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Sequence comparison between three geographically distinct *Spodoptera frugiperda* multiple nucleopolyhedrovirus isolates: Detecting positively selected genes ☆

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ABSTRACT

The complete genomic sequence of a Nicaraguan plaque purified *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) genotype SfMNPV-B was determined and compared to previously sequenced isolates from United States (SfMNPV-3AP2) and Brazil (SfMNPV-19). The genome of SfMNPV-B (132,954 bp) was 1623 bp and 389 bp larger than that of SfMNPV-3AP2 and SfMNPV-19, respectively. Genome size differences were mainly due to a deletion located in the SfMNPV-3AP2 *egt* region and small deletions and point mutations in SfMNPV-19. Nucleotide sequences were strongly conserved (99.35% identity) and a high degree of predicted amino acid sequence identity was observed. A total of 145 open reading frames (ORFs) were identified in SfMNPV-B, two of them (*sf39a* and *sf110a*) had not been previously identified in the SfMNPV-3AP2 and SfMNPV-19 genomes and one (*sf57a*) was absent in both these genomes. In addition, *sf6* was not previously identified in the SfMNPV-19 genome. In contrast, SfMNPV-B and SfMNPV-19 both lacked *sf129* that had been reported in SfMNPV-3AP2. In an effort to identify genes potentially involved in virulence or in determining population adaptations, selection pressure analysis was performed. Three ORFs were identified undergoing positive selection: *sf49* (*pif-3*), *sf57* (*odv-e66b*) and *sf122* (unknown function). Strong selection for ODV envelope protein genes indicates that the initial infection process in the insect midgut is one critical point at which adaptation acts during the transmission of these viruses in geographically distant populations. The function of ORF *sf122* is being examined.
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1. Introduction

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is a severe pest of maize, sorghum and rice in tropical and subtropical areas of the Americas. Isolates of *S. frugiperda* multiple nucleopolyhedrovirus (SfMNPV) are known to cause natural epizootics of disease and have been recognized as potential control agents against this pest (Escribano et al., 1999; Fuxa, 1982; Shapiro et al., 1991). A Nicaraguan isolate (SfMNPV-NIC) was selected for formulation and field trails in Honduras and Mexico (Williams et al., 1999), and has been extensively studied at the genomic and phenotypic levels (López-Ferber et al., 2003; Simón et al., 2004, 2005b, 2008c). SfMNPV-3AP2 is a single genotype isolate from Missouri (USA) with a fast killing phenotype that was tentatively attributed to its lack of the *egt* gene (Harrison et al., 2008). The SfMNPV-19 isolate, purified from a single larval cadaver collected in Paramá State, southern Brazil, was selected from 22 SfMNPV isolates collected in Brazil due to its high pathogenicity against fall armyworm larvae (Barreto et al., 2005; Wolff et al., 2008).

Adaptation of viruses to their local host populations has been described in various occasions. Comparative studies have reported that a Honduran population of *S. frugiperda* was most susceptible to a Central American isolate (Escribano et al., 1999), whereas insects from a Colombian population were most susceptible to a SfMNPV isolate from Colombia (O. Simón, unpublished data). Similar findings have been observed by others working on different insect-nucleopolyhedrovirus systems elsewhere (Erlandson et al., 2007; Erlandson, 2009). This pattern is likely to be generated through processes of host–pathogen co-evolution, in which selection favors viruses with high transmissibility in the local host population. A consequence of this is that the choice of an isolate as the basis for a biological insecticide requires the assessment of a range of geographical isolates, including those from the region in which the biological control program is required.

☆ The GenBank/EMBL/DDBJ accession number of the sequence reported in this paper is HM595733.

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A number of genes intervene in the species-specific virulence or host range of NPVs by affecting the ability to infect and replicate in cells and thereby influencing the dose required for host mortality, or the survival time of infected larvae from a given host population or species (Chen and Thiem, 1997; Chen et al., 1998; Popham et al., 1998). In most cases, species-specific effects of a gene on virulence or host range have been elucidated by ORF disruption or deletion. Eliminating or reducing virulence against one species following knock-out of a particular gene suggests that acquisition of new genes via recombination has the potential to shape NPV host range. However, very small changes such as individual amino acid replacements in particular viral genes have also proven influential in expanding host range or modifying virulence (Argaud et al., 1998; Kamita and Maeda, 1997; Yang, 1998). Additionally, nucleotide substitutions may lead to alterations in the activity of the encoded protein that facilitate adaptation to a new host or effectively overcome the defenses of a current host. Such mutations would confer a selective advantage and would be fixed in the population at a higher rate than silent or neutral substitutions (Harrison and Bonning, 2004).

The availability of the complete sequences of isolates from the same virus species from different geographical areas offers the possibility of another kind of analysis, based on a bioinformatics approach. In the present study, the complete sequence of an SfMNPV-NIC genotype was compared with those of SfMNPV-3AP2 and SfMNPV-19. Maximum-likelihood models of codon substitutions were used to test for selection on ORFs. The non-synonymous to synonymous substitution rate ratio ($\omega = d_N/d_S$) provides a sensitive measure of selection on amino acid sequences. We reasoned that comparative analyses of SfMNPV genomes from geographically distinct sources could highlight genetic differences involved in the marked phenotypic variation in virulence that has been reported in these isolates, and thereby focus attention on the key genes involved in traits that are considered desirable for the development of effective bioinsecticidal products.

2. Materials and methods

2.1. Virus

The Nicaraguan SfMNPV isolate (SfMNPV-NIC) was initially collected from larval cadavers collected from a maize field in Nicaragua. The isolate was propagated in *S. frugiperda* larvae reared in the laboratory and subjected to an uncertain number of passages. SfMNPV-NIC genotypic variants were isolated by plaque purification (Simón et al., 2004) and the predominant genotype that possessed the largest genome (SfMNPV-B) was selected for bacmid construction (Simón et al., 2008a).

Occlusion bodies (OBs) obtained from larvae infected with SfMNPV-B were extracted filtered through cheesecloth, washed twice with 0.1% SDS and once with 0.1 M NaCl and finally resuspended in bidistilled water. OB suspensions were quantified using a bacterial counting chamber and stored at 4 °C until used.

2.2. Viral DNA isolation and cloning

SfMNPV-B bacmid DNA was sequenced to ensure that the nucleotide sequence originated from a single genotype and was not the result of contamination from a persistent infection present in the insect colony (Simón et al., 2010). The complete SfMMPV-B genome was cloned in a pBACe3.6 vector that includes a chloramphenicol resistance gene (Frengen et al., 1999). The pUC19 plasmid was removed from pBACe3.6 by treatment with *Bam*HI. pBluescript KS-I with a modified polylinker that included the *Asc*I restriction site, was inserted using T4 DNA ligase (New England Biolabs, Ipswich, MA). To include the *Asc*I restriction site in pBluescript KS-I polylinker, the polylinker was amplified by PCR using two specific primers. One of the primers included a *Sac*I and an *Asc*I restriction site sequence, and a 20 nt homolog to the right arm of the polylinker, whereas the second primer included a 20 nt homolog to the left arm of the polylinker, and *Asc*I and *Kpn*I restriction site sequences. The PCR product was double digested with *Sac*I and *Kpn*I and cloned into pBluescript KS-I previously digested with these two enzymes. Once the modified pBACe3.6 was obtained, this and the SfMNPV-B DNA purified by CsCl were digested with *Asc*I and ligated overnight at 16 °C using the T4 DNA ligase (Simón et al., 2008a). The ligation reaction was dialyzed for 4 h against TE buffer. After dialysis, the ligation was used to transform DH10B GeneHogs electrocompetent cells. Transformed cells were incubated at 37 °C for 1 h in SOC medium and colonies were selected in the presence of chloramphenicol. Colonies were selected on chloramphenicol medium and bacmid DNAs were purified by alkaline lysis. DNA was digested with *Pst*I for comparison with the restriction profiles of each of the Nicaraguan SfMNPV genotypes (Simón et al., 2004). The bacmid with the complete genotype was selected for sequencing. Alkaline lysis and CsCl gradient purified bacmid DNA was used for sequencing (Sistemas Genómicos S.L., Paterna, Valencia, Spain).

2.3. DNA sequencing and sequence analysis

Shotgun sequencing was performed using a genomic library of SfMNPV-B bacmid DNA constructed into a sequencing vector. Sequence information was generated from 2304 reactions performed on 1152 clones. The depth of sequence coverage across the genome was 4–8x. Sequencing reactions were set up using the ABI Prism Big Dye Terminator Cycle Sequencing Reaction kit on a 9600 of PE model thermocycler. The reaction products were loaded in an automated DNA sequencer ABI PRISM. The DNA and deduced amino acid sequences were compared against the updated GenBank/EMBL, SWISS-PROT and PIR databases using BLASTn, (Altschul et al., 1990; Pearson, 1990). SfMNPV-3AP2 (accession number EF035042) and SfMNPV-19 (accession number EU258200) genomic sequences were obtained from the GenBank database and published sources. Sequence alignments and gene-parity plots were performed using NCBI BLAST alignment tools (Altschul et al., 1990) to examine genome organization and order of homologous ORFs. SignalP (Bendtsen et al., 2004), TargetP (Emanuelsson et al., 2000) and Virus-Ploc tools (Chou and Shen, 2008) were also used to determine the nature of selected proteins of unknown function.

2.4. Analysis of selection pressures

PAML software package version 4 (Yang, 2007; http://abacus.gene.ucl.ac.uk/software/paml.html) was used to estimate positive selection acting on SfMNPV ORFs and to infer amino acid sites subject to this process. This software uses a maximum-likelihood approach to determine the numbers of non-synonymous (amino acid changing) substitutions per non-synonymous site (d_N) and of synonymous (silent) substitutions per synonymous site (d_S). The ratio of d_N to d_S, ω, is a measure of the magnitude of selection acting on a gene. Genes with a value of $\omega = 1$ are deemed to be subjected to neutral selection, so that that non-synonymous mutations have no effect on fitness. Genes with $\omega < 1$ are undergoing negative or purifying selection, in which non-synonymous mutations are eliminated at a faster rate than synonymous mutations because of their deleterious influence on fitness. Finally, genes for which $\omega > 1$ are undergoing positive or diversifying selection in which non-synonymous mutations are fixed at a faster rate than synonymous mutations as they positively influence fitness. To achieve this, sequences were examined for the presence

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