al.*, 2003; Fédère *et al.*, 2004). As in *JcDNV*, *GmDNV*, *MIDNV* and *PfDNV* (Yamagishi *et al.*, 1999), one promoter, P1, controls transcription of the structural genes in the *BgDNV* genome. The genome of *MpDNV* (van Munster *et al.*, 2003), as in *BgDNV*, has two ORFs encoding structural proteins, but each ORF is under the control of its own promoter. Possibly, in *BgDNV*, two mRNAs are synthesized from the P1 promoter. Transcription starts from a common site and is terminated downstream of the polyadenylation site at nt 937–942 in the case of the ORF2 mRNA or at nt 2818–2823 in the case of the other, larger mRNA. Translation of the large mRNA may be initiated at several codons; one of the proteins may also be synthesized from the ORF2 mRNA. To explain such an unusual genome structure, it is reasonable to assume that the protein encoded by ORF2 is prevalent in the *BgDNV* capsid.

Transcription of the non-structural ORFs in *BgDNV* is controlled by two promoters, P2 and P3 (Fig. 7). The polyadenylation signal is at position 2810–1815, exactly at the end of ORF3. Possibly, one mRNA is synthesized from P2 and its translation starts at different sites to yield non-structural proteins. Moreover, it is possible that regulation of the P2 and P3 promoters is different and that, at certain

stages of the virus life cycle, only P3 is active and only ORF3 and ORF4 are expressed. It should be noted that such a structure is not common for all DNVs. Thus, the *PfDNV* (Yamagishi *et al.*, 1999) genes for non-structural proteins are transcribed from two promoters, but in *JcDNV*, *GmDNV*, *MpDNV* and *MIDNV* (Bergoin & Tijssen, 2000; Tijssen *et al.*, 2003; van Munster *et al.*, 2003; Fédère *et al.*, 2004), genes for non-structural proteins are transcribed from one promoter. Clearly, comparative genome analysis of insect DNVs may contribute greatly to our understanding of DNV evolution.

Densonucleosis pathology and genomic features (single-stranded DNA, ITRs and two strands that encode regulatory and structural proteins, respectively) suggest that the new pathogen that we discovered in *B. germanica* is a member of the genus *Densovirus* in the subfamily *Densovirinae*, family *Parvoviridae*. Phylogenetic analysis of the deduced amino acid sequences of a highly conserved region of NS-1 confirmed this taxonomic placement: *BgDNV* clustered with other members of the genus *Densovirus*, whereas *Iterovirus* and *Brevidensovirus* clustered separately as more distantly related genera (Fig. 8).

B. germanica is an important pest of residential and other structural environments and a major aetiological agent of allergic disease and asthma, especially among inner-city children (Rosenstreich *et al.*, 1997). The tendencies of *B. germanica* to aggregate and to engage in coprophagy and cannibalism (Kopanic *et al.*, 2001) might make it especially susceptible to horizontal *BgDNV* transmission. We initiated

screening of cockroach populations in the USA and Russia. Total DNA was extracted from cockroaches collected at three separate swine farms (NC, USA) and at 27 separate apartments in Moscow, Russia. Thirty individuals were analysed from each population. Two primers (see Methods) were used for PCR amplification of an approximately 500 nt fragment, spanning the middle region of the virus genome. We found virus-infected cockroaches in two swine farms, with 50 and 20 % infected insects (data not shown). Virus-infected cockroaches were not found in the third swine farm or in any of the Moscow populations. The mechanisms of BgDNV spreading into uninfected cockroaches are unknown. Two ways of virus transmission are conceivable: via virus particles (epidemiologically) and via chromosomes with integrated virus (genetically), similar to retroviruses and retroelements. We are presently investigating whether BgDNV integrates into the host genome and the possible mechanisms of virus activation.

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REFERENCES

- Afanasiev, B. & Carlson, J. (2000). Densovirinae as gene transfer vehicles. *Contrib Microbiol* **4**, 33–58.
- Afanasiev, B. N., Galyov, E. E., Buchatsky, L. P. & Kozlov, Y. V. (1991). Nucleotide sequence and genomic organization of *Aedes* densovirus. *Virology* **185**, 323–336.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Altukhov, Y. P. (2003). *Genetic Processes in Populations*, 3rd edn, p. 431. Moscow: Akademkniga.
- Bando, H., Choi, H., Ito, Y. & Kawase, S. (1990). Terminal structure of a densovirus implies a hairpin transfer replication which is similar to the model for AAV. *Virology* **179**, 57–63.
- Bergoin, M. & Tijssen, P. (2000). Molecular biology of *Densovirinae*. *Contrib Microbiol* **4**, 12–32.
- Bloom, M. E., Alexandersen, S., Perryman, S., Lechner, D. & Wolfenbarger, J. B. (1988). Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and a pathogenic strain of ADV. *J Virol* **62**, 2903–2915.
- Bossin, H., Fournier, P., Royer, C., Barry, P., Cérutti, P., Gimenez, S., Couble, P. & Bergoin, M. (2003). *Junonia coenia* densovirus-based vectors for stable transgene expression in Sf9 cells: influence of the densovirus sequences on genomic integration. *J Virol* **77**, 11060–11071.
- Bucher, P. (1990). Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J Mol Biol* **212**, 563–578.
- Bucher, P. & Trifonov, E. N. (1986). Compilation and analysis of eukaryotic POL II promoter sequences. *Nucleic Acids Res* **14**, 10009–10026.
- Carlson, J., Afanasiev, B. & Suchman, E. (2000). Densovirus as transducing vectors for insects. In *Insect Transgenesis: Methods and Applications*, pp. 139–159. Edited by A. M. Handler & A. A. James. New York: CRC Press.
- Chao, Y. C., Young, S. Y., III & Kim, K. S. (1984). Cytopathology of the soybean looper, *Pseudoplusia includens*, infected with the *Pseudoplusia includens* icosahedral virus. *J Invertebr Pathol* **45**, 16–23.
- Chao, Y. C., Young, S. Y., III, Kim, K. S. & Scott, H. A. (1985). A newly isolated densovirus from *Pseudoplusia includens* (Lepidoptera: Noctuidae). *J Invertebr Pathol* **46**, 70–82.
- Corsini, J., Afanasiev, B., Maxwell, I. H. & Carlson, J. O. (1996). Autonomous parvovirus and densovirus gene vectors. *Adv Virus Res* **47**, 303–351.
- Deiss, V., Tratschin, J.-D., Weitz, M. & Siegl, G. (1990). Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini. *Virology* **175**, 247–254.
- Dumas, B., Jourdan, M., Pascaud, A.-M. & Bergoin, M. (1992). Complete nucleotide sequence of the cloned infectious genome of *Junonia coenia* densovirus reveals an organization unique among parvoviruses. *Virology* **191**, 202–222.
- Dykstra, M. J. & Reuss, L. E. (2003). *Biological Electron Microscopy: Theory, Techniques, and Troubleshooting*, 2nd edn. New York: Springer.
- Fédière, G. (2000). Epidemiology and pathology of *Densovirinae*. *Contrib Microbiol* **4**, 1–11.
- Fédière, G., El-Far, M., Li, Y., Bergoin, M. & Tijssen, P. (2004). Expression strategy of densovirus from *Mythimna loreyi*. *Virology* **320**, 181–189.
- Garzon, S. & Kurstak, E. (1976). Ultrastructural studies on the morphogenesis of the densovirus (parvovirus). *Virology* **70**, 517–531.
- Gross, O., Tijssen, P., Weinberg, D. & Tal, J. (1990). Expression of densovirus *GmDNV* in *Galleria mellonella* larvae: size analysis and *in vitro* translation of viral transcription products. *J Invertebr Pathol* **56**, 175–180.
- Hu, Y., Zheng, J., Iizuka, T. & Bando, H. (1994). A densovirus newly isolated from the smoky-brown cockroach *Periplaneta fuliginosa*. *Arch Virol* **138**, 365–372.
- Kopanic, R. J., Jr, Holbrook, G. L., Sevala, V. & Schal, C. (2001). An adaptive benefit of facultative coprophagy in the German cockroach *Blattella germanica*. *Ecol Entomol* **26**, 154–162.
- Kurtti, T. J. & Brooks, M. A. (1977). Isolation of cell lines from embryos of the cockroach, *Blattella germanica*. *In Vitro* **13**, 11–17.
- Kurtti, T. J., Simser, J. A., Baldridge, G. D., Palmer, A. T. & Munderloh, U. G. (2005). Factors influencing *in vitro* infectivity and growth of *Rickettsia peacockii* (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Acari, Ixodidae). *J Invertebr Pathol* **90**, 177–186.
- Landureau, J.-C. & Jollès, P. (1969). Etude des exigences d'une lignée de cellules d'insectes (souches) EPA. I. Acides aminés. *Exp Cell Res* **54**, 391–398 (in French).
- Meynadier, G., Matz, G., Veyrunes, J.-C. & Bres, N. (1977). Virose de type densovirus chez les orthoptères. *Ann Soc Entomol Fr (N S)* **13**, 487–493 (in French).

- Mukha, D. V., Sidorenko, A. P., Lazebnaya, I. V. & Zakharov, I. A. (1995).** Structural variation of the ribosomal gene cluster in the class Insecta. *Genetika* **31**, 1249–1253 (in Russian).
- Munderloh, U. G. & Kurtti, T. J. (1989).** Formulation of medium for tick cell culture. *Exp Appl Acarol* **7**, 219–229.
- Poland, D. (1974).** Recursion relation generation of probability profiles for specific-sequence macromolecules with long-range correlations. *Biopolymers* **13**, 1859–1871.
- Reese, M. G. (2001).** Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. *Comput Chem* **26**, 51–56.
- Rosenreich, D. L., Eggleston, P., Kattan, M. & 8 other authors (1997).** The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N Engl J Med* **336**, 1356–1363.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* **74**, 5463–5467.
- Shade, R. O., Blundell, M. C., Cotmore, S. F., Tattersall, P. & Astell, C. R. (1986).** Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. *J Virol* **58**, 921–936.
- Shike, H., Dhar, A. K., Burns, J. C., Shimizu, C., Jousset, F. X., Klimpel, K. R. & Bergoin, M. (2000).** Infectious hypodermal and hematopoietic necrosis virus of shrimp is related to mosquito brevidensoviruses. *Virology* **277**, 167–177.
- Siegl, G. & Tratschin, J. D. (1987).** Parvoviruses: agents of distinct pathogenic and molecular potential. *FEMS Microbiol Lett* **46**, 433–450.
- Smith, R. F., Wiese, B. A., Wojzynski, M. K., Davison, D. B. & Worley, K. C. (1996).** BCM Search Launcher – an integrated interface to molecular biology data base search and analysis services available on the World Wide Web. *Genome Res* **6**, 454–462.
- Srivastava, A., Lusby, E. W. & Berns, K. I. (1983).** Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J Virol* **45**, 555–564.
- Steger, G. (1994).** Thermal denaturation of double-stranded nucleic acids: prediction of temperatures critical for gradient gel electrophoresis and polymerase chain reaction. *Nucleic Acids Res* **22**, 2760–2768.
- Suto, C., Kawamoto, F. & Kumada, N. (1979).** A new virus isolated from the cockroach, *Periplaneta fuliginosa* (Serville). *Microbiol Immunol* **23**, 207–211.
- Tanada, Y. & Kaya, H. K. (1993).** *Insect Pathology*. New York: Academic Press.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tijssen, P. & Bergoin, M. (1995).** Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. *Semin Virol* **6**, 347–355.
- Tijssen, P., Li, Y., El-Far, M., Szelei, J., Letarte, M. & Zádori, Z. (2003).** Organization and expression strategy of the ambisense genome of densonucleosis virus of *Galleria mellonella*. *J Virol* **77**, 10357–10365.
- Vago, C., Duthoit, J. L. & Delahaye, F. (1966).** Les lésions nucléaires de la “Virose à noyaux denses” du lépidoptère *Galleria mellonella*. *Arch Virol* **18**, 344–349 (in French).
- van Munster, M., Dulleman, A. M., Verbeek, M., van den Heuvel, J. F. J. M., Reinbold, C., Braut, V., Clériver, A. & van der Wilk, F. (2003).** A new virus infecting *Myzus persicae* has a genome organization similar to the species of the genus *Densovirus*. *J Gen Virol* **84**, 165–172.
- Wang, J., Zhang, J., Jiang, H., Liu, C., Yi, F. & Hu, Y. (2005).** Nucleotide sequence and genomic organization of a newly isolated densovirus infecting *Dendrolimus punctatus*. *J Gen Virol* **86**, 2169–2173.
- Yamagishi, J., Hu, Y., Zheng, J. & Bando, H. (1999).** Genome organization and mRNA structure of *Periplaneta fuliginosa* densovirus imply alternative splicing involvement in viral gene expression. *Arch Virol* **144**, 2111–2124.
- Zuker, M. & Stiegler, P. (1981).** Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information. *Nucleic Acids Res* **9**, 133–148.