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Application of the PCR–RFLP method for the rapid differentiation of *Spodoptera exigua* nucleopolyhedrovirus genotypes

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Abstract

Quality control during mass production of *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV), and studies on environmental fate following the use of this virus as a biological pesticide, would be facilitated by a rapid method for the detection and identification of isolates. A molecular biology tool was developed that combined the polymerase chain reaction and restriction fragment length polymorphism (PCR–RFLP) to differentiate SeMNPV isolates. Oligonucleotide primers were designed to amplify five variable SeMNPV genomic regions (V01, V02, V03, V04, V05). Four wild-type SeMNPV strains isolated from the United States (US2) and Spain (SP1, SP2, and SP3), and a laboratory cloned genotype (US1A), were analyzed with 36 different primer-endonuclease combinations. *Bgl*II digestion of the variable region 1 (V01) amplicon was the only combination that differentiated each of the five virus isolates tested, although genetic heterogeneity limited the discriminatory power of the technique. Six novel SeMNPV isolates originating from greenhouse soils in southern Spain were successfully identified using this method. As judged by sequence analysis, the V01 region, which comprises the homologous region 1 (*hr*1), is the most variable genomic region among the genotypes present in the Spanish isolates. This method constitutes a useful tool for processing large number of environmental samples and could be used to address environmental biosafety concerns.

Keywords: *Spodoptera exigua* multiple nucleopolyhedrovirus; Diagnostic PCR–RFLP method; Environmental monitoring; Quality control

1. Introduction

Baculoviruses are double-stranded DNA virus pathogens of arthropods, mostly insects of the order Lepidoptera (Gröner, 1986). These viruses are embedded in proteinaceous occlusion bodies (OB) adapted for horizontal transmission and persistence in the environment (Rohrmann, 1986). Infection occurs when phytophagous insect larvae consume leaves contaminated with OBs. Death ensues several days after acquiring an infection whereupon progeny OBs are released into the environment for transmission to susceptible insects.

The beet armyworm, *Spodoptera exigua,* is a major pest of worldwide importance, especially in covered crops. In southern Spain, the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) is commonly found in the environment and frequently causes a high prevalence of infection in *S. exigua* populations feeding on crop and natural plant hosts (Caballero et al., 1992a; Gelernter and Federici, 1986). Certain isolates of SeMNPV have been marketed as a biological insecticide in several countries. Mass production and research on field application of SeMNPV would be facilitated by the development of a rapid diagnostic method for quality control and environmental monitoring.

Studies involving the detection of baculovirus OBs from environmental samples have generally focused on isolation from OB reservoirs in the soil (de Moraes et al., 1999), leaf litter and aquatic habitats (Ebling and Holmes, 2002; England et al., 2001). Such studies have usually employed insect bioassays to amplify OBs isolated from environmental samples. Detection of viral DNA is now possible using highly sensitive techniques based on the polymerase chain reaction (PCR) as a means to detect and identify insect DNA viruses (Lupiani et al., 1999; Williams, 1993), particularly the NPVs (Burand et al., 1992; Christian et al., 2001; de Moraes and Maruniak, 1997; Faktor and Raviv, 1996; Woo, 2001).

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Previous approaches have involved detection and identification of NPVs based on the amplification of the gene for the conserved polyhedrin protein, the principal component of the viral OB. PCR amplification has been combined with sequence analysis (Woo, 2001), DNA–DNA hybridization (Faktor and Raviv, 1996), or the use of restriction endonucleases (Christian et al., 2001; de Moraes and Maruniak, 1997; Faktor and Raviv, 1996). PCR procedures coupled with restriction endonuclease analysis of the amplified product provide a rapid diagnostic tool for genus level (de Moraes and Maruniak, 1997), or species specific studies (Christian et al., 2001). The present study aimed to develop a molecular tool combining PCR and restriction fragment length polymorphisms (PCR–RFLP) to differentiate SeMNPV genotypes.

2. Materials and methods

2.1. Virus sources

Four SeMNPV field-collected isolates and a laboratory cloned genotype were employed for the development of a PCR–RFLP diagnostic method. The first of these was the Se-US2 strain, isolated in Florida, which constitutes the active component of the baculovirus based bioinsecticide Spod-X (Kolodny-Hirsch et al., 1993). The other three isolates, Se-SP1, Se-SP2, and Se-SP3 were collected from groups of infected cadavers at three different locations in southern Spain during the course of virus epizootics. All the Spanish isolates have been characterized by RFLP analyses of viral DNA (Caballero et al., 1992b; Muñoz et al., 1999; Murillo et al., 2001). Additionally, a genotypic variant, Se-US1A, cloned from the Californian isolate Se-US1 (Gelernter and Federici, 1986), was included as a reference. The Se-US1A genome has been entirely sequenced (Ijkel et al., 1999). Each isolate was amplified in fourth instar *S. exigua* reared on a wheatgerm semisynthetic diet (Greene et al., 1976), under controlled conditions (26 ± 2 °C, 70 ± 5% RH, and photoperiod 16 h:8 h L:D). OBs were purified from cadavers as described by Muñoz et al. (1997). Titers were determined using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase-contrast microscopy and stored at 4 °C until use.

The second part of the study involved validating the technique for closely related genotypes. For this, we used six SeMNPV isolates (SeSP03-17, SeSP03-18, SeSP02-13, SeSP202-14, SeSP03-09, SeSP03-16) originating from soil samples collected in greenhouses in Almeria, southern Spain (Murillo et al., submitted). Each soil isolate was obtained by feeding second instar *S. exigua* with mixtures of soil and semisynthetic diet, as described previously (Murillo et al., submitted). Larvae that died of polyhedrosis disease were used as inoculum and fed to fourth instars. RFLP analysis of viral DNA using *Bgl*II and *Pst*I showed that each isolate consisted of a single predominant genotype: Se-G24 (SeSP03-17), Se-G25 (SeSP03-18), Se-G26 (SeSP02-13), Se-G27 (SeSP02-14), Se-G28 (SeSP03-09), and Se-G29 (SeSP03-16). However, as cloning was not performed, it is possible that additional minority genotypes were present in these isolates.

2.2. DNA Isolation

Viral DNA was obtained by dissolving the OB matrix in an alkaline solution (0.3 M Na_2CO_3, 0.5 M NaCl, 0.03 M EDTA; pH 10.5), and by incubating the virions with proteinase K, followed by phenol/chloroform DNA extraction and alcohol precipitation (Muñoz et al., 1997). The final DNA pellet was suspended in 50 μl of sterile milli-Q water and stored at 4 °C until use.

To confirm virus identity, 0.5–1 μg of viral DNA was incubated with 1–5 units of restriction enzymes *Pst*I or *Bgl*II for 4–12 h. Reactions were stopped by addition of loading buffer (0.25% w/v bromophenol blue, and 40% w/v sucrose in water), loaded in 0.7% agarose gels with TAE buffer (40 mM Tris–Acetate; 1 mM EDTA), and electrophoresed at 20–40 V for 6–12 h. Ethidium bromide stained gels were then photographed on an UV transilluminator using the Chemi-doc software (Bio-Rad, Madrid, Spain).

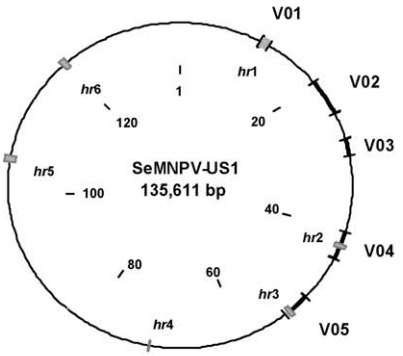
2.3. Design of the PCR primers

Five regions of high variability in the SeMNPV genome (V01, V02, V03, V04, and V05) (Fig. 1a), were identified from published studies (Dai et al., 2000; Heldens et al., 1996; Muñoz et al., 1998). Some of these regions contain large deletions (V02) or homologous regions *hr*1, *hr*2, and *hr*3 (V01, V04, V05) (Ijkel et al., 1999). PCR primers for these regions were designed using the complete genomic sequence of Se-US1A (Ijkel et al., 1999; Gene Bank accession no *NC*002169), and the sequence of SeSP2A *Pst*IMB region (Muñoz et al., 1999), with the aid of Primer 3 (http://frofo.wi.mit.edu/egibin/primer3/primer3_www.cgi) and Clone Manager software (Scientific & Education Software, USA).

2.4. PCR amplification and RFLP analysis of viral DNA

The specific primer pairs used for each genomic region (V01, V02, V03, V04, and V05) are given in Fig. 1a and b. PCR reactions were performed in a thermal cycler (RoboCycler Grand 96; Stratagene, Forest City, USA) under the following conditions. A 3-min denaturation step at 94 °C, followed by a number of amplification cycles: 30 sec at 94 °C, 1 min at the annealing temperature adjusted for each primer pairs (see Fig. 1b), and an elongation step at 72 °C, which varied according to the predicted length of the amplicon, and an extra extension step of 10 min at 72 °C (Fig. 1b). For amplification of regions V01, V03, V04, and V05, reactions were carried out in a total volume of 50 μl containing 300 μM of each deoxynucleoxide triphosphate, from 0.4 to 0.6 μM of each primer, 2.5 mM of $MgCl_2$, 1/5 volume of polymerase buffer, 0.5–20 ng DNA-template, and 2 units of Biotaq Polymerase (Bioline, London, UK). Amplification of the V02 region (≈4.9 kb) was carried out using 2.5 units of the "Elongase" thermostable *Taq* polymerase Takara LA and 1/5 volume of polymerase buffer following the manufacturer's instructions (Takara Bio Europe, Gennevilliers, France). A 5 μl volume of each PCR product (amplicon) was electrophoresed through 1 or 1.5% agarose gels and visualized by staining with

(a) (b)



Variable Region	Sequence of primer	Location in Se-US1A genome (bp)	Amplicon size (kb)	PCR reaction conditions		Number of cycles
				Anneal	Elongation	
V01	5′ ctttgtcatcgtcacctacg 3′ 5′ gagatcatcatcgatgaaatc 3′	10255/10374 11744/11764	1.5	58ºC 1′	72ºC 2′	20
V02	5'-gatccagtagggcacgttgt-3' 5'-gataccggtgcaggctttta-3'	19104/19123 24015/24034	4.9	63ºC 1′	72ºC 5′	30
V03	5'-atacgcccaccattcaagag-3' 5'-agtcgccatgagcaagagtt-3'	27655/27474 29918/29937	2.2	60ºC 1′	72ºC 2′30"	30
V04	5'-accgccaacattctatcgac-3' 5'-gcgacgaaacttgaaaaagc-3'	40169/40188 43915/43934	3.7	53ºC 1′	72ºC 4′	30
V05	5'-gcgcaacgtaaagcagtaca-3' 5'-gaccgtcaactcatccacct-3'	50128/50147 53678/53697	3.5	60ºC 1′	72ºC 4′	30

Fig. 1. (a) Location of variable regions (V01, V02, V03, V04, and V05) (thickened segments) within the Se-US1A genome. Grey bars indicate homologous region (*hr*s), 1–6 and numbers denote genomic nucleotide positions in kpb. (b) PCR primers, location of target region, predicted amplicon size, and reaction conditions for annealing and elongation.

ethidium bromide in a UV transilluminator using the Chemidoc software (Bio-Rad, Madrid, Spain). The conditions for each variable region that yielded positive amplification were tested two to three times to assure consistency of the results. A negative control lacking DNA-template was always included.

Between 100 and 200 ng of amplified DNA were digested with the enzymes *Aat*II, *Acc*I, *Bam*HI, *Bgl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Pst*I, *Pvu*I, *Sal*I, *Sau*3AI, and *Ssp*I in a total reaction volume of 10 μl, according to the manufacturer's instructions (Amersham, Little Chalfont, United Kingdom). Electrophoresis was performed as described above.

2.5. DNA sequencing and sequence analysis

Amplicons yielded by isolates SeSP03-17, SeSP03-18, SeSP02-13, SeSP02-14, SeSP03-09, and SeSP03-16 for the V01 region (*hr*1) were purified using the GFX PCR DNA kit following the manufacturer's instructions (Amersham, Little Chalfont, United Kingdom). DNA samples were sequenced in an ABI PRISM® 3100 sequencer (Applied Biosystems, USA) in a commercial laboratory (Systemas Genomicos, Valencia, Spain). Sequencing was performed from both amplicon ends using the primers previously described for the V01 region (Fig. 1b), and an additional internal primer designed at the 262 nucleotide position from the 5′ end (5′-CCAATGACGGTTGATGCAAA-3′). After a single readings with each of these primers, the sequences were assembled using Clone Manager 5.0 software and compared with those of Se-SP2A and Se-US1A. A *hr*1 using NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/tools) for alignment and MultAlin software tools (http://prodes.toulouse.inra.fr/multalin/multalin.html) for obtaining the consensus sequence (Corpet, 1998).

3. Results

3.1. Optimal amplicon-enzyme combination for SeMNPV isolates

The most abundant amplicons were obtained with 1 ng of DNA template and 20 reaction cycles. The wild-type SeMNPV isolates Se-US2, Se-SP1, Se-SP2, and Se-SP3 and the Se-US1A genotype, could be distinguished from each other by the V01 amplicon size and by the *Bgl*II restriction endonuclease profiles corresponding to these amplicons (Fig. 2, Table 1). The profiles that appear in Fig. 2 were reproducible, including the presence of minor bands, most evident in the Se-SP2 isolate, that indicated the presence of genetic heterogeneity. As a result, most of the isolates could be characterized as individual haplotypes (*sensu* Christian et al., 2001), whereas the complex composition of mixed genotype isolates (e.g., Se-SP1, Se-SP2) precluded assigning specific haplotypes but did allow a certain degree of differentiation. The rest of the PCR primer and restriction

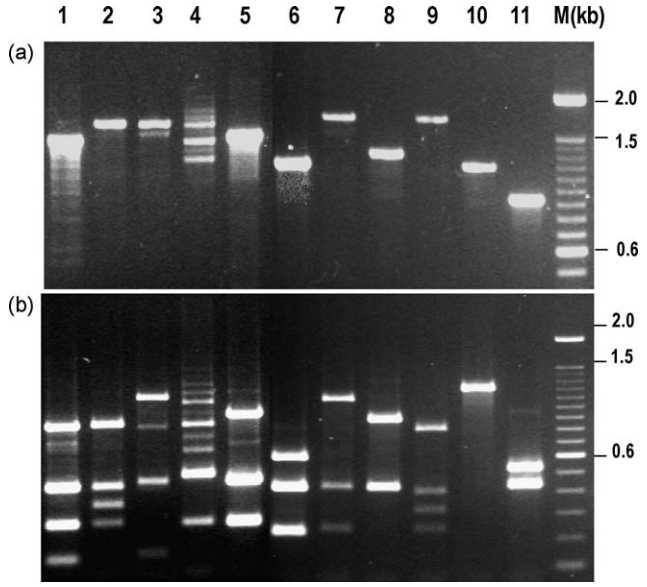


Fig. 2. (a) Electrophoresis of the V01 amplificon from Se-US1A (1), Se-US2 (2), Se-SP1 (3), Se-SP2 (4), Se-SP3 (5) Se-G24 (6), Se-G25 (7), Se-G26 (8), Se-G27 (9), Se-G28 (10), and Se-G29 (11) and (b) REN profiles resulting from *Bgl*II digestion of the above amplicons. M, molecular size marker lane (M) containing 100 bp DNA ladder (Invitrogene), with the size of three fragments indicated in kb. The results of PCR reactions involving single and mixed genotypes were found to be highly consistent and repeatable, indicating that multiple products were not PCR artefacts.

Table 1
Amplicon sizes from the five SeMNPV variable regions (V01, V02, V03, V04, and V05) and haplotypes resulting upon amplicon digestion with the enzymes *Aat*II (Aa), *Acc*I (Ac), *Bam*HI (Ba), *Bgl*I (BI), *Bgl*II (B2), *Cla*I (Ca), *Eco*RI (E1), *Eco*RV (E5), *Hae*III (Ha), *Hin*dIII (Hi), *Pst*I (Ps), *Pvu*I (Pv), *Sal*I (Sa), *Sau*3AI (S3), and *Ssp*I (Ss)

Isolate (Genotype)	V01		V02					V03			V04						V05					
	Amplicon size (kb)	Haplotype	Amplicon size (kb)	Haplotype				Amplicon size (kb)	Haplotype		Amplicon size (kb)	Haplotype					Amplicon size (kb)	Haplotype				
		B2		Ac,B1,E1 Ha, Ss	Pv	Ba	S3		B1,B2, E1,Hi, Ps, Pv, S3, Ss	Ha		Ac, Aa, PS, E5	Pv, Ss	B1	Ha	B2		Ac, C, E1, Pv, Sa	Ha	B2	E5	S3
Se-US1A	1.5	a	4.9	A	A	A	A	2.3	A	A	3.7	A	B	A	A	A	3.5	A	A	A	A	A
Se-US2	1.6	b	4.9	A	A	A	A	2.3	A	A	3.7	A	A	A	B	B	3.3	B	B	B	B	A
Se-SP1	1.6; 1.5	c[a]	4.9	A	B	A	B	2.3	A	B	3.7	A	B	B	C	B	3.4	C[a]	D[a]	B	B	A
Se-SP2	1.3; 1.4; 1.6	d[a]	4.9	A	B	B	C	2.3	A	A	3.7	A	B	B	C	B	3.4	C[a]	D[a]	B	A	A
Se-SP3	1.5; 1.4	e[a]	4.9	A	B	C	D	2.3	A	A	3.7	A	B	B	C	B	3.5	A	C	B	A	A
SeSP03-17 (Se-G24)	1.2	f	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SeSP03-18 (Se-G25)	1.7	g	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SeSP02-13 (Se-G26)	1.3	h	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SeSP02-14 (Se-G27)	1.7	b	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SeSP03-09 (Se-G28)	1.2	i	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
SeSP03-16 (Se-G29)	0.9	j	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Number of Haplo-types	–	10	–	1	2	3	4	–	1	2	–	1	2	2	3	2	–	3	4	2	2	1

For each variable region, five wild type strains (Se-US1A, Se-US2, Se-SP1, Se-SP2, and Se-SP3) were tested. An additional six Spanish isolates originating from soil (SeSP03-17, SeSP03-18, SeSP02-13, SeSP02-14, SeSP03-09, and SeSP03-16) were analysed for the V01 region alone.–, not tested.

[a] Presence of submolar fragments.

endonuclease combinations did not fully discriminate between the isolates. Of the 36 amplicon-enzyme combinations tested, 18 were monomorphic in that they produced a single unique profile for all five isolates. The remaining 18 combinations produced between two and four profiles per combination. The amplicons obtained from the variable regions V02, V03, and V04 were of identical size for the five SeMNPV isolates analyzed, but restriction endonuclease treatment resulted in eight polymorphic profiles, of which five were bimorphic, two trimorphic, and one tetramorphic. For V05, three different amplicon sizes were obtained, and their corresponding restriction profiles yielded two bimorphic, one trimorphic and one tetramorphic profile. Some of the polymorphic profiles grouped the isolates according to their geographical origin. For example, the *Pvu*I and *Bgl*II digestions of the V02 and V04 amplicons, respectively, distinguished the North American from the Spanish isolates.

3.2. Characterization of SeMNPV soil isolates by PCR–RFLP

A collection of six isolates from soil (SeSP03-17, SeSP03-18, SeSP02-13, SeSP02-14, SeSP03-09, and SeSP03-16) could also be identified by *Bgl*II digestion of the V01 amplicons obtained from each of them (Fig. 2b). PCR products for all isolates presented a unique fragment, which confirmed that they comprise a single predominant genotype. The name given to any particular genotype was based on the insect host (Se for *S. exigua*), followed by the letter G (Genotype), and an Arabic number indicating the order in which they were identified. Thus, in isolate SeSP03-17 we identified genotype Se-G24 as the only dominant genotype present, and so on for the remaining isolates, as mentioned above. All genotypes differed in the V01 amplicon size except Se-G25 and Se-G27, with a similar amplicon size of ca. 1.7 kb (Table 1; Fig. 2a). Upon *Bgl*II digestion, they yielded different profiles but the haplotype of Se-G27 was identical to that of the isolate Se-US2 (Table 1; Fig. 2b).

3.3. Comparison of the hr1 sequence for SeMNPV genotypes

The nucleotide sequence of genotypes Se-G24, Se-G25, Se-G26, Se-G27, Se-G28, and Se-G29 *hr*1 were compared with those of Se-SP2A (Muñoz et al., 1999) and the corresponding region (*Pst*IMB fragment produced by *Spe*I digestion) of the Se-US1A genome (Ijkel et al., 1999) (Fig. 3a). The amplicon sizes of the genotypes varied from 896 to 1661 base pairs (bp), principally due to the different number of repeats within each genotype (Fig. 3). All genotypes shared 100% identity within the first (5′) 300 and the final (3′) 110 nucleotides. The Se-G24 *hr*1 is formed by six direct repeats of ca. 100 bp (repeats I, II, III, IV, V, and VI) that are tandemly repeated and by eight imperfect palindromes, of which six were embedded within the direct repeats and two were flanking these (palindromes PI and PII). This genotype *hr*1 shares 98% identity to that of Se-SP2A at the nucleotide level (Muñoz et al., 1999). The only differences between Se-G24 and Se-SP2A *hr*1 consisted of a 10 bp gap between the nucleotide positions 424 and 434 bp in Se-G24, and three mismatches (at nt positions 402, 769, and 770), of which the last two resulted in the lost of a *Cla*I restriction site (Fig. 3).

Se-G25 *hr*1, had four additional palindromes with respect to Se-G24 of which two were embedded within direct repeats (VII and IX) and one within an inverted repeat (VIII) (Fig. 3). The Se-G27 *hr*1 was very similar to that of Se-G25, showing a small deletion of 20 bp, an additional *Bgl*II site with respect to this, and a 12 bp additional sequence between PIII and repeat IX. Se-G26 and Se-G28 *hr*1s differed from that of Se-G25 in that direct repeats III, IV, V and VI were missing in the two of them. In Se-G28 *hr*1, repeat II was also missing and an extra *Bgl*II site appeared in repeat I. Finally, Se-G29 *hr*1 was the shortest at 896 bp long. This *hr*1 differed from that of Se-G25 in that in the former, repeats III, IV, V, VI, VII and VIII and palindrome PII were missing. Comparison of the *hr*1 sequences from the Spanish genotypes with that of Se-US1A revealed a high level of nucleotide homology in between repeats VII, VIII, and XIV, and palindrome PIII (Fig. 3). Sequence alignment of PI, PII, and PIII within the *hr*1 from all genotypes revealed that most of the nucleotides are highly conserved, with a *Pvu*I restriction site at the core. A few mismatches were observed at positions 5, 8, and 21 to left and 4, 5, 18 and 21 to the right of the core (Fig. 3c).

4. Discussion

PCR amplification of a genomic variable region combined with RFLP successfully differentiated SeMNPV isolates from North America and Europe. One of the most important advantages of PCR–RFLP as a diagnostic method is the tiny amount of target DNA required. As a consequence, environmental samples from soil (de Moraes et al., 1999; Ebling and Holmes, 2002), water, detritus and forest litter (England et al., 2001), individuals from natural insect populations (Burand et al., 1992; Faktor and Raviv, 1996), or insect predators (Li et al., 1999), are readily processed by PCR methods. For instance, Burand et al. (1992) reported positive amplification with as little as 5 NPV genomes of gypsy moth NPV present on the surface of egg samples. However, the PCR reaction may be inhibited by some components of the environmental sample, that may have to be eliminated by dilution (Christian et al., 2001; de Moraes et al., 1999). For baculoviruses, this problem may be overcome by employing a bioassay screen in which soil samples are incorporated into artificial diet and fed to insect larvae (Richards and Christian, 1999). This method was recently validated for the *S. exigua* MNPV (Murillo et al. submitted).

The accuracy of PCR amplification in this study was influenced by the nucleotide sequence structure of template DNA and heterogeneity of template sequences. Repeat sequences (homologous regions) present in the template DNA tended to induce artificial fragment production. We verified that primer hybridization specificity can be corrected by raising annealing temperatures or reducing the initial concentration of template (Henson and French, 1993). An increase in non-specific amplifications became apparent when the concentration of target DNA was raised. The optimal DNA template concentration oscillated between 0.5 and 1 ng, which is 10-fold less than that reported for amplification of the polyhedrin gene region (de Moraes and

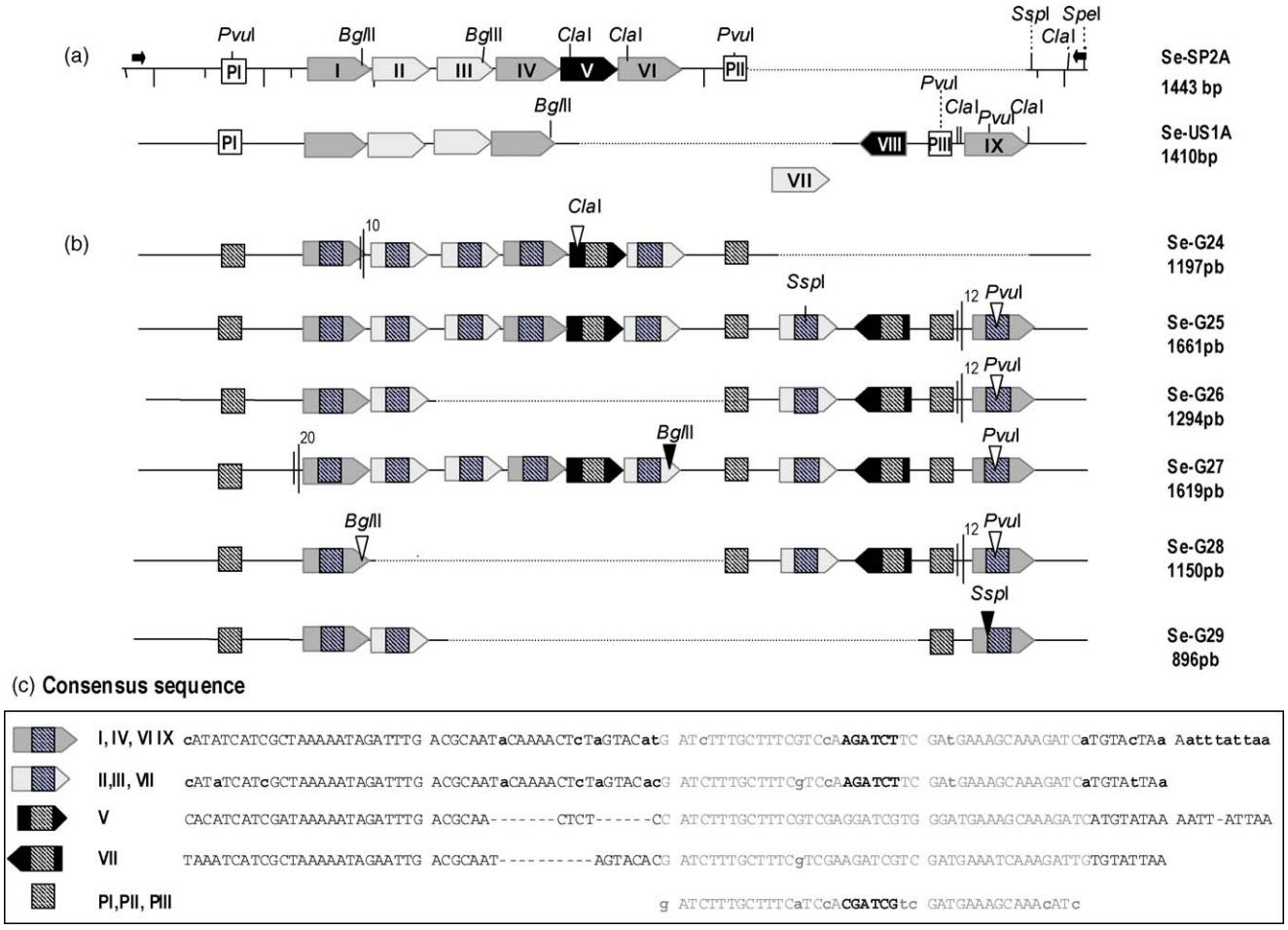


Fig. 3. (a) Schematic representation of the *hr*1 sequence structure of Se-SP2A and Se-US1A genotypes. Arrows pair indicated the location of amplification primers on Se-SP2A. (b) Comparison of amplification sequence of genotypes Se-G24, Se-G25, Se-G26, Se-G27, Se-G28, and Se-G29 indicating differences respect to Se-SP2A and Se-US1A. Restriction sites lost or gained are indicated by solid/soft inverted triangles labeled with the endonuclease name; deletions by a pair of vertical lines headed by the number of nucleotides lost. (c) Consensus sequence for repeats and palindromes for new Spanish genotypes indicating mismatches by small letters. Consensus sequences were aligned and the palindromes located inside are written in grey letters, and the restriction site at the core are remarket (*Sau*3AI -AGATCT or *Pvu*I -CGATCG).

Maruniak, 1997). The second factor affecting amplicon fidelity was the genomic variability within DNA templates. Natural NPV isolates almost invariably comprise a diversity of genotypic variants (Cory and Myers, 2003). A high degree of heterogeneity within a template interferes during the PCR reaction, producing false amplifications. We observed a greater capacity to discriminate between isolates from soil that comprised single dominant genotypes than genetically complex isolates Se-SP1, Se-SP2, and Se-SP3, originating from pooled groups of infected insects. Presumably, soil samples containing a low abundance of OBs reduce the probability of obtaining highly genotypically variable isolates, in contrast with isolates originating from infected larvae or mixtures of cadavers, each containing millions of OBs. This is because larvae fed with soil-diet mixtures most probably die as a consequence of an infection originating from a single or a few viral particles, as occurs during end-point dilution infections performed as a method of in vivo cloning of genotypes (Smith and Crook, 1988). Notably, Christian et al. (2001) also reported equimolar profiles for *Helicoverpa armigera* NPV (HaNPV) isolates obtained by the soil-diet incorporation method. Another source of the observed heterogeneity could be that strains Se-SP1, Se-SP2 and Se-SP3 have been amplified many times in laboratory studies and may have undergone reorganization in regions like the *hr*1. Whatever the reason, limited genomic variability within DNA samples improves the accuracy of the method, although previously reported PCR amplifications of the *hr*1 region with 1:3 or 1:1 genotype proportions did not detect evidence of inhibition by dominant genotypes at the expense of the rarer genotypes (Murillo et al., in press).

Christian et al. (2001) proposed amplification of genomic variable regions for detection and identification of variants of HaNPV collected in Australia. Sequencing studies have revealed that polymorphic regions appear to be confined to several hypervariable regions rather than the genetic variation being spread evenly throughout the NPV genome (García-Maruniak et al., 1996; Li et al., 2005; Muñoz et al., 1999). In the case of SeMNPV, the regions V01 and V05 tended to show higher levels of polymorphism than the other regions tested. Indeed, the V01 region had the highest power of resolution among the isolates included in our study. Interestingly, V01 and V05 contain the homologous regions *hr*1 and *hr*3 of the SeMNPV genome, respectively. Baculovirus *hr*s are characteristically composed of direct repeat

sequences and imperfect palindromic sequences (Possee and Rorhmann, 1997), although the number, size, restriction sites and genomic position is specific for each virus. The *hr* sequences are important as origins of DNA replication and as transcriptional enhancers (Friesen, 1997; Lu and Miller, 1997). The *hr*1 has been reported as a recombination hot-spot wherein DNA undergoes continuous arrangements, duplications, and deletions (García-Maruniak et al., 1996; Majima et al., 1993; Muñoz et al., 1999). Recently, *Mamestra configurata* NPV-A *hr*1 was reported as one of the regions with the greatest nucleotide divergence among two completely sequenced genotypic variants (Li et al., 2005). Together, these findings suggest that *hr*1 is one of the most important genomic regions for generating diversity in NPV populations.

The genetic diversity of NPVs is difficult and time-consuming to quantify, but the PCR–RFLP approach provides a rapid tool for detection of spatial and temporal variation within SeMNPV populations. The comparison of *hr*1 composition of six SeMNPV genotypes collected in 2002–2003, revealed important changes compared with those genotypes cloned from the isolate Se-SP2 collected in the same geographical area in 1990. With the exception of Se-G24, recently isolated Spanish genotypes did not present a variable number of 98 repeats within *hr*1, which has been a characteristic of Spanish genotypes described to date (Muñoz et al., 1999). Instead, they contained a set of repeats that are high conserved among the North American genotypes Se-US1A (Fig. 3), Se-G13 and Se-G19 (SeUS2B and Se-US2H; D. Muñoz, unpublished data). Presumably, North American genotypes have been introduced into southern Spain over the past 10 years by use of a commercial biopesticide product based on the SeMNPV-US2 strain. Recently isolated genotypes may have incorporated part of the North American *hr*1 via in vivo recombination during co-infections of *S. exigua* larvae. High frequencies of recombination during co-infection of host cells has been demonstrated between closely related genomes (Bull et al., 2001), and is likely responsible for generating genotypic heterogeneity in natural NPV populations (Croizier and Ribeiro, 1992).

The PCR–RFLP method developed here should facilitate the processing of large numbers of environmental samples and should also provide a monitoring system for analysis of the distribution and dispersal of SeMNPV after field applications. The technique may also be applied to studies on the impacts of SeMNPV innundative use on non-target organisms, or to detect and quantify covert infections in host populations.

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