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SHORT REPORT

Genetic variants in Argentinean isolates of *Spodoptera frugiperda Multiple Nucleopolyhedrovirus*

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Abstract

The fall armyworm, *Spodoptera frugiperda* (JE Smith) is a key pest in the Americas. Control strategies are mainly carried out by use of chemical insecticides and transgenic crops expressing *Bacillus thuringiensis* toxins. In the last years, resistance of *S. frugiperda* populations to transgenic corn was reported in different Latin American countries. The baculovirus *Spodoptera frugiperda Multiple Nucleopolyhedrovirus* (SfMNPV) is a pathogenic agent for the fall armyworm and a potential alternative for its control in integrated pest management strategies. In this work, we analyze some characteristics of two baculovirus isolates collected from maize (SfMNPV-M) and cotton (SfMNPV-C) fields from Argentina. The isolates were compared by restriction enzymes patterns and the analysis reveals the presence of genotypic variants in the SfMNPV-M isolate. We confirmed a deletion by sequencing fragments encompassing *egt* gene and most part of its contiguous gene (*orf A*) in a SfMNVP-M genotypic variant. Additionally, we estimated the 50% lethal dose and median survival time of each isolate in bioassays with *S. frugiperda* larvae.

Keywords Fall armyworm · SfMNPV isolates · Biological control · Baculovirus · Agricultural pest

The fall armyworm, *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is an important pest of maize and other crops in the Americas [1, 2] and has been recently reported in Africa [3]. Management of the fall armyworm is mainly carried out through the use of chemical insecticides and transgenic crops expressing *Bacillus thuringiensis* toxins [4]. In recent years, resistance of *S. frugiperda* populations to transgenic corn was reported in Puerto Rico, Brazil and Argentina [5–7]. Therefore, the use of biological control agents as an alternative for the integrated management of the fall armyworm has gained renewed interest.

The baculovirus *Spodoptera frugiperda* Multiple Nucleopolyhedrovirus (SfMNPV) is the main pathogen causing epizootic outbreaks in natural populations of the fall armyworm [8, 9]. Several studies have shown that baculoviruses adapt to their local host populations and do not present the same performance when applied to host populations from different regions [10, 11]. Therefore, it is required to study different isolates to rationally select the best biocontrol agent to a specific location. Currently, there are five complete SfMNPV genomes available in GenBank [12–15]. The in silico analysis of their restriction endonuclease (REN) patterns evidences variation observed experimentally in SfMNPV isolates reported in early studies [9, 16–18].

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In this work, we explore some properties of two indigenous SfMNPV isolates from Argentina that we found readily distinguishable by REN analysis: the isolate hereinafter referred to as SfMNPV-M (reported previously as SfMNPV-AR [16]) and SfMNPV-C.

To multiply the SfMNPV isolates, larvae of *S. frugiperda* were reared at the Institute of Microbiology and Agricultural Zoology (IMYZA), INTA. Larvae were raised on a maize-based artificial diet [19] at 25 ± 1 °C, 16:8 h L:D, 50–70% RH. Isolates SfMNPV-M and SFMNPV-C were obtained from single dead larvae collected in a maize field of the central region of the country, and in a northeastern cotton field, respectively. The viruses were multiplied by infecting fourth instar larvae with artificial diet contaminated with OBs purified from infected larvae using the methodology described by Berretta et al. [16]. Purified OBs were dissolved by alkali and DNA extraction was performed as described by Parola et al. [20]. For restriction enzyme analysis, 2.5 µg DNA was incubated with 10U BamHI (Fermentas) for 2 h at 37 °C. DNA fragments were visualized by electrophoresis using 0.8% agarose gels in TAE buffer. In silico BamHI digestion profiles were performed with Vector NTI program (Invitrogen) on SfMNPV genomes retrieved from GenBank: 3AP2 (EF035042), 19 (EU258200), Nic B (HM595733) and Col (KF891883).

The BamHI restriction pattern of SfMNPV-M genomic DNA was identical to that of NicB containing seven major fragments (designated A to G), and the majority of the bands were also coincident with those found in the patterns of other exotic isolates [16]. On the other hand, the pattern of SfMNPV-C DNA revealed differences with that of SfMNPV-M (Fig. 1a): SfMNPV-C lacks band F of around 5.1 kbp and contains a large fragment predicted to be "A+F", resulting from the lack of a BamHI site that separates fragments A and F (as previously seen in Col and confirmed for Arg C in this study). Interestingly, the BamHI pattern of SfMNPV-M unveiled the presence of a submolar band of around 3.4 kbp, suggesting the presence of more than one genotype in this isolate.

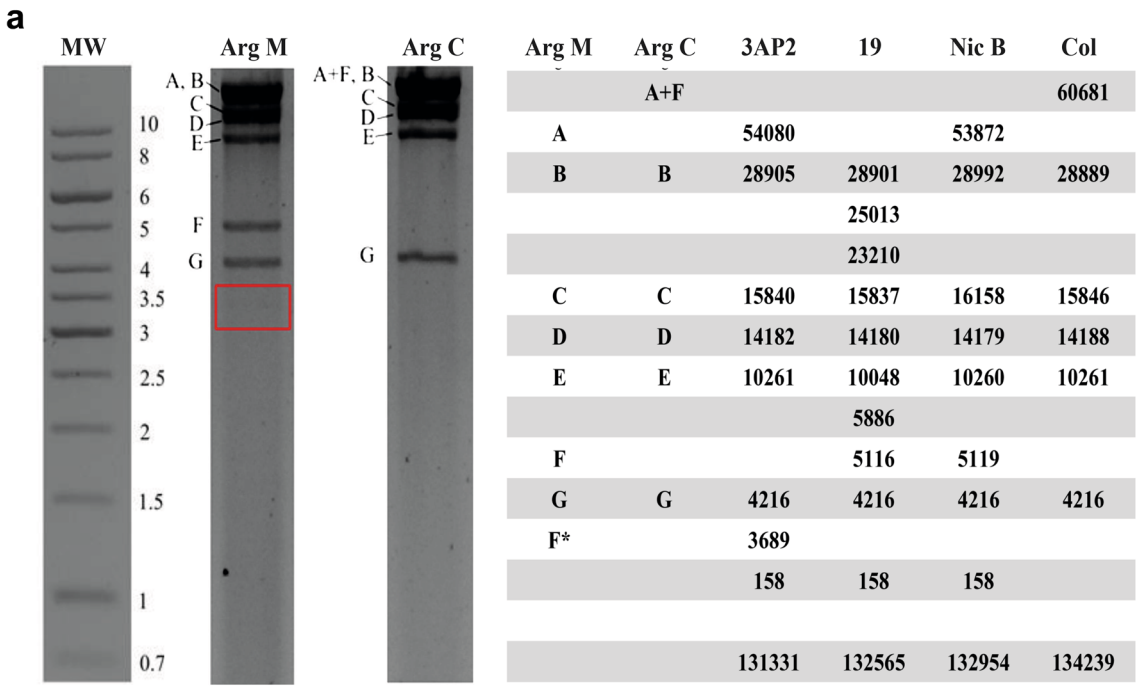
BamHI fragments of SfMNPV-M DNA were cloned into pcDNAII plasmid (Invitrogen) and transformed in *E. coli* DH5α. Several clones were end-sequenced and fragments of two different lengths were identified sharing sequence identity with the ends of BamHI fragment F of SfMNPV-Nic B. Two clones were further sequenced to completeness by the Sanger method with a primer-walking strategy and were designated M1 and M11 after their recorded colony number, with fragment lengths of 5121 bp and 3429 bp, respectively (GenBank MN494087 and MN494088). Using the SfMNPV-Nic B genome as a reference, we found that clone M1 had the sequence corresponding to the complete locus presented in Fig. 1b(1), while clone M11 presented a deletion encompassing a significant portion of both *egt* gene and its contiguous gene (*orf A*) (Fig. 1b(2)). Interestingly, the *egt* gene has been reported as a hot spot for deletions in other SfMNPV isolates [13, 14]. Homologs of *orf A* have been found in group II NPVs but their function remains unknown. Bioinformatic analysis did not retrieve known predicted domains (supplementary material). These results confirmed the existence of two haplotypes in SfMNPV-M, which we designated SfMNPV-M1 and SfMNPV-M11.

To differentially amplify SfMNPV-M haplotypes, the following PCR primers were designed and synthesized: BamHI-Fdel fw: 5´GAAGTCGTAGTCGTCAAATC3´ and BamHI-Fdel rv: 5´TCCAAATCTGAAGACAGTTG3´ (Fig. 1b(1)). M1 was the most abundant genotype as judged by the intensity of F band in the gel (Fig. 1b(2)). We were able to detect both haplotypes in ODVs of polyhedra purified from larvae infected with SfMNPV-M through amplification of the expected DNA fragments of 2096 bp (M1) and 406 bp (M11) (Fig. 1b(2)). The relative intensity of these PCR products is in accordance with the M11 haplotype being the most abundant, as predicted by the restriction pattern. An intermediate band (ca. 1500 bp) of very low intensity could be observed in the electrophoresis of the PCR products, suggesting the existence of another, previously undetected haplotype (red arrow in Fig. 1b(2)). In contrast, a single 2096 bp fragment was obtained through amplification of DNA purified from ODVs of SfMNPV-C, suggesting that there are no haplotypes with major deletions involving *egt* gene in this isolate.

The replication of the haplotypes in cell culture was also investigated. *S. frugiperda* Sf9 cells obtained from American Type Culture Collection (ATCC) were cultured at 27 °C as a monolayer with Grace's insect medium (Invitrogen) supplemented with 1% fetal bovine serum (Internegocios, Argentina) and gentamycin. Sf9 cells were transfected using Cellfectin® reagent (Invitrogen) mixed with SfMNPV-M DNA according to the manufacturer's instruction. The supernatants of Sf9 cells were collected at different time points and were analyzed by PCR with primers BamHI-Fdel-fw and BamHI-Fdel-rev.

The relative intensity of all three PCR bands might reflect the relative abundance of three haplotypes in the BV progeny (Fig. 1b(3)). There might be variations in the proportions of these haplotypes in BVs as when produced in cell culture or propagation in vivo in *S. frugiperda* larvae.

To evaluate the biological activity of SfMNPV isolates bioassays were carried out by feeding of 50, 100, 150 and 300 OBs doses to newly molted 3rd instar *S. frugiperda* larvae, after 12 h starvation, using the droplet-feeding method described by Hughes and Wood [21]. To prior estimate the volume ingested by one larva, 220 larvae were fed a colored solution (1% sucrose, 0.1% Coomassie Brillant Blue), weighted individually before and after ingestion (as determined by the observation of the colored digestive tract), and the mean



Arg M	Arg C	3AP2	19	Nic B	Col
	A+F				60681
A		54080		53872	
B	B	28905	28901	28992	28889
			25013		
			23210		
C	C	15840	15837	16158	15846
D	D	14182	14180	14179	14188
E	E	10261	10048	10260	10261
			5886		
F			5116	5119	
G	G	4216	4216	4216	4216
F*		3689			
		158	158	158	
		131331	132565	132954	134239

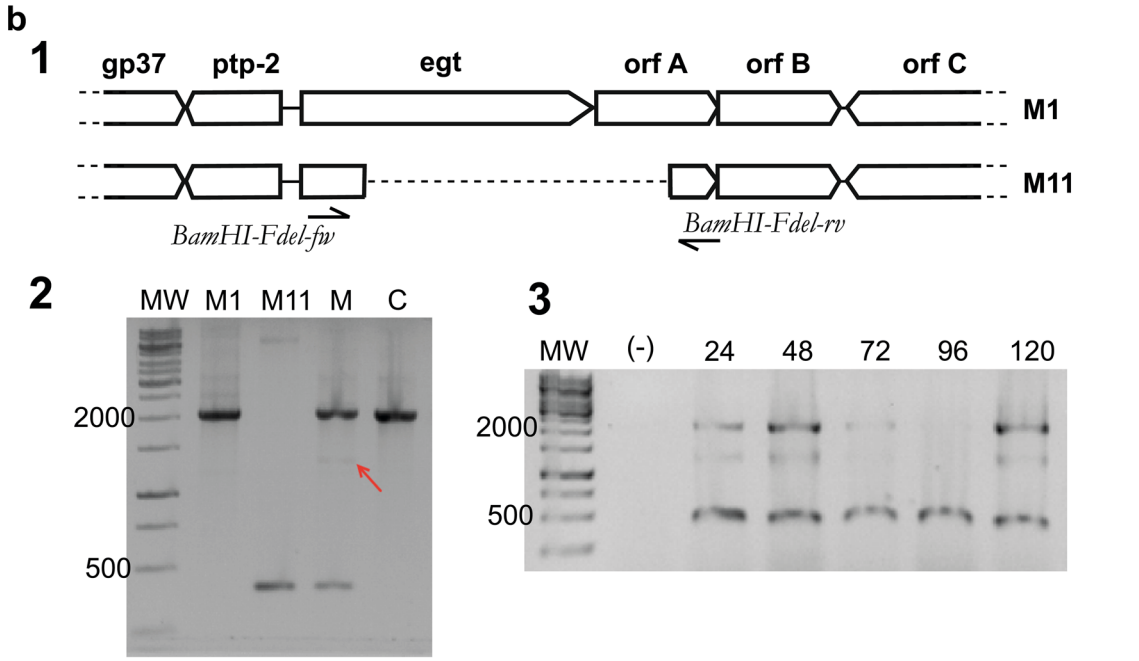


Fig. 1 **a** BamHI restriction profiles of SfMNPV-M and C. Bands were named alphabetically with decreasing size (A–G). Left panel: Submolar band detected in SfMNPV-M is boxed in red. Right panel: Length of fragments (bp) obtained with in silico BamHI restriction analysis of SfMNPV genomes 3AP2 (EF035042), 19 (EU258200), Nic B (HM595733) and Col (KF891883). Homologous fragments are shown in the same row. Total length of each genome is shown in the bottom row. The 3689 bp fragment (F*) in 3AP2 is homologous to F band. **b** Haplotype polymorphism of SfMNPV isolates. *1* BamHI-F restriction fragment of SfMNPV-M haplotypes M1 and M11. The dotted line represents the deletion encompassing most part of *egt* and *orf A* in M11. The sites for annealing of primers BamHI-Fdel-fw and BamHI-Fdel-rev are indicated. *2* Differential amplification of SfMNPV haplotypes using the following DNA templates: M1 and M11, BamHI-F fragment of M1 and M11 haplotypes, respectively, cloned in plasmid vector pcDNAII; M, DNA from OBs of SfMNPV-M. Arrow indicates an undescribed haplotype; C, DNA from OBs of SfMNPV-C. *3* PCR amplification of supernatant of Sf9 cells transfected with SfMNPV-M DNA, collected at 24–120 h post transfection

Table 1 LD_{50} and ST_{50} values for the two SfMNPV isolates in third instar of *S. frugiperda*

SfMNPV-ARG isolates	LD50 (OBs)	Fiducial limits		Slope	Intercept	Chi^2	ST_{50} (h)
		Lower	Upper				
SfMNPV-Arg M	167.56	27.026	242.407	2.6	2.6	3.45	116.64
SfMNPV-Arg C	116.42	80.28	140.59	4.9	4.9	6.83	113.28

volume ingested was calculated. The volume ingested by each larva was estimated as 0.23 µl and was used to calculate the virus doses. Bioassays were performed with 30 larvae per virus dose and 30 larvae used as control. The experiments were replicated three times and larval mortality was recorded along 2 weeks. The lethal doses 50 (LD_{50}) data were analyzed using the Probit Method [22] and the Pearson goodness-of-fit chi-square statistic was used to test that the model adequately fits the data. LD_{50} values of the two virus isolates were compared using *t* tests at $p = 0.05$. Median survival times (ST_{50}) were calculated for SfMNPV isolates in *S. frugiperda* using Vistat software (Cornell University, Ithaca, NY).

The bioassays results showed that LD_{50} for SfMNPV-M and SfMNPV-C isolates was 167.56 and 116.42 OBs/larvae, respectively, and this difference was not statistically significant (Table 1). The median survival times using the lowest dose that yields 100% mortality were 116.64 and 113.28 h for SfMNPV-M and SfMNPV-C isolates, respectively, and the difference was not statistically significant. Typical baculovirus-induced liquefaction signs were observed in dead larvae infected with both SfMNPV isolates (data not shown).

In this work, we compared two Argentinian isolates of SfMNPV. We found differences in the BamHI restriction pattern of their genomes, with a polymorphic DNA fragment involving the *egt* region. Besides, SfMNPV-C was slightly more virulent than SfMNPV-M, although the difference was not significant.

Escribano et al. [11] also characterized an Argentinian isolate of SfMNPV. Its banding pattern appears different from the ones presented here. Variations in BamHI restriction patterns in the 4–6 kb migrating zone seem to be common to SfMNPV isolates [18]. Besides variation between native SfMNPV isolates, genetic differences can also be detected within an isolate, indicating a mixed population of different genotypes (haplotypes), in a single-infected larva. Moreover, different haplotypes may be occluded in the same OB and recruited as nucleocapsids of a single occluded-derived virus (ODV) wrapped with a common virion envelope [23].

We detected a submolar band in the BamHI restriction pattern of SfMNPV-M; this finding led to the identification of at least two genotypes within this isolate: M1 and M11. Some haplotype variants were detected in SfMNPV isolates of USA and Nicaragua, and their genomes were found to present deletions with variable length, encompassing 1–14 genes, within the same genomic region [13, 14]. In accordance to this, we found that variant M11 lacks genes *egt* and *orf A* (orf *A* = *sf27* in [13]).

PCR analysis permitted us to detect the presence of M1 and M11 haplotypes in ODVs produced in larvae and viruses budded from cultured cells. Due to the high sensitivity of PCR technique, we could infer a third putative genotype in SfMNPV-M but further analysis is required to confirm this. In summary, we could detect deletion haplotypes in SfMNPV-M but not in SfMNPV-C. However, we cannot rule out the possibility of the existence of deleted haplotypes in such a low proportion that cannot be detected, or with deletions encompassing the binding site for one or both primers.

Genotypic variants present in a SfMNPV virus population may play an important role in pathogenicity. It has been shown that mixed genotypes have improved killing efficiency compared to that of individual genotypes, even non-defective genotype, containing the complete genome [24, 25]. In addition, it has been proposed that the occlusion of different genotypes in the same OB is an adaptive mechanism that ensures genetic variability in viral transmission [23]. Characterization of indigenous baculovirus isolates specific for relevant local pest populations is an important issue in the rationale use of these biocontrol agents for pest management. It was demonstrated that viruses adapt to local populations of susceptible host. For example, Escribano [11] and collaborators found that *S. frugiperda* larvae from a Honduran population were more susceptible to a SfMNPV variant from Nicaragua (neighbor to Honduras) compared to variants from USA or Argentina. Similarly, Barrera et al. [12] demonstrated that the Colombian isolate of SfMNPV was better suited as a biocontrol agent towards a Colombian population of the fall armyworm than the Nicaraguan isolate.

Further research would need to be undertaken to evaluate the potential use of SfMNPV-M and SfMNPV-C isolates as biocontrol agents of local *S. frugiperda* populations.

Author contributions JN and MB: Conceptualization; JN, MB, MLF and RS: Methodology and investigation; MLF and MB: Writing-original draft preparation; VR and ASC: Administration and supervision. MB, RS, MLF and VR: Writing-reviewing and editing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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