



A homolog of the vaccinia virus D13L rifampicin resistance gene is in the entomopoxvirus of the parasitic wasp, *Diachasmimorpha longicaudata*

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Abstract

The parasitic wasp, *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), introduces an entomopoxvirus (DIEPV) into its Caribbean fruit fly host, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), during oviposition. DIEPV has a 250–300 kb unipartite dsDNA genome, that replicates in the cytoplasm of the host's hemocytes, and inhibits the host's encapsulation response. The putative proteins encoded by several DIEPV genes are highly homologous with those of poxviruses, while others appear to be DIEPV specific. Here, a 2.34 kb sequence containing a 1.64 kb DIEPV open reading frame within a cloned 4.5 kb *EcoRI* fragment (designated R1-1) is described from a DIEPV *EcoRI* genomic library. This open reading frame is a homolog of the vaccinia virus rifampicin resistance (*rif*) gene, D13L, and encodes a putative 546 amino acid protein. The DIEPV *rif* contains two *EcoRV*, two *HindIII*, one *XbaI*, and one *DraII* restriction sites, and upstream of the open reading frame the fragment also contains *EcoRV*, *HindII*, *SpeI*, and *Bsp106* sites. Early poxvirus transcription termination signals (TTTTTnT) occur 236 and 315 nucleotides upstream of the consensus poxvirus late translational start codon (TAAATG) and at 169 nucleotides downstream of the translational stop codon of the *rif* open reading frame. Southern blot hybridization of *HindIII*-, *EcoRI*-, and *BamHI*-restricted DIEPV genomic DNA probed with the labeled 4.5 kb insert confirmed the fidelity of the DNA and the expected number of fragments appropriate to the restriction endonucleases used. Pairwise comparisons between DIEPV amino acids and those of the *Amsacta moorei*, *Heliothis armigera*, and *Melanoplus sanguinipes* entomopoxviruses, revealed 46, 46, and 45 % similarity (identity + substitutions), respectively. Similar values (41–45%) were observed in comparisons with the chordopoxviruses. The mid portion of the DIEPV sequence contained two regions of highest conserved residues similar to those reported for *H. armigera* entomopoxvirus rifampicin resistance protein. Phylogenetic analysis of the amino acid sequences suggested that DIEPV arose from the same ancestral node as other entomopoxviruses but belongs to a separate clade from those of the grasshopper- infecting *M. sanguinipes* entomopoxvirus and from the Lepidoptera-infecting (Genus B or Betaentomopoxvirus) *A. moorei* entomopoxvirus and *H. armigera* entomopoxvirus. Interestingly, the DIEPV putative protein had only 3–26.4 % similarity with RIF-like homologs/orthologs found in other large DNA non-poxviruses, demonstrating its closer relationship to the Poxviridae. DIEPV remains an unassigned member of the Entomopoxvirinae (<http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm>) until its relationship to other diptera-infecting (Gammaentomopoxvirus or Genus C) entomopoxviruses can be verified. The GenBank accession number for the nucleotide sequence data reported in this paper is EF541029.

Keywords: DIEPV *rif* gene, wasp virus, symbiotic entomopoxvirus

Abbreviations: DIEPV: *Diachasmimorpha longicaudata* entomopoxvirus; **Rif:** rifampicin resistance gene; **RIF:** putative rifampicin resistance protein

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Introduction

The Entomopoxvirinae Subfamily (Family: Poxviridae) is comprised of three genera based on morphology, host range, and genome size of viruses infecting Coleoptera (Genus A or Alphaentomopoxvirus), Lepidoptera (Genus B or Betaentomopoxvirus), and Diptera (Genus C or Gammaentomopoxvirus).

The Orthoptera-infecting *M. sanguinipes* entomopoxvirus is currently a temporary species within the Betaentomopoxvirus (ICTVdB 2004). Although entomopoxviruses have been isolated from the Hymenoptera, they have yet to be assigned a genus (King et al. 1998).

Evidence for a distant relationship between chordopoxviruses and entomopoxviruses was initially based on DNA sequence comparisons of genes encoding thymidine kinase (Gruidl et al. 1992), DNA polymerase (Mustafa and Yuen 1991), and nucleoside triphosphate phosphohydrolase I (Hall and Moyer 1991; Yuen et al. 1991). The rifampicin resistance gene (*rif*) [and the putative protein (RIF) it encodes] found in chordopoxviruses such as vaccinia (Niles et al. 1986), variola (Shchelkunov et al. 1993), and swinepox (Massung et al. 1993), also occurs in several entomopoxviruses (Winter et al. 1995; Osborne et al. 1996; Afonso et al. 1999; Bawden et al. 2000). The *rif* gene was considered to be highly conserved within, and characteristic of, the Poxviridae and thus, a unique monophyletic origin was suggested (Osborne et al. 1996). However, RIF-like sequences and certain other proteins assumed to be unique to poxviruses occur in some large double stranded eukaryotic DNA non-poxvirus families, suggesting that poxviruses and these double stranded DNA viruses share the same ancestry (Iyer et al. 2001),

and probably that RIF is not characteristic of the Poxviridae alone.

In vaccinia, the RIF protein (D13L) (Moss 1996, 2001) localizes predominantly on the concave surface of the membrane cisternae of viral crescents and is presumed to be essential as a scaffold for the formation of the Golgi-derived membranes, characteristic of the early stages of virion assembly (Sodiek et al. 1994). Morphologically similar structures are highly conserved within the Poxviridae (Nile et al. 1986; Shchelkunov 1993; Massung et al. 1993; Winter et al. 1995; Moss 1996, 2001; King et al. 1998) and likely, serve a similar function.

We report here the sequencing and comparative analysis of a complete open reading frame within a partially sequenced clone (designated RI-1) derived from an *EcoRI* library of the *Diachasmimorpha longicaudata* entomopoxvirus (DIEPV) DNA. DIEPV was first described from the parasitic wasp *D. longicaudata* (= *Biosteres* = *Opius longicaudatus*) (Hymenoptera: Braconidae) and was shown to be transmitted to the larvae (hosts) of the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) during oviposition by the wasp (Lawrence and Akin 1990). DIEPV invades the host's hemocytes where it replicates and exhibits the immature virus, intracellular mature virus, cell-associated virus, and extracellular enveloped virus forms (Lawrence 2002, 2005) known to occur in members of the Poxviridae (Moss 2001). DIEPV inhibits encapsulation by the host's hemocytes, thereby protecting the wasp's eggs and as such, is the first symbiotic entomopoxvirus described to date (Lawrence 2005). We show that the DIEPV D13L homolog is more closely related to entomopoxviruses and chordopoxviruses than

to orthologs/paralogs of other large double stranded DNA viruses.

Few viruses or virus-like particles that are symbionts of parasitic wasps that attack dipteran hosts have been reported. The first virus-like particles from the *Leptopilina* parasitic wasp were reported from parasitized *Drosophila melanogaster* larvae and like DIEPV, were found to disrupt the cellular encapsulation ability of the host (Rizki and Rizki 1990). However, neither the nucleic acid composition nor family of these virus-like particles has been identified (Rizki and Rizki 1990). A rhabdovirus is also injected into *A. suspensa* larvae by the *D. longicaudata* female (Lawrence and Matos 2005) but its genes have also not been sequenced. Therefore, DIEPV is the first dipteran-infecting viral symbiont of a parasitic wasp for which any gene sequence is known.

Materials and Methods

Construction of the DIEPV EcoRI library

Details of the *EcoRI* DIEPV DNA library construction and sequencing of cloned fragments have been described (Lawrence 2002). Briefly, DIEPV DNA was extracted from virions that were harvested from female wasp venom glands and purified by sucrose density gradient centrifugation (Lawrence 2002). Upon digestion with *EcoRI* (Roche Molecular Biochemicals, www.roche.com), the resulting DIEPV DNA fragments were cloned into the pBluescript® II KS (+/-) cloning vector (pBS; Stratagene, www.stratagene.com) using T4 DNA ligase (Roche) and the manufacturer's and standard (Sambrook et al. 1989) protocols. The clones were used to transfect supercompetent DH5- α *Escherichia coli* cells (Gibco-BRL, www.lifetech.com/www.invitrogen.com), amplified, and selected on ampicillin - Xgal (Gibco- BRL) agar plates at 37 °C for 18 h as previously described (Lawrence 2002). Recombinant plasmids were isolated from bacterial cells by alkaline lysis (Sambrook et al. 1989) and the presence of the DIEPV DNA inserts verified by *EcoRI* digestion and subsequent electrophoresis (Lawrence 2002). The clones (RI) were arbitrarily numbered and the RI-1 clone was selected for further analysis.

DNA labeling, hybridization, and detection

To verify the fidelity of the RI-1 DNA insert to the DIEPV genome, a 3 μ g sample of the isolated

insert was labeled with digoxigenin (DIG) by random priming using the DIG-High Prime® labeling protocols (Roche). DIEPV genomic DNA was digested with *EcoRI*, *HindIII*, and *BamHI* (Roche) and the resulting fragments electrophoresed into a 0.8% agarose gel at 30 V for 18 h and transferred to nitrocellulose membrane by the capillary method. The DNA was then fixed to the membrane by UV cross-linking at 50 mJoules. The blot was probed with 100 ng of the DIG-RI-1 insert diluted in 5 μ l hybridization buffer [5x SSC (750 mM NaCl, 75 mM sodium citrate solution, pH 7.0), 0.1% (w/v) N-lauroylsarcosine, 0.2% (w/v) SDS, 1% blocking reagent (Roche)] at 65°C for 16 h. Hybridization was followed by two 5 min washes at RT with 2x washing buffer (2x SSC, 0.1% SDS) and two 15 min washes with 0.5x washing buffer. The hybridization signal was visualized using the DIG chemiluminescent detection protocol and exposure to LumiFilm (Roche).

Sequencing of the open reading frame within the DIEPV RI-1 clone

Forward and reverse sequencing of the open reading frame within the RI-1 clone were accomplished by primer walking, with fluorescence-labeled dideoxynucleotides and *Taq* DyeDeoxy terminator cycle sequencing protocols (Applied Biosystems, Perkin-Elmer Corp., home.appliedbiosystems.com) and the extension products analyzed with a model 377A DNA sequencer (Applied Biosystems), as previously described (Lawrence 2002). Sequences were assembled and further analyzed with the Sequencher 3.0 software (Gene Codes Corp., www.genecodes.com).

Sequence analysis of the RI-1 open reading frame

The amino acids deduced from the partial sequence of RI-1 by the Sequencher program were compared with homologs in the GenBank, PIR, and SWISS-PROT databases using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). A multiple sequence alignment of the RI-1 open reading frame protein and its homologs was performed using the CLUSTALW 1.81 program (Thompson et al. 1994), with gap initiation and extension penalties of 10 and 0.2, respectively. Aligned sequences were imported into the Phylogenetic Analysis Using Parsimony (PAUP*®) program (Swofford 1998) to generate a phylogenetic tree using the neighbour joining method and 1,000 bootstrap trials to assess tree

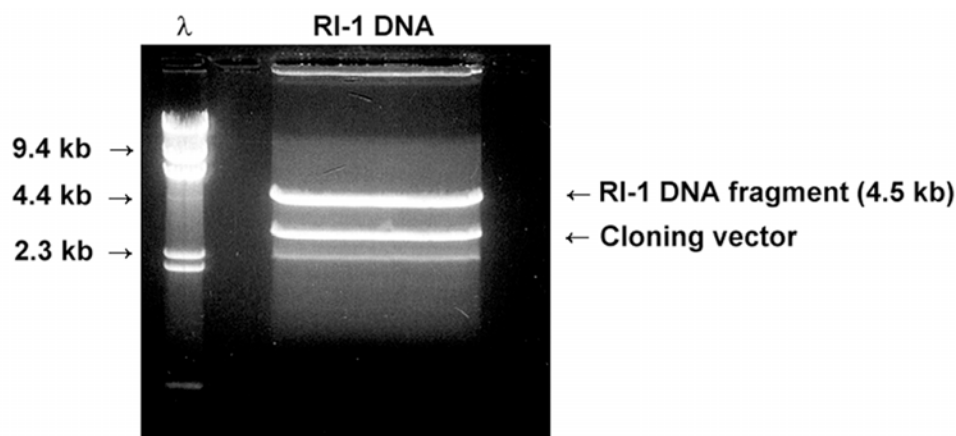


Figure 1. Electrophoretic analysis of the *EcoRI* digested DIEPV RI-1 clone. A 75 μ l aliquot of the digested clone was applied to the gel. DNA fragment sizes were verified using a BioRad® λ high molecular weight DNA size standard (λ). The upper band corresponds to the RI-1 insert of approximate 4.5 kb. The lower band is the pBluescript® cloning vector of 2.96 kb.

reliability. Pairwise comparisons of the DIEPV RI-1 open reading frame nucleotides and deduced amino acids with those of homologs identified by BLAST, were expressed as percent nucleotide identities, amino acid identities, or amino acid similarities [identities + homologous (conservative, *sensu*Mount 2001) substitutions].

Rifampicin-like proteins occur in other large DNA non-poxvirus families including the insect-infecting Iridoviridae and Ascoviridae (Iyer et al. 2001; Stasiak et al. 2001 Stasiak et al. 2003). Thus pairwise amino acid comparisons, separate from those made with the poxviruses, were performed between the RIF sequence of DIEPV, orthologs/homologs from the insect iridovirus IIV-6, the *Diadromus pulchellus* ascovirus 4a (DpAV4a) from a parasitic wasp of the same name, and other non-pox DNA viruses.

Results

Purification, sequencing and analysis of the RI-1 insert

The size of the RI-1 insert was verified to be ~ 4.5 kb (Figure 1). Hybridization of the DIG-probe to the insert and the restricted DIEPV genomic DNA in the Southern blot, verified their fidelity to the DIEPV genome (Figure 2). The single hybridized fragment, with the same size as the positive control (~4.0), obtained with the *EcoRI* digested genomic DNA confirmed the absence of an *EcoRI* restriction site within the fragment (Figure 2). The four bands detected in blots of the *HindIII* digest (Figure 2) were also consistent with the presence of three *HindIII* sites within the sequence (Figure 3). Although no *BamHI* sites (therefore one band) were predicted, two bands

were observed (Figure 2), suggesting the presence of a second site in the unsequenced portion of the clone. Sequencher also predicted *XbaI*, *DraII*, *SpeI*, and *Bsp106* restriction sites within the RI-1 fragment (Figure 3) but these enzymes were not evaluated.

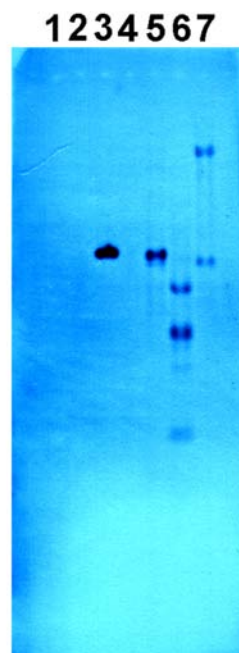


Figure 2. Autoradiograph of Southern hybridization of digested DIEPV genomic DNA with a 4.5 kb specific probe generated from the DIEPV RI-1 insert. Lanes 1–2: empty; Lane 3: 1 μ l of the DIEPV RI-1 undigested 4.5 kb insert (positive control); Lane 4: 2 μ l salmon sperm DNA (negative control); Lane 5: 5 μ l *EcoRI* digested DIEPV genomic DNA; Lane 6: 5 μ l *HindIII* digested DIEPV genomic DNA; Lane 7: 5 μ l *BamHI* digested DIEPV genomic DNA.

The sequenced portion of the RI-1 fragment was determined by Sequencher to contain one complete open reading frame of 1,640 bases, encoding a putative protein of 546 amino acids and an apparent partial open reading frame. The *rif* open reading frame had 529 bases (5') and 174 bases (3') immediately flanking its translational start and stop codons, respectively (Figure 3). Thus, the sequenced portion of R1-1 comprised 2.34 kb (GeneBank accession # EF541029) of the ~4.5 kb R1-1 insert. The analyses below will focus only on the complete open reading frame and sequences immediately flanking it (Figure 3).

The translation initiation codon (ATG) of the open reading frame starts at 530 nucleotides from the 5' end of the fragment and the translational stop codon (TAA) starts at 2,168 nucleotides (Figure 3). Immediately preceding the translational initiation codon is a highly A/T rich (87%) 30 nucleotide sequence. Three of these bases immediately preceding the ATG and in combination with it, form the consensus poxvirus late transcriptional start signal (TAAATG) (Rosel et al. 1986; Moss 1996, 2001) (Figure 3). Potential poxvirus early transcription termination signals (TTTTnT) occur at 236 and 315 nucleotides upstream of the late translational start codon and 168 nucleotides downstream of the translational stop codon of the open reading frame (Figure 3).

revealed almost no conserved amino acids within the first 253 amino acids of the DIEPV sequence, except for a short region [LPE(I)/(V)KG] between amino acids 53–58 in which valine was substituted in the chordopoxviruses for isoleucine in the entomopoxviruses (Figure 4a). Two additional motifs, HTN(L)/(I)/(V)L(M)/(V)/(S)F(GT)/(SR)/(TR)R and GD(N)/(L)RS, occur within DIEPV amino acids 326–370 (region I) and 383–441 (region II) respectively (Figure 4a). These regions of 43 and 58 amino acids have ~28 and 26% conserved residues respectively, and correspond to the same two regions in the *H. armigera* entomopoxvirus RIF that had 56 and 53% conserved amino acids respectively, when that virus was aligned with vaccinia and swinepox (Osborne et al. 1996). When only entomopoxviruses were aligned, the conserved amino acids in regions I and II of the DIEPV RIF increased to ~44 and 38% respectively (Figure 4b). Interestingly, when each entomopoxvirus sequence was individually aligned with DIEPV, the percent conserved residues increased even further to as high as 79 and 41% in regions I and II respectively (alignment not shown). In addition at least 10% of 40 residues at the N-terminus and 20% of 50 residues toward the C-terminus were conserved between DIEPV and each of the other (beta) entomopoxviruses (data not shown).

Alignment of all deduced poxvirus sequences

Regions I and II had motifs common to both

Table 1. Pairwise comparison of amino acids and nucleotides of the rifampicin resistance homologs of DIEPV and other poxviruses. The lower left triangle represents the percent similarities (= amino acid identities plus homologous substitutions). Numbers in parentheses represent percent amino acid identities. The upper right triangle represents percent nucleotide identities.

	DIEPV	AmEPV	HaEPV	MsEPV	MolCV	SPV	MyxV	VaccV	VarV
DIEPV	100%	32	15	49	0	12	2	10	5
AmEPV	46 (25)	100%	78	68	0	27	10	18	18
HaEPV	46 (24)	88 (77)	100%	67	1	18	10	11	11
MsEPV	45 (26)	72 (56)	74 (53)	100%	1	18	4	16	16
MolCV	41 (16)	44 (23)	44 (23)	44 (22)	100%	51	59	54	54
SPV	44 (19)	46 (26)	44 (24)	47 (23)	79 (57)	100%	68	70	65
MyxV	44 (17)	45 (26)	45 (25)	48 (21)	79 (57)	89 (76)	100%	63	70
VaccV	45 (19)	45 (26)	44 (25)	44 (22)	80 (59)	85 (70)	84 (68)	100%	98
VarV	45 (19)	45 (25)	44 (24)	44 (22)	80 (59)	85 (69)	84 (68)	99 (99)	100%

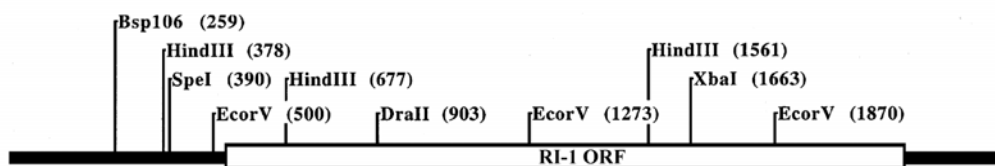


Figure 3a. Locations of restriction enzyme recognition sites within a ~2.54 kb sequenced portion of the RI-1 DNA fragment predicted by the Sequencher 3.0 program.

N K D E H P F L F H K A K S E E I F S T
Y I I

841 CAACGAATAT CACTCGTTAA ACTATTTTAC CAACAAAGAT GTTTTTCTGA CAACCAAAGA
AGGGACCCAC

N E Y H S L N Y F T N K D D F L T T K E
G T H

911 GCTGATTGCA TAATTTTCCC TAAAAAAGAA ATATCTATTC CATTGGATTC GTTGCTTTCT
GCTTTTAAAA

A D C I I F P K K E I S I P L D S L L S
A F K I

981 TCTTTAAAGA TACCGAAATT ATTTTCAATT TCAAATCCA TAACATTGAA GAAATTATAG
CCTATGATGT

F K D T E I I F N F K F H N I E E I I A
Y D V

1051 AGAATTTAGA CGTCATTCAC TAGAACAAC CAAGAAAAAC TTTTCTGAAA CATCATTGAA
TATCAGATTC

E F R R H S L E Q L K K N F S E T S L N
I R F

1121 CAATTTTGA ATGTTCCAAT AATTTATCA GCAGAACTCA CAGCAACTAA CGTAATTACC
AAAAAGGATG

Q F L N V P I I S S A E L T A T N V I T
K K D V

1191 TGATTGGTAA AGATAATACT CAAATGATGA ATACATCAGA CTTCTCAAAC ACTATTGCTG
TAAGTTTCCA

I G K D N T Q M M N T S D F S N T I A V
S F H

1261 TTCTAAAAGC GATATCTTTA ATCACGAAAA TCGTTATATT ATTAATCCGG GTGTAGATTA
TTCCGAAGAT

Figure 3b. DNA sequence of the RI-1 open reading frame and an immediately preceding region (539 nt) containing putative poxvirus early transcriptional stop (TTTTnT) and late promoter (TAAATG) sequences (highlighted in black). Restriction enzyme recognition sites, shown in (a), are underlined. The putative translational stop codon (TAA) is indicated by an asterisk (*). The sequence has been assigned GeneBank accession # EF541029.

S K S D I F N H E N R Y I I N P G V D Y
 S E D

1331 **GTGCTTGTC AGAAATGGGT TTAAATATT TTAAAAGATT TGCTTATTGT GACCACAAA**
GATATGTCCC
 V L V Q K W V L N I L K D L L I V T T K
 D M S L

1401 **TGTCAGAAA TAAAAAGCT CTGGGTTTCA AAGACGAAGC TGTGTTCCAT GAAATTACTA**
AAAATACTAT
 S E N K K A L G F K D E A V F H E I T K
 N T M

1471 **GACTTTC AAT AAACTCGAAA AAAGGTTCTG TAAGATCACA ATCGAAAATA TCCCAGAAGA**
TCACAAACTT
 T F N K L E K R F C K I T I E N I P E D
 H K L

1541 **TATTATCATA CAAATATTCT AAGCTTCACC AGACGTTTCC AACACACCAA AGCACTCAAT**
GTTTCCACAC
 Y Y H T N I L S F T R R F Q H T K A L N
 V S T L

1611 **TTTTTAAGAA AATCACGGGT GTTTATCTTC CCAATCAAAA AGTAATCAAT TTTCTAGATA**
TAGATCATAG
 F K K I T G V Y L P N Q K V I N F I S I
 W L D

1681 **TATAGATATT AAAATTGTAA GTTTACCTAT TAGTATTTGG GATCATGAAT TGAATAGTCA**
TCCAGGTGAT
 I D H S I D I K I V S L P D H E L N S H
 P G D

1751 **TTAAGATCCA ATGCCATGAA AGAACGTGAT TTTTCTTTA AGAATAGATT TTTGCTTGGA**
ATGGACTTCA
 L R S N A M K E R D F F F K N R F L L G

Figure 3b (con't). DNA sequence of the RI-1 open reading frame and an immediately preceding region (539 nt) containing putative poxvirus early transcriptional stop (TTTTnT) and late promoter (TAAATG) sequences (highlighted in black). Restriction enzyme recognition sites, shown in (a), are underlined. The putative translational stop codon (TAA) is indicated by an asterisk (*). The sequence has been assigned GeneBank accession # EF541029.

M D F N

1821 **ATTGCAAAGA TAGAGGATAT GAACGTATTT CACTTAAAGG TGGTAAAGAT ATCTTTGAAA**
ACCTTCTTCG

C K D R G Y E R I S L K G G K D I F E N
 L L R

1891 **GGAAAGAAAA CCCTTTCTTC GTAAACTTCC CATTATCGAA TTTGATCCAG CTATGCAAAG**
AGGTATTTTCG

E R K P F L R K L P I I E F D P A M Q R
 G I S

1961 **TTATATACAA CCTTCATAAG CCCATCTCTC ATGATATACG CAGATCCCTC TATCAACTTT**
ACAAATTTCT

L Y T T F I S P S L M I Y A D P S I N F
 T N F L

2031 **TAGTCGAGAT CCAATGGAAA GAATATGATG AGTGTGATCC TCTAAATCTA TTA AACGTT**
TCCCATGTGT

V E I Q W K E Y D E C D P L N L L K R F
 P C V

2101 **GGACTTATAT GAGATGCAAA AAATCACACA AAATCCTGAT ACACAACGTA TTAGTATTGA**
ATCTATATAA

D L Y E M Q K I T Q N P D T Q R I S I E
 S I *

2171 **ATGCTTGACT TTTTAATATT TCATTCTCAA CCCTTTGTTCG TTCAGCTTTC AAAAAAGCGA**
AACCCCATTT

2241 **GATTGATTCA CTTGAGGGCA AATTTTGGAA CACAGGAGTA TTGGCACTTA CTATGGTATT**
ATTTTGGGAA

2311 **GATTTTATAA CATGTCTTTC TGGTAAC**

Figure 3b (con't). DNA sequence of the RI-1 open reading frame and an immediately preceding region (539 nt) containing putative poxvirus early transcriptional stop (TTTTnT) and late promoter (TAAATG) sequences (highlighted in black). Restriction enzyme recognition sites, shown in (a), are underlined. The putative translational stop codon (TAA) is indicated by an asterisk (*). The sequence has been assigned GeneBank accession # EF541029.



Figure 4a. ClustalW 1.81 multiple sequence alignment of the deduced amino acid sequence of the putative rifampicin resistance protein homologs from *Amsacta moorei* entomopoxvirus (AmEPV), *Heliothis armigera* entomopoxvirus (HaEPV), *Melanoplus sanguinipes* entomopoxvirus (MsEPV), *Molluscum contiguosum* poxvirus (MOLCV), swinepox virus (SPV), Myxoma poxvirus (MYXV), vaccinia virus (VACV), variola virus (VARV), and *Diachasmimorpha longicaudata* entomopoxvirus (DIEPV). A colon (:) represents amino acid homologous (“conservative”, sensu Mount 2001) substitutions. A period (.) identifies amino acid non-homologous substitutions. Asterisks indicate identical amino acids conserved in all sequences. Underlined sequences represent regions I and II in HaEPV and DIEPV with the highest percent conserved amino acids previously identified for HaEPV by Osborne et al. (1996). For the three motifs identified within the RIF sequence, Blue = conserved in all poxviruses; Red = conserved only among chordopoxviruses; Green = conserved only among EPVs. Other colors = conserved in some members of a subfamily.

VACV	NKFISYPGYSQDEKDYIDAYVSRLLDDLVTIVSDGP-----PTGYPEA-AEIVEVPEP	318
VARV	NKFISYPGYSQDEKDYIDAYVSRLLDDLVTIVSDGP-----PTGYPEA-AEIVEVPEP	318
SPV	NKFISYPGYSQDEKDYICVFERLLDDLVTIVCDTS-----PKWFPET-AELVEVPNS	319
MYXV	NRFISYPGYNQTERDYICAFVERLLEDLVTIVSDIV-----PSTFPDS-AEIVEVPPD	319
MOCV	NKFIAYPGFSQSEQSYVCAFVERLLEDLIRISDAE-----PSGFPEA-AELVEVPPG	317
AmEPV	NMFISYPDPYPETEENFIKTYVVKLLKDLIIISDDENFI----KSKGFSDK-CKFKKIDPC	324
HaEPV	NMFISYPDPYPETEKEYIKSFIDRIISDLIIISPDEDFL----KHRGFNEK-SKFKKLYY	341
MsEPV	KSFISYPNYPETEESFIKSYVDKILQDLLIVDFNNFY----AKRKFNDKCKFVEIKPF	329
DlEPV	NRYIINPGVDYSEDVLVQKWLNLKDLLIVTTKDMLSLSENKALGFKDEAVFHEITKNT	313
	: * * . * : : : . : * * : :	
VACV	GIVSIQD-ADVVKIDNVPDNMSVYLHINILMFGR-KNSFTYNISSKFSAITGTYSDAT	376
VARV	GIVSIQD-ADVVKIDNVPDNMSVYLHINILMFGR-KNSFTYNISSKFSAITGTYSDAT	376
SPV	GIVTIQD-VDIFVRIDNVPDNMSVYFHINILVFGTR-KNSVTYNISSKFTITITGTYSEST	377
MYXV	GIVNIQD-VDVFKIDNVPDKMAVYFHINILVFGTR-KNSVYNISSKFSITITGTYSEST	377
MOCV	GLVSIQD-VDVLRIDGVPAGKTVFFHINILVFGTR-RNSFMYNISSKFSVIAGCFSPAT	375
AmEPV	DKIVFDVNNNCEINIMNVEGFDLYYHINILSFSRR-NNPNDYNISSKFSKISGTYIPNE	383
HaEPV	DEIKFDVNNNCTVNIINVPENHNIYHINILSFSRR-NNPNEYNISSKFNIIIGTYIPEE	400
MsEPV	DVVKHDVNNQCIINIKIPEGMKLYYHKNILSFSRR-NKNEYNISSKFKYILGEYLEKE	388
DlEPV	MTFNKLEKRFCKITTIENIPEDHKLYYHINILSFTWRFOHTKALNVSTLFFKKITGVYLPQ	373
	. : * . * . : : * * * * * . : * * * . * * :	
VACV	KRTIFAHISHSINI IDTSIPVSLWTSQRNVYNGNRSAESKAKDLFINDPFIKGDIFKNK	436
VARV	KRTVFAHISHSINI IDTSIPVSLWTSQRNVYNGNRSAESKAKDLFINDPFIKGDIFKNK	436
SPV	NRIMFHVSHSINI TDVSI PVSWTQRNTYNGNRSSESSKNKDLFINDPFIKGDIFKNK	437
MYXV	KRIMFHVSHSINI TDVSI PVSWTQRNTYNGNRSSESSKNKDLFINDPFIKGDIFKNK	437
MOCV	GKLIPTSVQHTVSVTDASIPVGFWSPPKNVYHGNRSCSSRAKDFVNDPFLKGVDFLNK	435
AmEPV	DKILIEHVKHTINI SDVSIPLSIWNANENTSTGDRSKSKSDIYVNDPFFVGLDFLSK	443
HaEPV	DKILIEHVKHTINI TDVSI PVSWTQRNTYNGNRSSESSKNKDIYDDPFFVGLDFLSK	460
MsEPV	DRITYFDVSHDISI SDVSIPLSIWNANENTSTGDRSKSKSDIYVNDPFFVGLDFLSK	448
DlEPV	KVINFLDIDHSIDIKIVSLPISIDWHELNSHPGDRSNAMKERDFFKRNRLGDMFNCK	433
	: . * : : . * * : * * * * * : * . . : : * * * *	
VACV	TDIISRLEVRFGNDVLYSENGPISRIYNELLTK-----SNNGTRITLTFNFTPK	484
VARV	TDIISRLEVRFGNDVLYSENGPISRIYNELLTK-----SNNGTRITLTFNFTPK	484
SPV	TDIISRLEVRFGNDVLYSETSPISKVYNDLLSN-----HKCGMRTLRFNFTPK	485
MYXV	MDLISRLEVRFGNDVLYSETAPISKIYNDLLSG-----CDSGIRMLRFNFTPK	485
MOCV	AEVISRMEVRFGNDVMEYSEIAPISRVIYQVLHG-----AHCGRKLLRFNFTPK	483
AmEPV	ELGIIISRISISSANESIAEFNSDIVNIDSYFSSDALYAVSKTSDHSNPSIFLYRPNLHNI	503
HaEPV	<u>ELGIIISRISISSANESIAEFNSDIVNIDSYFSSDALYAVSKTSDHSNPSIFLYRPNLHNI</u>	520
MsEPV	DLGIFTSTLKTNSNETIHDINSRPNYEFYLNNSCVYVPTINDESYPISIFHRFNQHSI	508
DlEPV	<u>DRGYERISLKGKGDIFENLLRERKPFRLKLPIT-----EFDPMQRGISLTYTT</u>	481
	: . : : . : : : . * . : : : : :	
VACV	IFFRPTTITANVSRGKDKLSRVVYSTMVNHPIYVQQLVVCNDLYKVSYDQGSIT	544
VARV	IFFRPTTITANVSRGKDKLSRVVYSTMVNHPIYVQQLVVCNDLYKVSYDQGSIT	544
SPV	TFFKPTTIVANPSRGKDKLSRVVYSTMVNHPIYVQQLVVCNDLYKVSYDQGSIT	545
MYXV	TFFKPTTIVANPSRGKDKLSRVVYSTMVNHPIYVQQLVVCNDLYKVSYDQGSIT	545
MOCV	AFRPTTITANVSRGKDKLAVRVYSSMDPNPISYVQQLVVCNDLYKVSYDQGSIT	543
AmEPV	IFIEPSRLIADAAKNFRCVNLSDWKEFPEVDPRSLFNKELQICQITIVKKISYDNNIITV	563
HaEPV	IFVEPSRLIADVGNFRCVNLAVDWKDFSEVDPRSLFNKELQICQITIVKKISYDNNIISV	580
MsEPV	LLSEPSRLIADNKNFRFRISICINWKHYPTDPRSLFKQYMIIGMTIVKKIYDNNIINV	568
DlEPV	FISPSLMIYADPSINFNTNLFVEIQWKEYDECDPLNLLKRFPCVDLYEMQKITQNPDTQRI	541
	: . : : . : : : . * . : : : : :	
VACV	KIMGDNN-- 551	
VARV	KIMGDNN-- 551	
SPV	KIIGEL-- 551	
MYXV	KITDDVKNK 554	
MOCV	KVSE---- 547	
AmEPV	HILE---- 567	
HaEPV	HILE---- 584	
MsEPV	HIVDERK-- 575	
DlEPV	SIESI---- 546	
	:	

Figure 4a (con't). ClustalW 1.81 multiple sequence alignment of the deduced amino acid sequence of the putative rifampicin resistance protein homologs from *Amsacta moorei* entomopoxvirus (AmEPV), *Heliothis armigera* entomopoxvirus (HaEPV), *Melanoplus sanguinipes* entomopoxvirus (MsEPV), *Molluscum contiguosum* poxvirus (MOLCV), swinepox virus (SPV), Myxoma poxvirus (MYXV), vaccinia virus (VACV), variola virus (VARV), and *Diachasmimorpha longicaudata* entomopoxvirus (DlEPV). A colon (:) represents amino acid homologous (“conservative”, sensu Mount 2001) substitutions. A period (.) identifies amino acid non-homologous substitutions. Asterisks indicate identical amino acids conserved in all sequences. Underlined sequences represent regions I and II in HaEPV and DlEPV with the highest percent conserved amino acids previously identified for HaEPV by Osborne et al. (1996). For the three motifs identified within the RIF sequence, Blue = conserved in all poxviruses; Red = conserved only among chordopoxviruses; Green = conserved only among EPVs. Other colors = conserved in some members of a subfamily.

Discussion

An *EcoRI* (RI-1) clone selected from a DNA genomic library of DIEPV from the parasitic wasp *D. longicaudata*, contains a complete open reading frame that was shown by BLAST search to be a homolog of the vaccinia *rif* (D13L) gene. Upstream of the *rif* open reading frame were characteristic poxvirus early transcription termination signals (TTTTnT) (Moss 1996, 2001) (Figure 3). The presence of the characteristic poxvirus consensus late transcriptional start signal (TAAATG) and stop codons confirm that the DIEPV open reading frame is a late gene (Rosel et al. 1986). An 87% A/T rich region immediately before the DIEPV *rif* putative translational initiation site (Figure 3) is similar to the 91% adenylated sequence upstream of the translational start site in the *rif* of the *H. armigera* entomopoxvirus (Osborne et al. 1996).

The DIEPV RI-1 open reading frame is 1,641 base pairs and potentially encodes a 546 amino acid polypeptide that shares considerable similarity with RIFs of both chordopoxviruses and entomopoxviruses (Figure 4, Table 1). In vaccinia, RIF has been shown to be involved in the formation of the Golgi-derived crescent-shaped membranes characteristic of the early stages of virion assembly (Sodiek et al. 1994). Similar crescents also occur during DIEPV morphogenesis (Lawrence and Akin 1990). Because morphologically similar structures are conserved within the poxvirus family (Moss 1996, 2001) and are presumed to arise through similar mechanisms, RIF was considered to be unique to poxviruses (Osborne et al. 1996). However, there are reports of *rif*-like genes in certain other large DNA non-poxvirus families with which poxviruses are suspected to share a common ancestry (Iyer et al. 2001) but it is not clear whether they are functionally similar (Table 2). Amino acid comparisons between DIEPV and the

insect-infecting non-pox DNA (asco- and irido-) viruses revealed $\leq 26.4\%$ amino acid similarity among their RIF-like proteins, far less than the similarities between DIEPV and other poxviruses (Table 1). Thus while DIEPV RIF, like those of other poxviruses, may be distantly related to RIF-like proteins from non-pox large DNA viruses, it is closer to homologs of entomopoxviruses and chordopoxviruses (Table 2). These results, along with previously published phylogenetic comparisons of other DIEPV genes with those of other poxviruses (Lawrence 2002; Mwaengo and Lawrence 2003; Hashimoto and Lawrence 2005), further support our hypothesis that DIEPV is an entomopoxvirus.

The sequence alignment shows two highly conserved internal regions within DIEPV RIF that correspond to those described for the *H. armigera* entomopoxvirus (Osborne et al. 1996). Within these regions, two apparent motifs were evident but exhibited amino acid substitutions that were unique to their respective virus subfamilies (Figure 4a). Conserved inner regions of poxvirus RIFs have been hypothesized to interact with eukaryotic subcellular elements (Osborne et al. 1996). It has been further hypothesized that protein function may depend on their 'head to tail' interaction (Baldick and Moss 1985). The DIEPV deduced protein sequence showed very low amino acid conservation within its terminal regions in alignments with all poxviruses (Figure 4a) but had at least 10 and 20% conserved amino acids within 40 and 50 residues respectively, of the N- and C- termini in alignments with individual entomopoxviruses (data not shown). It is not clear whether or how these conserved amino acids at the DIEPV RIF termini may influence protein function within the host.

The present study demonstrates that DIEPV, a unique viral symbiont of a parasitic wasp of tephritid fruit flies, possesses yet another

Table 2. Percent similarity between DIEPV D13L vaccinia homolog and orthologs/homologs from large enveloped double stranded DNA viruses from non-poxvirus families.

Virus family	Genus	Virus name	Acronym [Accession #]	Percent Homology
Asfaviridae	<i>Asfavirus</i>	African swine fever virus	ASFV [NP_042775]	22.2
Iridoviridae *	<i>Lymphocystivirus</i>	Lymphocystis disease virus 1	LDV-1 [NP_044812]	20.8
	<i>Iridovirus</i>	Invertebrate Iridescent virus	IIV-6 [NP_149737]	19.9
Phycodnaviridae *	<i>Chlorovirus</i>	<i>Paramecium bursaria</i> chlorella virus 1	PBCV-1 [NP_048978]	3.4
	<i>Phaeovirus</i>	<i>Ectocarpus siliculosus</i> virus	ESV [NP_077601]	10.2
Ascoviridae **	<i>Ascovirus</i>	<i>Diadromus pulchellus</i> ascovirus 4a	DpAV4a [CAC84483]	26.4

* Iyer et al., 2001

** Stasiak et al., 2003

homolog of a poxvirus gene. While several DIEPV genes remain to be sequenced and characterized, almost 50% of sequences published to date (Lawrence 2002; Mwaengo and Lawrence 2003; Hashimoto and Lawrence 2005), collectively have the highest homology with those of entomopoxviruses. However, these DIEPV genes and deduced proteins exhibit sufficient differences from the lepidopteran and *M. sanguinipes* entomopoxviruses, that they were placed in a different entomopoxvirus clade (Figure 5), suggesting that DIEPV belongs to a different genus. DIEPV is designated as an unassigned species within the subfamily [00.058.2.00.001.00.001. *Diachasmimorpha entomopoxvirus* (DIEV) (ICTVdB 2004)] but its pathogenicity to dipterans (Shi et al. 1999; Lawrence 2005) suggests that it is likely a member of the Gammaentomopoxvirus genus. Its true phylogenetic position within the subfamily is hampered by the lack of sequences from known dipteran entomopoxviruses and therefore awaits further clarification.

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Correction

Figure 3b was originally published in a truncated form; the corrected version is shown below.

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1      TTGTCAATGA TGGGGTTAAA ATGGTTTCGA TGAGTAAATA ACTATATATC CTAGTCAATA ATGTATGATA
71     TGGAAATGCG TACTCCAGTA TAATATTTTCG TAAAAAGTC TATAAATTCT GTAAAGATTA TTTTAATATA
141    CTCTTTTTTC GTTCGGCACA AGTAGAAATC TATATTATTG TAACGATTCA AAAGAAACAT AACTGTTACA
211    AAATTTTTGT GTCCTTCTTG TTTAAAGTAA TCATGTATAT AGGTCGTATC GATAAAGTCG TCGTCTAATT
281    CTTTCGTATC GATTTTTTTT AATAAATTCT CATATAATTC ATCTAAAAGG AACATATCAA CAGTCTCCAT
351    GGATTTAAGA ACATTGTTC AATGTATAAG CTTATTTTTA CTAGTTTCGG CGAAAGTGT CAAATTCGAA
421    ATGTGTTTAC CACAGCGGTT ACAAAGAGA ACCTTTTTCG TATATATCAA ATCTTCACTG TCACATTTTCG
491    AACATTTGAT ATCTTTCATT TATATTTTTT CCTTTTTAAA TGGAGCTAAC CACTTTTAAT ACCAATCATC
                                         M E L T T F N T N H P

561    CATTCATCCA TTCGGCGTAC CCCAAACTT TTTCATATGT TCCAAAAAT GAAAATGATA TATATTTCTGT
      F I H S A Y P K T F S Y V P K N E N D I Y S V

631    GAATGTAACC GATGTACGTG TAGAAGCAAT CAGTTCTCCT GAAATTAAGC TTATTTTACC GGAGATTAAA
      N V T D V R V E A I S S P E I K L I L P E I K

701    GGCAAAGGAC GTGTGTCTTA TCTCAAAAAT TACCAGTTTC TTCTTTTAGA CTATTTTGAA ATCTGGTTAA
      G K G R V S Y L K N Y Q F L L L D Y F E I W L K

771    AAAATAAAGA CGAACATCCA TTTTTGTTC ATAAAGCCAA AAGTGAGGAA ATTTTTTCAA CTTATATTAT
      N K D E H P F L F H K A K S E E I F S T Y I I

841    CAACGAATAT CACTCGTTAA ACTATTTTAC CAACAAAGAT GTTTTTCTGA CAACCAAAGA AGGGACCCAC
      N E Y H S L N Y F T N K D D F L T T K E G T H

911    GCTGATTGCA TAATTTTCCC TAAAAAAGAA ATATCTATTC CATTGGATTC GTTGCTTTCT GCTTTTAAAA
      A D C I I F P K K E I S I P L D S L L S A F K I

981    TCTTTAAAGA TACCGAAATT ATTTTCAATT TCAAATTC TAACATTGAA GAAATTATAG CCTATGATGT
      F K D T E I I F N F K F H N I E E I I A Y D V

1051   AGAATTTAGA CGTCATTCAC TAGAACAAC CAAGAAAAAC TTTTTCTGAA CATCATTGAA TATCAGATTC
      E F R R H S L E Q L K K N F S E T S L N I R F

1121   CAATTTTGA ATGTTCCAAT AATTTTCAAT GCAGAACTCA CAGCAACTAA CGTAATTACC AAAAAGGATG
      Q F L N V P I I S S A E L T A T N V I T K K D V

1191   TGATTGGTAA AGATAATACT CAAATGATGA ATACATCAGA CTTCTCAAAC ACTATTGCTG TAAGTTTCCA
      I G K D N T Q M M N T S D F S N T I A V S F H

```

Figure 3b. DNA sequence of the RI-1 open reading frame and an immediately preceding region (539 nt) containing putative poxvirus early transcriptional stop (TTTTnT) and late promoter (TAAATG) sequences (highlighted in black). Restriction enzyme recognition sites, shown in (a), are underlined. The putative translational stop codon (TAA) is indicated by an asterisk (*). The sequence has been assigned GeneBank accession # EF541029.

1261 **TTCTAAAAGC GATATCTTTA ATCACGAAAA TCGTTATATT ATTAATCCGG GTGTAGATTA TTCCGAAGAT**
 S K S D I F N H E N R Y I I N P G V D Y S E D

1331 **GTGCTTGTC AGAAATGGGT TTAAATATT TTAAGATT TGCTTATTGT GACCACAAA GATATGTCCC**
 V L V Q K W V L N I L K D L L I V T T K D M S L

1401 **TGTCAGAAAA TAAAAAGCT CTGGGTTTCA AAGACGAAGC TGTGTCCAT GAAATTACTA AAAATACTAT**
 S E N K K A L G F K D E A V F H E I T K N T M

1471 **GACTTTCAAT AAACGCGAAA AAAGGTTCTG TAAGATCACA ATCGAAAATA TCCCAGAAGA TCACAACTT**
 T F N K L E K R F C K I T I E N I P E D H K L

1541 **TATTATCATA CAAATATTCT AAGCTTCACC AGACGTTTCC AACACACCAA AGCACTCAAT GTTCCACAC**
 Y Y H T N I L S F T R R F Q H T K A L N V S T L

1611 **TTTTTAAGAA AATCACGGGT GTTTATCTTC CCAATCAAAA AGTAATCAAT TTTCTAGATA TAGATCATAG**
 F K K I T G V Y L P N Q K V I N F I S I W L D

1681 **TATAGATATT AAAATTGTAA GTTTACCTAT TAGTATTGG GATCATGAAT TGAATAGTCA TCCAGGTGAT**
 I D H S I D I K I V S L P D H E L N S H P G D

1751 **TTAAGATCCA ATGCCATGAA AGAACGTGAT TTTTTCTTTA AGAATAGATT TTTGCTTGGA ATGGACTTCA**
 L R S N A M K E R D F F F K N R F L L G M D F N

1821 **ATTGCAAAGA TAGAGGATAT GAACGTATTT CACTTAAAGG TGGTAAAGAT ATCTTTGAAA ACCTTCTTCG**
 C K D R G Y E R I S L K G G K D I F E N L L R

1891 **GGAAAGAAAA CCCTTTCTTC GTAAACTTCC CATTATCGAA TTTGATCCAG CTATGCAAAG AGGTATTTTCG**
 E R K P F L R K L P I I E F D P A M Q R G I S

1961 **TTATATACAA CCTTCATAAG CCCATCTCTC ATGATATACG CAGATCCCTC TATCAACTTT ACAAATTTCT**
 L Y T T F I S P S L M I Y A D P S I N F T N F L

2031 **TAGTCGAGAT CCAATGGAAA GAATATGATG AGTGTGATCC TCTAAATCTA TTAACCGTT TCCCATGTGT**
 V E I Q W K E Y D E C D P L N L L K R F P C V

2101 **GGACTTATAT GAGATGCAAA AAATCACACA AAATCCTGAT ACACAACGTA TTAGTATTGA ATCTATATAA**
 D L Y E M Q K I T Q N P D T Q R I S I E S I *

2171 ATGCTTGACT TTTAATATT TCATTCTCAA CCCTTGTCG TTCAGCTTTC AAAAAAGCGA AACCCATTT

2241 GATTGATTCA CTTGAGGGCA AATTTGGAA CACAGGAGTA TTGGCACTTA CTATGGTATT ATTTTGGGAA

2311 GATTTTATAA CATGTCTTTC TGTAACTTT TTCT

Figure 3b (con't).