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Purification and Partial Characterization of an Entomopoxvirus (DlEPV) from a Parasitic Wasp of Tephritid Fruit Flies.

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Abstract

An insect poxvirus [entomopoxvirus (EPV)] occurs in the poison gland apparatus of female *Diachasmimorpha longicaudata* , a parasitic wasp of the Caribbean fruit fly, *Anastrepha suspensa* and other tephritid fruit flies. The DlEPV virion is 250-300 nm in diameter, has a "bumpy" appearance and a unipartite double stranded DNA genome of 290-300 kb. DlEPV DNA restriction fragment profiles differed from those reported for *Amsacta moorei* EPV (AmEPV) and *Melanoplus sanguinipes* EPV (MsEPV), the only two EPVs whose genomes have been sequenced, and from those reported for vaccinia (Vac), a vertebrate poxvirus (chordopoxvirus, ChPV). Blast search and ClustalW alignment of the amino acids deduced from the 2316 nucleotides of a DlEPV DNA fragment cloned from an EcoR1 genomic library revealed 75-78% homology with the putative DNA-directed RNA polymerases of AmEPV, MsEPV, and two ChPV homologs of the Vac J6R gene. Of the deduced 772 amino acids in the DlEPV sequence, 28.4% are conserved/substituted among the four poxviruses aligned, 12.9% occur in at least one EPV, 6.5% in at least one ChPV, 3.1 % in at least one EPV and one ChPV, and 49.1% occur only in DlEPV. Although the RI-36-1 fragment represents a portion of the gene, it contains nucleotides that encode the NADFDGDE consensus sequence of known DNA-directed RNA polymerases. Western blots using a mouse polyclonal anti-DlEPV serum recognized six major protein bands in combined fractions of sucrose-purified DlEPV, at least one band in homogenates of male and female wasps, and at least two bands in host hemolymph that contained DlEPV virions. A digoxigenin-labeled DlEPV genomic DNA probe recognized DNA in dot-blots of male and female wasps. These results confirm that DlEPV is a true EPV and probably a member of the Group C EPVs. Unlike other EPVs, DlEPV does not express the spheroidin protein. Since it also replicates in both the wasp and fly, members of two different insect Orders, DlEPV may represent a new EPV Group, or a subgroup of the Group C viruses.

Keywords: *Anastrepha suspense, Biosteres longicaudatus, Diachasmimorpha longicaudata, Opius longicaudatus,* Caribbean fruit fly, Entomopoxvirinae, Extracellular virus, Hymenoptera: Braconidae, Poxviridae, Symbiotic virus

Abbreviation:

aa	amino acid(s)
AGF	accessory gland filament
AmEPV	EPV of *Amsacta moorei*
ChPV	chordopoxvirus
DlEPV	the entomopoxvirus of *Diachasmimorpha longicaudata*
ds	double stranded
EPV	entomopoxvirus
TEM	transmission electron microscopy
Vac	vaccinia

Introduction

Diachasmimorpha longicaudata (Dl) is a braconid wasp that parasitizes fruit flies including the Caribbean fruit fly, *Anastrepha suspensa* (Lawrence and Akin, 1990; Lawrence, 2000). An EPV-like virus replicates and undergoes morphogenesis in the poison gland apparatus (Fig. 1) of the female wasp, from which it is transmitted to the fruit fly larva host during parasitism (Lawrence and Akin, 1990; Lawrence, 2000). Since EPVs are commonly named after the insects from which they are first isolated or described (Granados, 1973), the virus from *D. longicaudata* is referred to as DlEPV (Lawrence, 2000). DlEPV is unusual in that it replicates in both the wasp and the dipteran host of the wasp but is pathogenic

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only to the dipteran. Furthermore, DlEPV does not express an occlusion body protein (spheroidin) as do all other EPVs (Goodwin et al., 1991; Hall and Moyer, 1991, 1993).

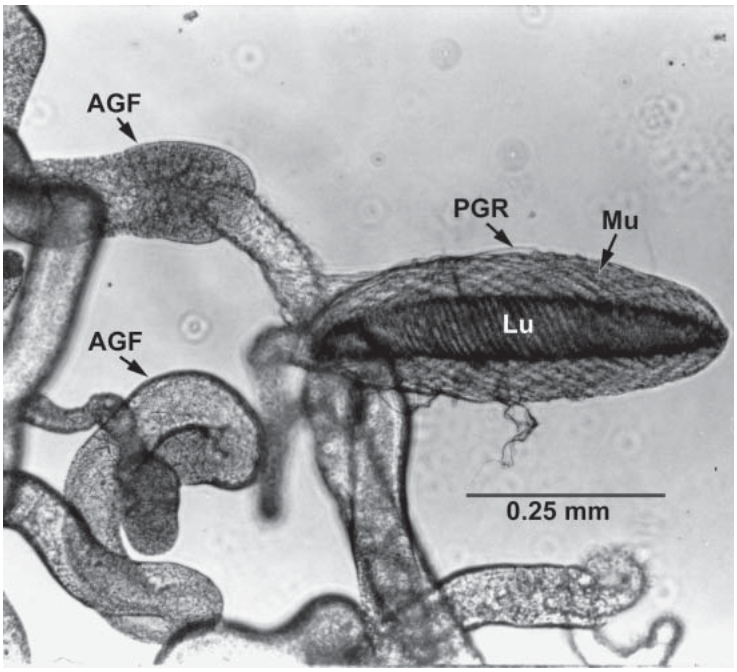


Figure 1. Accessory (poison) gland apparatus from female *Diachasmimorpha longicaudata*. AGF=Accessory (poison) gland filament; Lu=lumen, and Mu=muscles of the poison gland reservoir; PGR=poison gland reservoir.

Poxviruses of insects [SubFamily Entomopoxvirinae (EPVs)] have brick-or ovoid-shaped virions of 165-300 nm x 150-470 nm and double-stranded (ds) DNA of 124-242 kb (Arif, 1984; Adams and Bonami, 1991; Goodwin et al., 1991; Street et al., 1997). Three EPV categories and their prototype species are currently recognized: Group A (Coleoptera-infecting EPVs) - *Melolontha melolontha* (MmEPV); Group B (Lepidoptera- and Orthoptera-infecting EPVs) - *Amsacta moorei* (AmEPV); and Group C (Diptera-infecting EPVs) - *Chironomus luridus* (ClEPV) (Murphy et al., 1995). Viral cores may be unilaterally concave (Genus A), rectangular (Group B) or dumbbell-shaped (Genus C) (Goodwin et al., 1991). All EPVs described to date have proteinaceous (spheroidin) occlusion bodies (Hall and Moyer, 1991, 1993).

This paper describes the purification and partial characterization of DlEPV. The results reported here, together with the viral morphology (Lawrence and Akin, 1990; Lawrence, 2000) and our recent identification of a DlEPV homolog of the rifampicin resistance (rif) gene of poxviruses (unpublished), suggest that DlEPV is a new member of the Entomopoxvirinae. However, the absence of the expression of a spheroidin protein and occlusion bodies in DlEPV could indicate that this virus represents a new EPV Group or a subgroup of Group C. To my knowledge, this is the first symbiotic EPV from a parasitic wasp to be purified and characterized.

Materials and Methods

Rearing

Diachasmimorpha longicaudata (Ashmead) (= *Biosteres* = *Opius longicaudatus*) and *Anastrepha suspensa* (Loew) were reared at 25-27 ºC and 75-80% RH, as previously described (Lawrence et al., 1976; Lawrence, 1988). Mated 5-7-day-old female wasps deprived of hosts were homogenized and used in dot blot and Western blot experiments (see below), or dissected in cold TE (10 mM Tris and 1mM EDTA, pH 8.0) to remove the virus-containing poison gland, as previously described (Lawrence and Akin, 1990). Glands were stored at -80 ºC prior to sucrose density gradient centrifugation or DNA extraction, as described below.

DlEPV Purification by Sucrose Density Gradient Centrifugation

The glands were homogenized in TMN buffer (0.01 M Tris, 1.5 mM MgCl2, 0.1 M NaCl, pH 7.4) in a 0.1 ml Wheaton homogenizer (Fisher Scientific, www1.fishersci.com) and centrifuged at 4,000 x g. The supernatant was then overlaid on a 5-40% (w/w) sucrose gradient and centrifuged at 31,000 x g for 1.5 h at 4 ºC in a Beckman SW60 rotor (Beckman Instruments, www.beckman.com). The resulting bands were each resuspended in TMN then overlaid on a 40-63% (w/w) sucrose gradient and centrifuged at 100,000 x g (1 h at 4 ºC). Each band was collected into a 1.5 ml centrifuge tube, diluted in TE, and centrifuged at 31,000 x g (30 min at 4 ºC). The pellet was resuspended in TE and stored at -80 ºC. Aliquots of each pellet of the purified virus were viewed under the electron microscope after staining with 2% uranyl acetate to reveal salient features of the virion, as previously described (Lawrence and Akin, 1990).

Generation of Anti-DlEPV Polyclonal Antibodies

Three fractions (sucrose bands of ~41-45, 48-50, and 53- 55 %) of the purified virions in TE (450 µl containing 1.1 µg protein/ µl) were combined with an equal volume of PBS (8.0 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4 /L, pH 7.4), and 900 µl 2 x RIBI MPL-TDM emulsion (RIBI Immunochemical Research, Inc.) as adjuvant, and administered subcutaneously to three Balb-c mice (Jackson Labs). Each mouse received 2 x 50 µl initial injections at two ventral groin sites and 1 x 100 µl dorsally. Booster injections of 50 µl/mouse were administered at the same sites as above at three and one-half and six and one-half weeks after the initial injections. Test bleeds (by tail snip) of anaesthetized mice were performed at one and two and one-half weeks after each of the two booster injections. A final injection of 30 µl/mouse was given five weeks after the last booster injection.

Prior to ascites fluid collection, each mouse was primed with 0.5 ml pristane [2,6,10,14-tetramethylpentadecane (Sigma #T-7640, www.sigmaaldrich.com], injected intraperitoneally (IP), about 12 days after the final immunization (see above). Three days later, each mouse was IP-injected with 1 x 10^6 fused HL4 (Sp2/0) cells in 0.5 ml PBS. Ascites fluid was harvested by peritoneal tap, centrifuged for 10-15 min at 3000 rpm at 4 ºC in a Beckman GH 3.7 rotor (Beckman Instruments, www.beckman.com). The supernatant containing the anti-DlEPV polyclonal ascites fluid was then stored at -20 ºC for later use.

Electrophoresis and Western Blotting of DlEPV Proteins

Proteins (~5 µg) from the purified virus were denatured in a 2 x Laemmli (1970) buffer, boiled for 3-5 min, and resolved in a 10-15% gradient SDS-PAGE Phastgel (Pharmacia, www.Pharmacia.com) for 30 min at 40 V along with 25- and 50 kDa mouse IgGs (Sigma) as molecular weight markers. Pre-immune

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mouse serum (~4 µg) was run as a control. The unstained gels were electroblotted to nitrocellulose membranes (30 min) at 20 V. The membranes were then blocked overnight in filtered 5% non-fat dry milk in PBS- 0.2% sodium azide (PBS-AZ), washed 3 x 5 min in PBS-AZ and 0.05% Tween, and incubated for 60 min in anti-DlEPV serum diluted 1:100 in 1% BSA in PBS-AZ. After 3 x 5 min PBS-AZ-Tween washes, the membranes were incubated at room temperature for 60 min in a rabbit anti-mouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:1000 in 1% BSA in PBS-AZ. After 4 x 5 min washes in the same wash buffer as above, the bands were visualized with the phosphatase substrate, NBT/BCIP [nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (-Sigma)].

To identify viral proteins in male and female wasps, adults were homogenized in Laemmli (1970) buffer, boiled for 10 min, then centrifuged for 5 min at 4,000 x g at 4 °C, and the supernatant was electrophoresed in a 12.5% denaturing gel. Proteins were blotted to nitrocellulose membrane as previously described (Shi et al., 1999), probed with the mouse anti-DlEPV polyclonal serum (1:1000) followed by a goat anti-mouse IgG-alkaline phosphatase (1:10,000) secondary antibody, and visualized with NBT/BCIP as above. Hemolymph from 24-36-h-old unparasitized A. suspensa pharate pupae and those parasitized 48-52 h earlier by *D. longicaudata* served as the negative and positive controls, respectively. A single purified DlEPV fraction (~53-55 % sucrose) also served as a positive control. The antibody had a sensitivity of ~25 ng protein.

DNA Purification.

Homogenates of 600 glands/100 µl homogenization buffer [(HB) 10 mM Tris, 100 mM EDTA, 1% SDS] were centrifuged at 4,000 x g and 4 °C for 5 min, and the supernatant used directly for DNA purification in liquid (Sambrook et al., 1989) for dot blot experiments, or by pulse field gel electrophoresis (PFGE) for genome size estimation, as described below. DNA used for restriction enzyme digestion was obtained from sucrose purified virions as described above, followed by purification (Sambrook et al., 1989). The purified DNA was precipitated with isopropanol, washed with 70% ethanol, air-dried and resuspended in TE pH 7.0, and stored at -20 °C until further use.

Estimation of DlEPV Genome Size by Pulse Field Gel Electrophoresis

The size of the DlEPV genome was estimated by resolving the intact DNA in a PFGE CHEF DR II system as described by the manufacturer (BioRad, www.bio-rad.com). Basically, the poison gland homogenate (described above) was mixed 1:1 with 2% low melt SeaKem Gold agarose (www.cambrex.com/) in 0.5 x TBE (45 mM Tris, 45 mM borate, 1.0 mM EDTA, pH 8.3) and added to gel plug molds (BioRad). The plugs (1% agarose) were incubated in a solution of 0.5M EDTA pH 8.0, 10% SDS, and 1 mg/ml proteinase K at 37ºC for 24 h. The digested plugs were washed for 2 x 30 min in 10 mM Tris, 50 mM EDTA and 1 mM PMSF at 50ºC to inactivate proteinase K (Birren and Lai, 1993), and equilibrated in 0.5 x TBE. The plugs were then sealed in gel wells and electrophoresed into 1% low melt agarose in 0.5 x TBE at 14 °C for 16 h at 4 V/cm and an angle of 120°. Initial and final switch intervals were 50 and 90 sec, respectively. The DNA was visualized with 0.05 mg/ml ethidium bromide.

Restriction Endonuclease Digestion of Viral DNA

DlEPV DNA for restriction fragment profiles was incubated for 24 h at 37 °C in the digestion buffer appropriate to the restriction enzyme. Each reaction was terminated by the addition of 0.5 M EDTA (pH 8.0) for a final concentration of 10 mM EDTA (Sambrook et al., 1989). About 3.5 µg digested DlEPV DNA were electrophoresed into 0.7% Seaplaque GTG (www.cambrex.com/) agarose gel in 0.5 x TBE, and visualized with 0.05 mg/ml ethidium bromide.

DlEPV Genomic Library Construction

DNA for genomic library construction was obtained from virions purified in sucrose as described above. Virions were lysed in lysis-denaturation solution from the GNOME kit (Bio 101, www.qbiogene.com), incubated with RNAse (1 mg/ml) at 55 0 C for 15 min, followed by proteinase K treatment (1mg/ml) for 3h. The DNA was precipitated with ethanol, spooled on a Pasteur pipette, dried at room temperature, then resuspended in TE, pH 7.0. The DNA concentration was estimated in a 0.8% agarose gel using a mass ruler (Invitrogen).

About 10 µg of DlEPV DNA were digested with the EcoRI (RI) restriction enzyme (Roche Molecular Biochemicals) and cloned into the EcoRI site of the pBluescript II KS (+/-) phagemid cloning vector (pBS; Stratagene, www.stratagene.com/) using standard protocols (Sambrook et al., 1989). Aliquots of 60 ng of digested DNA and 20 ng linearized pBS were co-incubated with 1 µl ligation buffer and 1U T4 DNA ligase (Roche) at room temperature for 16 h. One microliter of the ligation mix was used to transform supercompetent DH5 alpha E. coli cells (Gibco-BRL, www.lifetech.com). The transformed cells were incubated in SOC broth for 1 h at 37 °C with agitation (225 rpm), plated on selective LB agar medium containing 100 µg/ml ampicillin and 50 µg/ ml Xgal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) (Gibco-BRL), and incubated overnight at 37 °C. Bacterial cells containing the recombinant plasmids were then selected and amplified in 50 ml LB medium containing ampicillin and Xgal, as above. The recombinant plasmids were harvested by the alkaline lysis method (Sambrook et al., 1989). The size of the DlEPV DNA insert in each clone was verified by EcoRI digestion of the recombinant plasmids followed by electrophoresis of the resulting fragments against molecular weight markers into 0.8% agarose gels. The fidelity of the fragments to the DlEPV DNA genome was verified by dot-blot hybridization using a digoxigenin (DIG) (Boehringer-Mannheim, www.roche.com)-labeled DlEPV genomic DNA probe, as described below.

Sequencing of Clones

Recombinant plasmid DNA was purified using the Qiagen miniprep kit (Qiagen, www.quigen.com). The forward and reverse strands of the template DNA were sequenced by the DNA Sequencing Core, University of Florida Interdisciplinary Center for Biotechnology, using commercially available primers and the fluorescence-labeled dideoxynucleotide and Taq dyedeoxy terminator cycle sequencing protocols (Applied Biosystems, home.appliedbiosystems.com). The labeled extension products were

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analyzed on a model 377 automated DNA sequencer (Applied Biosystems).

Sequence Analysis

Nucleotide sequences were aligned into contigs and open reading frames were identified using the Sequencher v4.0 program (Gene Codes Corp., www.genecodes.com). The DlEPV deduced amino acid sequences and their homologs were identified by the Blast database search programs (Altschul et al., 1997). Homologous proteins were retrieved from Genebank and aligned with the deduced DlEPV sequences using the ClustalW multiple alignment program (Thompson et al., 1994). Motifs and consensus sequences were identified through the PROSITE (Hofmann et al., 1999) and InterProScan v3.1 (ftp://ftp.ebi.ac.uk/pub/databases/interpro/iprscan/) database scanning programs.

Random Primed Labeling of DlEPV DNA Probe

Viral DNA was denatured by boiling then DIG-labeled by random priming, using the Genius 1 kit (Boehringer-Mannheim). DNA was incubated at 37 °C for 16 h in the reagents (hexanucleotide mix, dNTP labeling mix, sterile distilled water and Klenow enzyme) provided by the manufacturer, and the reaction was terminated by the addition of 200 mM EDTA (pH 8.0). Upon the addition of glycogen (20 mg/ml), the DNA was precipitated overnight at -20 °C with 3 M NaOAc and cold 100% ethanol. The DNA was pelleted, washed with 70% ethanol, dried, and resuspended in TE.

The labeling efficiency was estimated using an anti-DIG-alkaline phosphatase conjugate (AP) (Boehringer-Mannheim) to probe serial dilutions of DIG-labeled control DNA (provided with the Genius 1 kit) and the DIG-labeled DlEPV DNA. Basically, 1 µl aliquots of each sample was spotted on and bound to a nylon membrane in a UV GeneLinker oven (BioRad) at 125 mjoules for 3 min. The membrane was then incubated for 5 min at room temperature in Genius buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5) and then in a 2% blocking reagent (Genius buffer 2, Boehringer-Mannheim). After incubation of the nylon membrane in 1:5,000 AP in blocking reagent for 5 min at room temperature, the signal was visualized with NBT in Genius buffer 3 [(100 mM Tris, 100 mM NaCl, 50 mM MgCl, pH 9.5 (Boehringer-Mannheim)] in the dark for 30-90 min at room temperature. The labeled control DNA was used to estimate the concentration of the DIG-labeled DlEPV probe.

Detection of DlEPV DNA by Dot-Blot Hybridization

DNA was mixed 10:1 with denaturing solution (4 M NaOH, 100 mM EDTA) and incubated at room temperature for 10 min, then boiled for 2 min. Aliquots of each sample were applied to a nitrocellulose membrane under vacuum on a Bio-Dot microfiltration apparatus (BioRad). The membrane was then air dried, the DNA crosslinked (GeneLinker, BioRad) as described above, then prehybridized in 5 x SSC (750 mM NaCl, 75 mM sodium citrate, pH 7.0), 1% (w/v) blocking reagent, 0.1% N-laurosarcosine, and 0.02% SDS for 4 h at 70 °C, according to the manufacturer's guidelines (Boehringer-Mannheim). The membrane was then incubated in the boiled DIG-labeled DlEPV probe (sensitivity to 0.06 µg DNA) at 70ºC for 24 h, washed in a 2 x, then 0.5 x SSC solution containing 0.1% SDS, at 70 °C. Color development using NBT was as described above for "Random Primed Labeling of DlEPV DNA Probe."

Electron Microscopy

A drop of the purified virus was placed on a formvar-coated copper grid and stained with 1% (w/v) aqueous uranyl acetate, a commonly used negative stain, and viewed under a JOEL 100 CX electron microscope as previously described (Lawrence and Akin, 1990). Host hemocytes infected with DlEPV were prepared for transmission electron microscopy (TEM) following procedures described earlier (Lawrence, 1988; Lawrence and Akin, 1990). Briefly, host larvae and puparia were collected immediately after parasitism and at 4 h intervals until 144 h after parasitism. The puparia were transected under fixative [2% glutaraldehyde in 0.1 M sodium cacodylate (NaCAs), pH 7.2] and fixed for 20 h at 4 °C h. Following 3 x 15 min rinses in fresh NaCAs, specimens were postfixed in 1% osmium tetroxide buffered with 0.1 M NaCAs for 2 h at room temperature, rinsed in deionized water, and dehydrated in a graded series of ethanol-acetone, then infiltrated and embedded in Spurr's resin. Thin sections (60 nm) were stained with 5% acidic uranyl acetate and Reynold's lead citrate and examined in a JEOL 100 CX electron microscope at 80 kV.

Results

Mature DlEPV virions occur in a dense liquid within the lumen of the poison gland reservoir (Fig. 1) of female wasps. The lumen is separated from the surrounding muscles of the gland reservoir by a thick cuticular intima (Lawrence and Akin, 1990). The cuticle appears as oblique striations in the center and along the length of the gland (Fig. 1). Purified virions are 250-300 nm in diameter and intact mature particles have a loose envelope with a "bumpy" or mulberry-like appearance (Fig. 2) similar to that of the vaccinia (Vac) poxvirus (Noyes, 1962) and grasshopper EPVs (Street et al., 1997). TEM revealed a biconcave core with two lateral bodies (not shown, see Lawrence, 2000; Lawrence and Akin, 1990), a characteristic of poxviruses (Moss, 2001). Sucrose fractions of ~41-45, 48-50, and 53- 55 % contained about 50% intact virions, viral cores, and empty viral envelopes, ~25% viral cores plus numerous empty envelopes, and ~70% intact virions plus viral cores and empty envelopes, respectively.

Based on PFGE analysis (Fig. 3), DlEPV has a 290-300 kb double stranded unipartite DNA genome. However, a manual summation of electrophoresed DNA fragment sizes derived from single and double restriction enzyme digestions, suggests a more conservative estimate of the genome is 250-275 kb (Fig. 4). There were no similarities between the fragment profiles of HindIII-digested (not shown) or BamHI-digested (Fig. 4) DlEPV DNA and those published for Vac (Langridge, 1983), AmEPV (Hall and Hink, 1990), and various grasshopper and locust EPVs (Langridge, 1983; Erlandson and Street, 1997), suggesting that DlEPV is a distinctly different virus. The many fragments obtained with EcoRI (cuts at G | AATTC) and EcoRV (cuts at GAT | ATC) digestions compared with fewer obtained from BamHI (cuts at G | GATCC), Pst (cuts at CTGCA | G), and Xho (cuts at C | TCGAG) digestions (Fig. 4), suggest that DlEPV is A-T rich. Indeed, the sequence of the DlEPV RI-36-1 cloned fragment is about 68% A-T rich (Fig. 5a).

The Sequencher program showed that the entire R1-36-1

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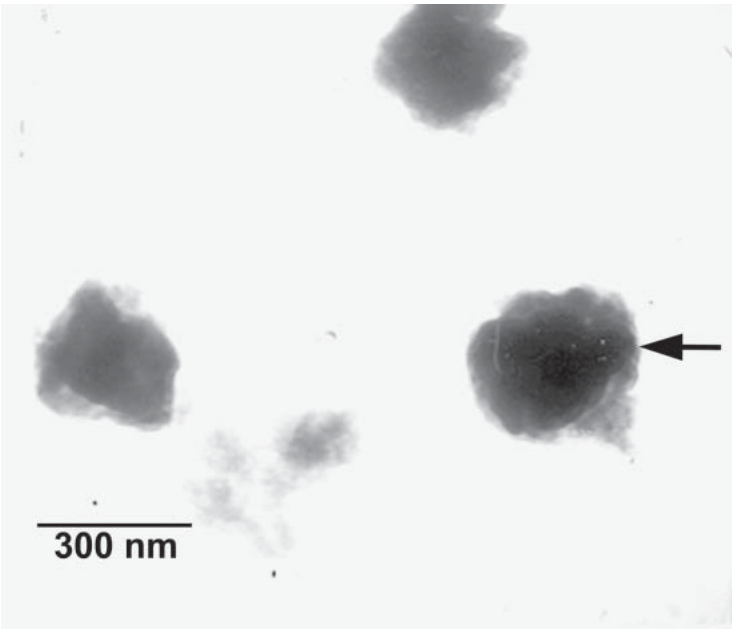


Figure 2. Electron micrograph of sucrose-purified DlEPV virions from the poison gland apparatus (Fig. 2.) of female *Diachasmimorpha longicaudata*. Virions were stained with uranyl acetate as described in Materials and Methods. Note loose mulberry-like viral membrane (arrow).

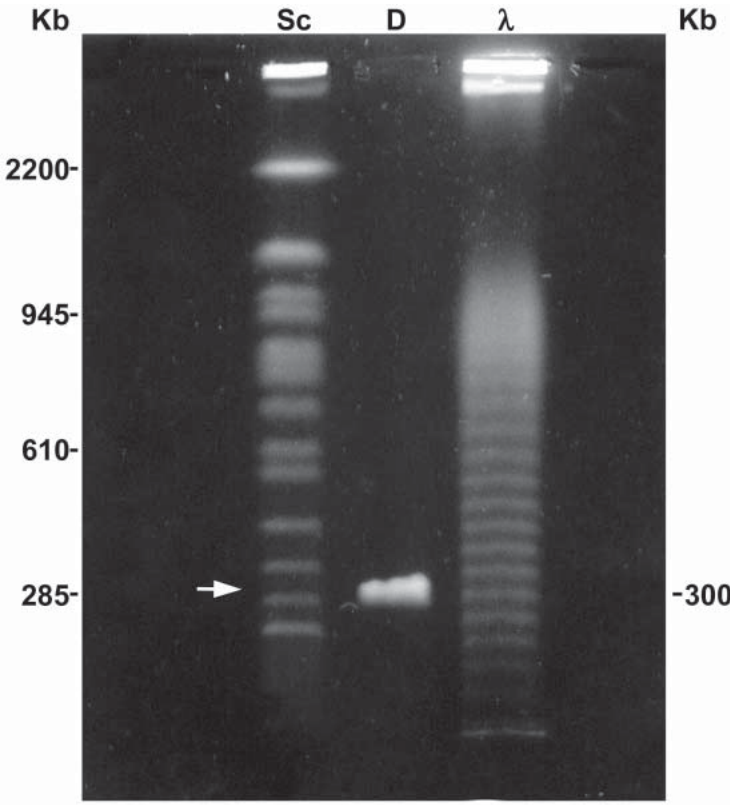


Figure 3. Pulse field gel electrophoresis of DlEPV genomic DNA (D). Virions were sucrose-purified, digested in 1% pulse field grade (PFG) agarose plugs (BioRad) with proteinase K, as described in Materials an Methods, and run into a 1% PFG agarose gel in a CHEF DR II (BioRad) system in 0.5 x TBE at 14ºC. The run time was 22h at 6V/cm with initial and final switch times of 60 and 90 sec., respectively, at an angle of 120º. The gel was stained with 0.25 mg/ml EtBr. The DlEPV genomic DNA is 290-300 kb (arrowhead). λ=Lambda concatemer of 50 Kb bands. Sc=*Saccharomyces cerevisiae* DNA.

fragment consists of 2316 nt that lack "start" and "stop" codons and represent a partial sequence from within one large gene (Fig. 5a). The nucleotide sequence AATGCTGATTTTGATGGAGATGAG (Fig. 5a) putatively encodes NADFDGDE that is recognized by the PROSITE database search programs, as the consensus sequence for the RNA polymerase gene family (Fig. 5b). Furthermore, Blast and InterProScan searches indicated that the R1-36-1 sequence is homologous with the DNA-directed RNA polymerases of AmEPV and MsEPV (78% for each) and the ChPVs lumpy skin disease virus and Yaba pox monkey tumor virus and Vac (75% for each). ClustalW alignment of the deduced DlEPV amino acids with sequences from four of the above-mentioned poxviruses that are homologous with the protein encoded by the Vac J6R gene (Fig. 5b) revealed that 28.4% of the DlEPV amino acids are conserved or substituted among all the EPVs and ChPVs evaluated, 12.9% are shared with at least one EPV but not with any ChPVs, 6.5% are found in at least one ChPV but not in the other EPVs, 3.1% occur in at least one ChPV and one EPV, and 49.1% are found only in DlEPV (Fig. 5b).

DlEPV DNA was detected in dot-blots of male and female wasps (Fig. 6) and in host hemocytes (not shown). The mouse anti-

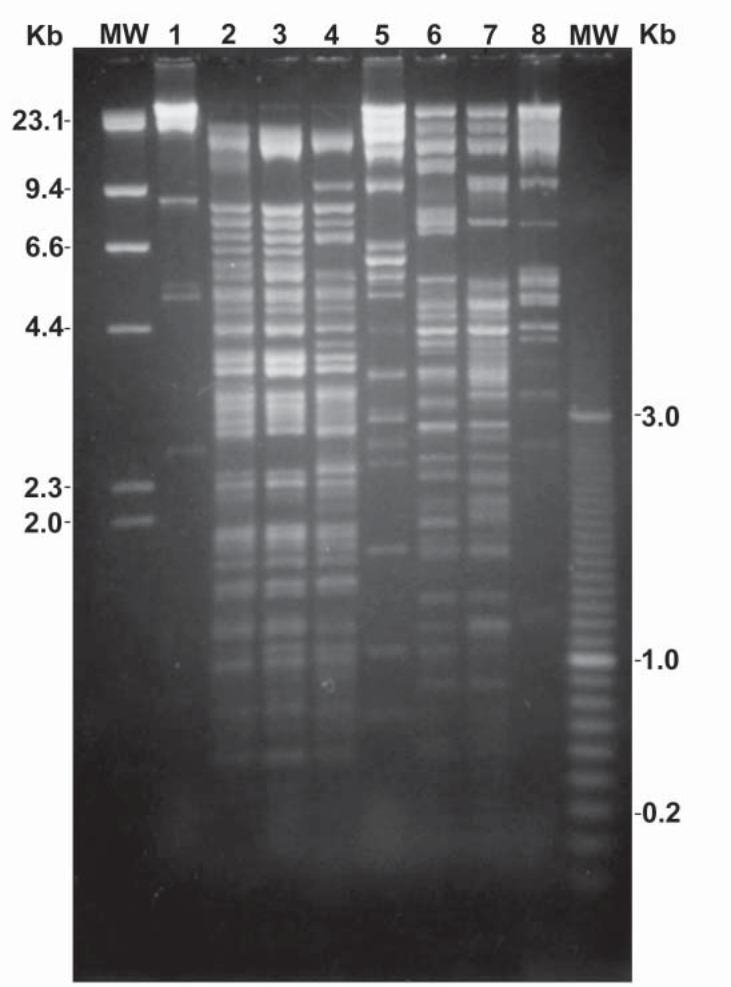


Figure 4. Fragment profile of restriction endonuclease digested DlEPV genomic DNA. About 3.5 µg of digested DlEPV were loaded in the respective lanes of a 0.7% Seaplaque GTG (FMC) agarose gel and electrophoresed in 0.5 x TBE at 40V. The gel was stained with 0.05 mg/ml EtBr. MW=molecular weight in kilobase pairs (Kb); 1=Pst I; 2=EcoRV/ Pst I; 3=EcoRV; 4=EcoRV/BamHI; 5=BamHI; 6=EcoRI; 7=EcoRI/Xho; 8=Xho.

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GAATTCTCTTTGGTATCCAATGAAGAAATCGAATCCATACCCATAGCTATAAAAAACAT
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TTAACCCCTTCGAATGCTCATCAAATCGTATCTCCCGAAAATGTCTTTTATGCTAATAG
TATCTTACTTCCACCACATAATCTACGAACTATCAATGTTTATGATGGTCAGGTTACGA
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CAAATCCCCATGTGCCAATCTCGAACAAGCTTTGATTATGAACTCACGAAATCTCTTCA
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CAAAGGGTTAACGTTTGAAGAATATGCTACAATCGTAAAACAAGAAGCTTTTCCACAAA
TTGTTAATGTTACAACTGGTACTTCACAAACAGGATTTTTGGGGAAAAAAATGGTTAAA
ATGGCTTCTCGAATTC

Figure 5a. Complete DNA and deduced amino acid sequences of the DlEPV EcoRI clone #36 (RI-36) that contains a partial open reading frame (RI-36-1) of a large gene that encodes a homolog of a DNA-directed RNA polymerase. The GenBank accession number for this sequence is AF500107. Complete DNA sequence of DlEPV RI-36-1 consisting of a 2316 nt partial open reading frame. The nucleotides in blue encode the consensus sequence of the DNA-directed RNA polymerase gene family. GAATTC = EcoRI restriction site.

DlEPV polyclonal antibodies recognized at least six (54-100 kDa) major and several minor protein bands in combined fractions (41-55% sucrose) of the purified virus (Fig. 7). Two closely migrating bands of about 100 kDa and two of ~54 kDa were detected in a 53-55% sucrose fraction of the purified virus. Three bands of similar mass (one of ~100 kDa, and two of ~54 kDa) were also detected in the host hemolymph (Fig. 8). Based on their relative migration, the ~54 kDa bands in the virus fraction and host (Fig. 8) corresponded to the two closely migrating bands of similar size in the combined virus fractions (Fig. 7). Male and female wasps had only one major (probably two closely migrating) DlEPV protein band(s) (~60-65 kDa) that also seemed to correspond to bands in the combined DlEPV fractions (Fig. 7). Interestingly, the band(s) detected in the adult wasps were not detected in the host hemolymph or in the DlEPV fraction (Fig. 8). Many of the bands in the combined fractions of the purified virus (Fig. 7) were not detected in the host hemolymph, adult wasps or single virus fraction (Fig.7, 8).

TEM (Fig. 9) confirmed the dot-blot results (Fig. 6) of the

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Sequence 1: gi|13876680|gb|AAK43568.1|  1210 aa-Lumpy skin disease virus=Homolog of Vac.J6R
Sequence 2: gi|6681610|dbj|BAA88785.1|   1285 aa-Yaba monkey tumor virus=Homolog of Vac J6R
Sequence 3: gi|9964535|ref|NP_065003.1|  1301 aa-AmEPV221
Sequence 4: gi|9631519|ref|NP_048113.1|  1319 aa-MsEPV043
Sequence 5: DlEPV36                       772 aa-DlEPV RI-36-1

CLUSTAL W (1.81) multiple sequence alignment

gi|13876680|gb|AAK43568.1|      ------------------------------------------MDGALC 6
gi|6681610|dbj|BAA88785.1|      -MAVISKVTYSLYNQEEINATDVLINHVKNDDDIGTVKDGRLGAMDGALC 49
AmEPV221                        ----MTTFKYTLLDNSTIDAIPIVIDSIGNDNEN-SVKSPKLGGTKFNVC 45
MsEPV043                        MAKVNTEILFSMIPNSIIDSIPIIINSISNDADN-NVKSTKLGGTKFNVC 49
DlEPV36                         --------EFSLVSNEEIESIPIAIKNISGENKTEAAQLG--AIDSFQKC 40
                                                                          .   *

gi|13876680|gb|AAK43568.1|      KTCGKT-ELQCFGHWGKVRLYETHIIKPEYIGEVIRILN--HICIRCGFL 53
gi|6681610|dbj|BAA88785.1|      KTCEKT-ELQCFGHWGKVRLYETHIVKPEYIGEVVRILN--HICIRCGFL 96
AmEPV221                        STCNLTRENGDMGHPGRTPLRDMCIVKSGCIKNVLDTLNTLKLCNSCFMI 95
MsEPV043                        STCRLTKDNGDLGHPGRTPLKKMAIIKPAFIKSVLDTLNALKICSNCKMF 99
DlEPV36                         ETCQLT--SACPGHFGKFHLTQP-LFKVAFKKLVENIFK--YTCSFCGAL 85
                                .**  *      ** *:  * .  :.*      *   ::    *  *  :

gi|13876680|gb|AAK43568.1|      RSREPYIEDVTKMSSHALRK-------LKDKILSKKK-------SCWNSK 89
gi|6681610|dbj|BAA88785.1|      RSREPYMEDITRMPLNSLKK-------LKDKILSKKK-------SCWNSK 132
AmEPV221                        KNNTIFSEIIEKYNSEYNIN-------LKKEILSLLKNNRQGGVKCNNEN 138
MsEPV043                        RDNFALYKILKKYNIDVQDNKIDPPTELKKEILTLIKLNKQSASKCNNIN 149
DlEPV36                         QNLELLELIKQIDERNTGIT-----VKDRAAFKKILEATKQSKFKCIAPN 130
                                :.             .   .       :  : .  :       .*   :

gi|13876680|gb|AAK43568.1|      CMQQYQKITFSKKK-VCFVN-KSDDITIPNALIYQKITSIYKRFWPLLEI 137
gi|6681610|dbj|BAA88785.1|      CMQPYQKITFSKKK-VCFIN-KSDEITIPNALIYQKVTSIYKRFWPLLEI 180
AmEPV221                        CQNITGTYKYNQKKSYFYVK-KQKDEIILNKTVYTMLLGIPDIIYKCVTV 187
MsEPV403                        CQLPIATYKYNTIKAQFYIK-VIKDKVISNEQIYKMLIGIPHIVYKCIKS 198
DlEPV36                         CQKQVSPLQYSKNNNFIYNSGTTKGIVLDNRHVFNILQNLPQTFKLLLTP 180
                                *       :.  :   : .   .   : *  ::  : .: . .   :

gi|13876680|gb|AAK43568.1|      HQKPENLFYKNFFPVPPFIIRPAISFWIDSIP-KETNELTYLLGMIVKYC 186
gi|6681610|dbj|BAA88785.1|      YQKPENLFYKNFFPVPPLIIRPAISFWIDSIP-KETNELTYLLGMIVKYC 229
AmEPV221                        PYADSQLQPYKAFYANNIIIPVLPSRPPNYFDNKESHVMTTKLGQLVGTS 237
MsEPV043                        PLSIGNFTPYEAFYTNNILIMVNPARPPNHYDNKDSHIMTTKLNQLVGSI 248
DlEPV36                         SNAHQIVSPENVFYANSILLPPHNLRTINVYDGQVTSLLTSDLNLIMRRV 230
                                      .   : * .  :::        :    : :  :*  *. ::

gi|13876680|gb|AAK43568.1|      NMNADEQVIQKAIIEYDDIKIISNNTI-----SINLSYITSGKNNMIRSY 231
gi|6681610|dbj|BAA88785.1|      NMNAEEQVIQKAVIEYDDIKIISNNTI-----SINLSYITSGKNNMIRSY 274
AmEPV221                        QKSRDESEVQKIYNDIDNVKPNSPYKTSNMLVTLNIQVGGNKKGSIVRSN 287
MsEPV043                        IKEKEIEDIQKIYNDIDHVKPKSPYSRGTLFDTLNTEVAGNKKEGILRSY 298
DlEPV36                         ANNETNAKIQKILDSIDNSRGANPYAIN-KKLTLDTLTGGHSKESYLRSY 279
                                  .     :**   . *. :  .         :::       * . :**

gi|13876680|gb|AAK43568.1|      IVARRKDHNARSVIGPDITLTINEVGVPFYIRNTLTEKIFVNPFTTDEVQ 281
gi|6681610|dbj|BAA88785.1|      IVARRKDQTARSVIGPDTSLTINEVGVPEYIRNTLTEKIFVNPFTVKYVK 324
AmEPV441                        IMARRADNTARCVAGP-TMDKIGYIYIPKIVAKTLTSSIYYNRFTENMIK 336
MsEPV043                        ILARRSDNTGRGVAGP-IIAKIGYLGIPEIVASTLTESIYYNRFTEQKVR 347
DlEPV36                         INGKRIPETARAVIEP-SMNKTGFIEVPSYILNKLRDVVFYNHVTKDNIL 328
                                * .:*  ...* *  *    . . : :*  : ..* . :: * .* . :

gi|13876680|gb|AAK43568.1|      RLFTNNEIKFYFNKRLN-------QLTRIKQGKFIKNKIHMLPGDWVEVP 324
gi|6681610|dbj|BAA88785.1|      KMFTNNEIKFYFNKRLN-------QLTRIKPGKFIKNKIHMLPGDWVEVS 367
AmEPV221                        DMLVNDNNKIKYILLYRYDQLKPTTLLKIKPQSRLNNLLKMKYGDRIEVE 386
MsEPV043                        DLIYNSPD-VKFIVLYNYEALKPATILEVRPEAKINNLLKIKYGDRIEIP 396
DlEPV36                         KSLQNEQAFLTYIKSDHNSENPYMVYDLAQKNGYLT--LAPNFGDIFEKR 376
                                  : *.   . :    .            :    :.  :    ** .*

gi|13876680|gb|AAK43568.1|      LKENTSIIFGRQPSLHRYNVIASTVKYIEGDTTKIPPGIANSQNADFDGD 374
gi|6681610|dbj|BAA88785.1|      LRENSSIIFGRQPSLHRYNVIASTVKYTEGDTIKIPPGIANSQNADFDGD 417
AmEPV221                        LEDNDVILFSRQPSLHKFNIQAGICKIWDNNTIATPTPIANSMNLDYDGD 436
MsEPV043                        LQNYSMILFSRQPSLHKFNIQCAFCLIHKAQTFSTPTAIANSMNLDYDGD 446
DlEPV36                         KEEGGFVTICRHPSIWLTNIQSGIIKRSEGFTINIPTTICTSFNADFDGD 426
                                 .:   : : *:**:   *: ..     .  *:  *. * .* * *:***
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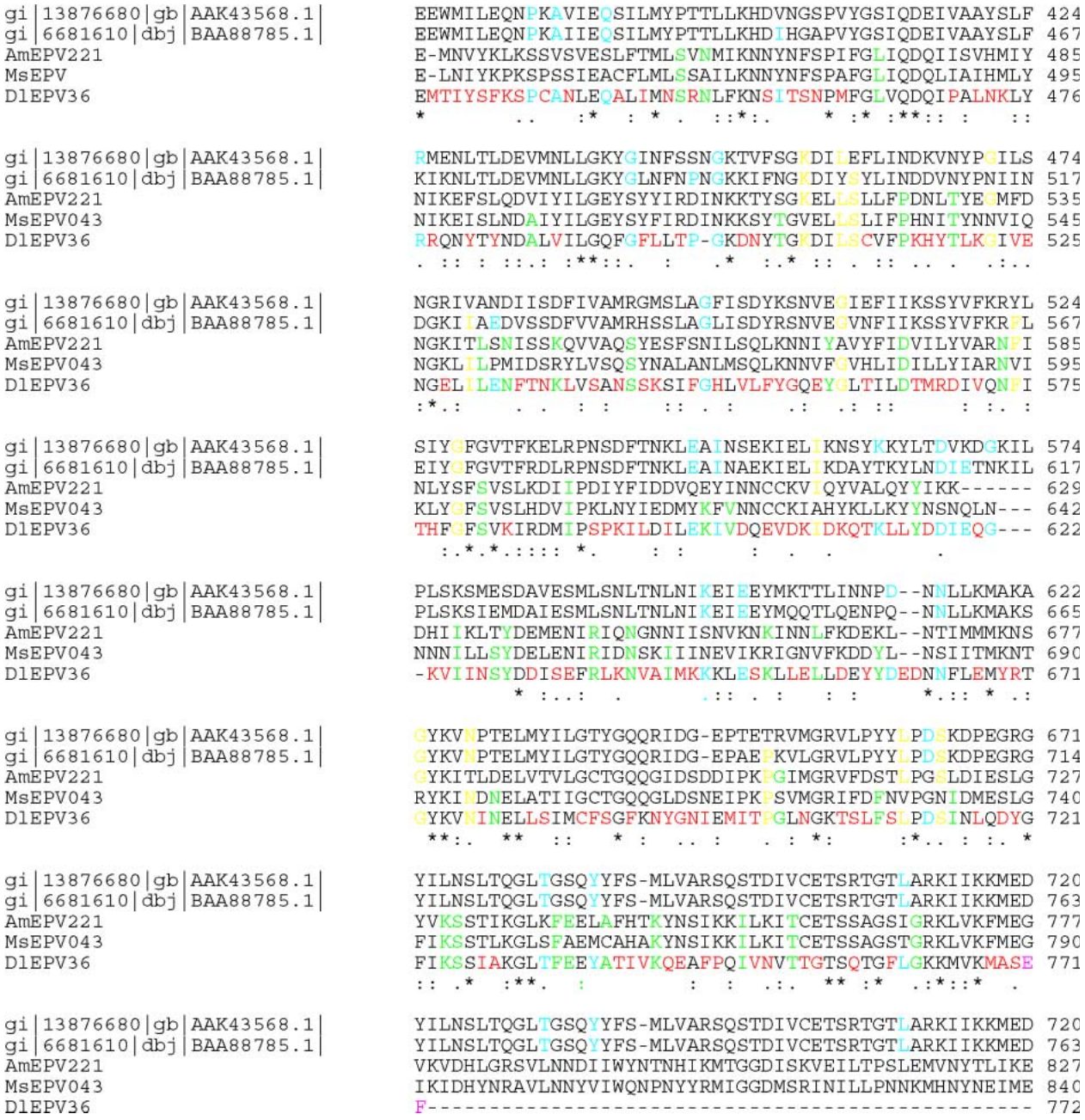


Figure 5b. Complete DNA and deduced amino acid sequences of the DlEPV EcoRI clone #36 (RI-36) that contains a partial open reading frame (RI-36-1) of a large gene that encodes a homolog of a DNA-directed RNA polymerase. The GenBank accession number for this sequence is AF500107.

Alignment of the deduced amino acid sequences of DlEPV R1-36-1, AmEPV221, MsEPV043, and two vertebrate poxvirus homologs of Vaccinia (Vac) J6R, lumpy skin disease virus and Yaba monkey tumor virus that encode a putative DNA-dependent RNA polymerase. Gold= aa shared between DlEPV and at least one of the EPV AND one of the vertebrate poxvirus sequences. Green = aa shared between DlEPV and at least one of the two entomopoxviruses, AmEPV and MsEPV, but not found in the two vertebrate poxvirus sequences; Pink = EcoR1 (FE translated from the GAATTC) restriction sites; Red= aa found only in the DlEPV sequence; Shadowed area = the NADFDGDE consensus sequence of RNA polymerases; Blue = aa shared between DlEPV and at least one of the vertebrate poxvirus sequences but not found in the two EPV sequences; asterisk (*), semicolon (:) and period (.) = identical, conserved and semiconserved substitutions respectively, among all five sequences.

presence of virus in host hemocytes. Indeed, as early as 48-52 hours after parasitism, extracellular enveloped virions were budding from the plasma membrane of host hemocytes into the hemolymph (Fig. 9) where viral maturation occurs (Lawrence and Akin, 1990). Fragments of cellular material also occur in the hemolymph (Fig. 9). Virogenic stroma are also evident within the infected hemocyte (Fig. 9).

Discussion

This is the first report of the purification and characterization of DlEPV, the only symbiotic EPV reported from a parasitic wasp. The discrepancy between the DlEPV genome sizes estimated by PFGE [290-300 kb (Fig. 3)] and by slab gel electrophoresis [250-275 kb (Fig. 4)] is apparently not unusual.

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Indeed, Hall and Hink (1990) found differences (242 and 225 kb, respectively) between PFGE and slab gel estimates for the AmEPV genome. In our hands, the AmEPV and DlEPV DNA resolved by PFGE were ~250 kb and > 290 kb, respectively (not shown). This suggests that the DlEPV genome is larger than that of AmEPV. At an estimated 250-300 kb (Fig.3,4), DlEPV is in the same size range as other EPV genomes reported to date (Moss, 2001).

A-T richness, the possession of the rifampicin resistance gene, and a cytoplasmic site of replication and assembly are characteristics of the Poxviridae (Osborne et al., 1996; Moss, 1996, 2001;Bawden et al., 2000). Based on the many restriction fragments obtained with EcoRI and EcoRV (versus Pst I and Xho) digestion of DlEPV DNA (Fig. 4) a high A-T: G-C ratio was assumed and subsequently confirmed by the 68% A-T richness of the DlEPV RI-36-1 DNA sequence (Fig. 5a). Its deduced protein, the putative DlEPV DNA-directed RNA polymerase (Fig. 5b), is 75-78% homologous with those of other poxviruses (Fig.5b). In separate studies, the sequences of other DlEPV clones were >66% A-T rich and encoded putative proteins that had >60% homology with the DNA ligases and helicases of AmEPV, MsEPV, Vac, and other ChPVs (unpublished), and with the rifampicin resistance protein (GenBank Accession #AF159588) (unpublished) known to occur in all poxviruses (Osborne et al., 1996).

Although DlEPV morphogenesis was previously documented in the female wasp (Lawrence and Akin, 1990) this is the first evidence of DlEPV DNA (Fig. 6) and proteins (Fig. 8) in the male. The DlEPV-related proteins are localized in the Hagen's glands (Khoo and Lawrence, in press), a tergal gland of male wasps known to secrete volatile compounds (Williams et al., 1988) presumed to have a role in mating, courtship and defense (Haramoto, 1957). The parasitism-specific protein (PSP24) (Lawrence, 1990; Rolle and Lawrence, 1994a, b) that is induced by DlEPV (Shi et al., 1999) also occurs in the Hagen's glands (Khoo and Lawrence, in press). The mechanism of DlEPV transmission to male and female offspring remains to be determined but could be as a provirus within



Figure 6. Dot blot of total DNA from male (A) and female (B) *D. longicaudata* and salmon sperm (C) (control) probed with DIG-labeled DlEPV DNA. Top row: 2µg DNA/lane; bottom row: 1µg DNA/ lane; D=DlEPV probe dilutions in descending concentrations 1, 0.5, 0.25, 0.12, and 0.06 µg DNA. The detection of DlEPV DNA in male and female wasps suggests that viral DNA occurs in males and also confirms earlier reports (Lawrence and Akin, 1990) that the virus occurs in female wasps.

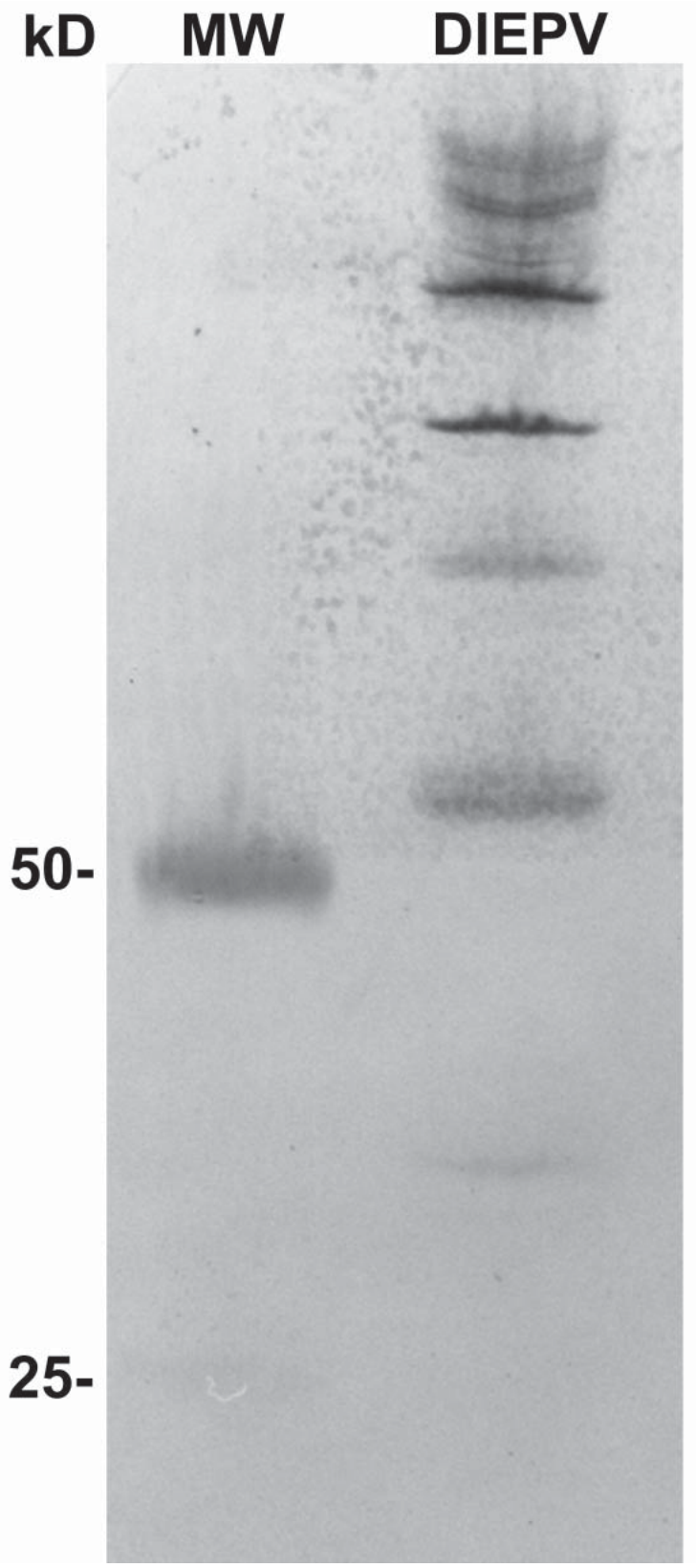


Figure 7. Immunodetection of DlEPV proteins using a mouse polyclonal anti-DlEPV serum generated against three (41-45, 48-50, and 53- 55 % sucrose) combined fractions of sucrose- purified virions and a rabbit anti-mouse second antibody. At least six major viral protein bands are detected. Pre-immunized mouse serum (lane not shown) did not react with the antibody. MW=mass in kiloDaltons (kD), 25 and 50 kD markers=purified mouse IgG.

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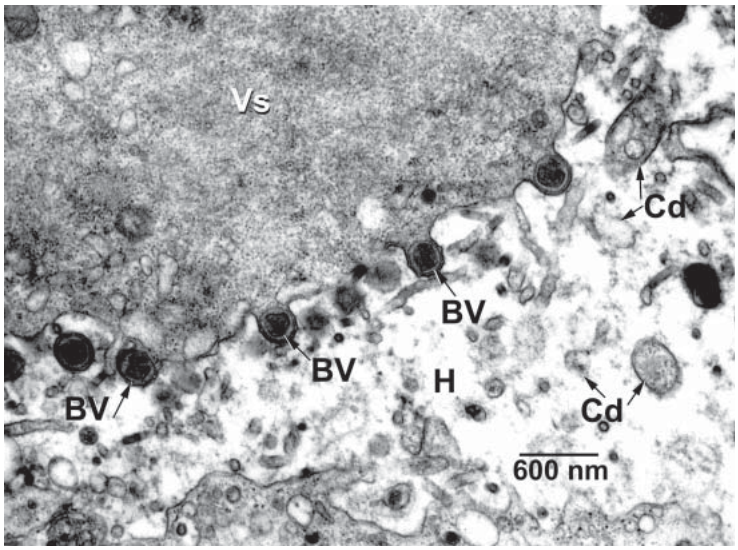


Figure 9. Transmission electron micrograph of a hemocyte of the host *Anastrepha suspensa* 48-52 hours after parasitism (96 hpp) by *D. longicaudata*. BV=budding virus; Cd=cell debris; H=hemolymph; Vs=cytoplasmic virogenic stroma.

the wasp genome, as extra-chromosomal DNA or intact virions within
the egg, or through larval ingestion of virions along with the host's hemolymph.

More protein bands were detected in Western blots of purified DlEPV combined from three sucrose fractions (Fig. 7) than from one fraction (Fig. 8) because the former contained more virions, viral cores, and envelopes (see Materials and Methods) that probably yielded different types and higher concentrations of proteins/peptides compared to the latter. This may also explain why the single DlEPV fraction did not contain bands corresponding with those in the wasps (Fig.8) despite
evidence that the virus occurs in the female wasp (Lawrence and Akin, 1990). The proteins detected in the host hemolymph
were similar to those in the DlEPV fraction (Fig. 8) but differed from those in the wasps (Fig. 8). Differential viral expression has been observed between wasp and host (unpublished
observations) and likely explains the different proteins in the two samples (Fig. 8).

When DlEPV infects host hemocytes, it is localized in a cytoplasmic virogenic stroma where it presumably replicates, and then buds into the host's hemolymph (Fig. 9). Since infected hemocytes are unable to encapsulate wasp eggs in vivo and in vitro (unpublished observation), DlEPV is beneficial (symbiotic) to the wasp. Unlike polydnaviruses that replicate in the wasp but not in the hosts (see Stoltz and Whitfield, 1992), DlEPV replicates in both wasp and host (Lawrence and Akin, 1990), that are members of two different insect orders. DlEPV extracellular enveloped virus buds from hemocytes into the host's hemolymph (Fig. 9) as occurs in other poxviruses (Moss, 1996, 2001). Interestingly, no DlEPV budding has been observed in the wasp to date. Instead, EM studies show the virus at different stages of morphogenesis [i.e. empty crescents and others with nucleoids or with a biconcave core and one or two lateral bodies (the mature virus)] within the extracellular lumen of the AGF of the female wasp's poison gland (Lawrence and Akin, 1990). The site of DlEPV replication and initiation of assembly within the wasp is not known.

Taken together, our previous report on DlEPV morphology and morphogenesis (Lawrence and Akin, 1990) and the data presented here on its genome size, A-T richness, cytoplasmic localization within host hemocytes, and the homology of its RI-36-1 deduced protein with other poxvirus DNA-directed RNA polymerases, indicate that DlEPV is a true poxvirus. This is bolstered by our identification of the DlEPV rifampicin resistance homolog (unpublished) that is considered to be a characteristic of the subfamily (Osborne et al., 1996). We also found other DlEPV homologs of the DNA ligases and helicases of poxviruses (unpublished), including MsEPV and AmEPV, the only two EPVs whose genomes have been sequenced (Afonso et al., 1999; Bawden et al., 2000) and which are Group B (Lepidoptera-infecting) EPVs (but see Bawden et al., 2000). However, since DlEPV is pathogenic to a dipteran host and has the biconcave core like the dipteran EPV (Goodwin et al., 1991), it probably is a Group C virus. DlEPV is unusual in that it (a) replicates in insects of two different orders although it is pathogenic only to the dipteran host, (b) does not express the spheroidin protein in the form of normal occlusion bodies as do other EPVs (Goodwin et al., 1991; Hall and Moyer, 1991; Hall and Moyer, 1993), and (c) is transmitted to its host through oviposition by a wasp (instead of per os as are other EPVs). This suggests that DlEPV could either be placed in a new group within the Entomopoxvirinae or in a subgroup of the Group C viruses.

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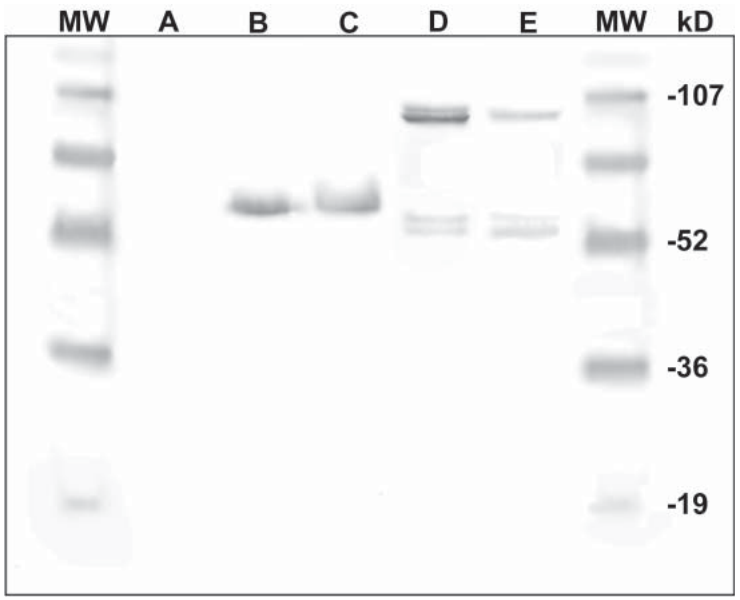


Figure 8. Immunodetection of DlEPV proteins in whole body homogenates (5µl) of male (B) and female (C) D. longicaudata. Molecular mass in kiloDaltons (kD). Unparasitized (A) and 48h-52 h-old parasitized (96 hpp) (E) respectively, pharate pupal hemolymph of *A. suspensa*. D= Purified DlEPV from one 53-55% sucrose fraction. One major (or two closely migrating) band(s) of H"60 kD is detected in male and female wasps, while four bands, two of H"100 kD and two of H"54 kD, occur in the hemolymph of parasitized *A. suspensa* and in the single DlEPV sucrose fraction (positive control).

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