Diversity of Iberian nucleopolyhedrovirus wild-type isolates infecting *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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ABSTRACT

Larvae of the tomato fruitworm, *Helicoverpa armigera*, were surveyed for nucleopolyhedrovirus (NPV) infection (referred to as HearNPV) in three different locations from the Iberian Peninsula: Olivenza and Toledo in Spain and the Oeste region in Portugal. Twenty HearNPV isolates were obtained from single field-collected larval cadavers. Restriction endonuclease (REN) profiles of the collected isolates with BglII and PstI allowed identification of six different *H. armigera* single-embedded NPV strains in Spain (referred to as HearSP3, HearSP4, HearSP5, HearSP6, HearSP7, and HearSP8) and two in Portugal (referred to as HearPT1 and HearPT2). No strains were shared by isolates from different geographical regions except HearSP5, which was found in isolates from Olivenza and Toledo. Cluster analysis based on the restriction fragment length polymorphisms of these strains in relation to two previously identified strains from Badajoz (HearSP1) and Cordoba (HearSP2) in Spain, showed no correlation among the strains and their geographical origin. The biological activity of HearSP2, HearSP4, HearSP7, HearSP8, HearPT1, and HearPT2 was compared in terms of pathogenicity (50% lethal concentration, LC_{50}) and virulence (mean time to death). HearPT2 and HearSP7 were significantly more pathogenic than HearSP2, with LC_{50} values 2.8 and 2.6-fold higher than the latter, respectively, on *H. armigera* second instars. HearSP4 and HearPT2 killed larvae significantly faster than HearSP8, whereas HearSP2, HearSP7, and HearPT1 showed intermediate mean time to death values.

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1. Introduction

Helicoverpa armigera (Hübner), the tomato fruitworm, is an important pest of several crops all over the world except on the American continents (Fitt, 1989). In the Iberian Peninsula, it is a key pest on numerous crops including field-grown tomato, pepper, cotton and maize, protected (i.e., glass and plastic houses) vegetable and ornamental crops such as tomato, sweet pepper, lettuce, roses, and carnations, summer cole crops and strawberry (Torres-Vila et al., 2002; Figueiredo et al., 2006).

Environmental and food residue problems associated with chemical control tactics have encouraged the search for more sustainable pest control measures. Baculoviruses are considered as one of the most interesting insect biological control agents and very promising as biopesticides for their high specificity, virulence, ability to persist in the environment and compatibility with other beneficial natural enemies (parasitoids, predators and pathogens) and chemical pesticides (Harper, 1986). Baculovirus-based insecticides, therefore, have the potential to play an important role in integrated pest management (IPM) and organic farming programs. Given the prevalence of *H. armigera* populations showing resistance to a range of chemical insecticides that have already been described worldwide (McCaffery, 1998; Torres-Vila et al., 2002), baculovirus-based products would likely prove useful in Iberia for tomato fruitworm control in several crop systems in rotation with *Bacillus thuringiensis* Berliner (Bt), and other biorational pesticides. Moreover, the use of nucleopolyhedroviruses (NPVs) (Baculoviridae), other biorationals and Bt in rotation could be a useful tool to retard the development of Bt resistance in pest populations (Raymond et al., 2006).

Genotypic variants of the same baculovirus, such as those commonly found at different geographical locations and sometimes even in the same host individual, have been reported to differ in their DNA restriction endonuclease (REN) profiles and also in their biological activities (Caballero et al., 1992; Takatsuka et al., 2003; Cory et al., 2005). These differences in pathogenicity and virulence due to natural intraspecific heterogeneity may represent an interesting tool to develop new baculovirus-based bioinsecticides (Moscardi, 1999).

Several NPVs of the single- (SNPV) and multiple-embedded nucleocapsid type (MNPV) have been isolated from *H. armigera* populations all over the world (Gettig and McCarthy, 1982; Figueiredo et al., 1999; Ogembo et al., 2005; Zhang et al., 2005). Some of them

have been characterized thoroughly, including two genotypes puri-fied from different Chinese isolates, and their genomes have been en-tirely sequenced (Chen et al., 2001; Zhang et al., 2005). A *H. armigera* NPV (HearNPV) isolated in the Chinese province of Hubei has been produced as a biopesticide and extensively used on cotton (Wang et al., 2001), whereas in Thailand, HearNPV isolates are produced lo-cally for control of *H. armigera* in tomato, cotton, and tangerine (Jones et al., 1998). As the use of foreign NPV isolates can have a neg-ative impact on the biological activity of native strains (Muñoz and Caballero, 2000) and different host biotypes can vary in their suscep-tibility to geographically distinct isolates (Milks, 1997), the selection of indigenous isolates that are suitable for development as biological control agents requires the characterization of the strains present in each geographical region.

Two NPV isolates from the Iberian Peninsula were identified previously (Figueiredo et al., 1999) and characterized as single-embedded NPVs (SNPVs) (Arrizubieta et al., unpublished data). In our present study, larvae of the tomato fruitworm were surveyed for NPV infection in several locations of the Iberian Peninsula. The collected isolates were compared genetically by restriction fragment length polymorphism (RFLP), and biologically by deter-mining their pathogenicity and mean time to death (MTD). In addi-tion, the RFLP profiles of the different distinct isolates were used for a cluster analysis of the isolates.

2. Materials and methods

2.1. Insect rearing

The *H. armigera* population was maintained at 25 ± 2 °C, 70–80% relative humidity and 16 h:8 h day–night photoperiod on a semi-synthetic diet based on that described by Poitout and Bues (1974).

2.2. Helicoverpa armigera larval sampling and virus isolation

NPV isolates were originally obtained from *H. armigera* larvae with clear signs of patent NPV disease collected in 1999 in two sweet field maize (maize plants and wild host plants) from Oliven-za and Toledo (Spain) and in field tomatoes and tomatoes and sweet peppers grown in greenhouses from Oeste (Portugal) (Table 1). Larvae were collected individually into 1.5 ml plastic Eppendorf tubes except some of the samples from Oeste (3–4 larvae per tube), and preserved at −18 or 4 °C in the dark until diagnosis was done. Each sample was macerated in double distilled water and the pres-ence of occlusion bodies (OBs) was verified by phase contrast microscopy of the homogenated tissue at 400×. A total of 49 sam-ples were examined (16 from Spain and 33 from Portugal).

2.3. OB amplification and purification

OB samples were multiplied in groups of 10–20 third instars of *H. armigera*. Only one virus sample was produced at each time to prevent cross-contamination. For this, larvae were starved for at least 12 h and then fed diet plugs that had been surface-contami-nated with crude extracts from homogenated NPV-infected larvae. Larvae were reared individually on semi-synthetic diet until death

or pupation. Infected larvae were frozen as soon as they died. To purify OBs, dead larvae were macerated in 300 μl distilled water, filtered twice through muslin-like tissue and centrifuged at 1600 g for 5 min at room temperature. Pellets were resuspended in 1 ml water and 0.5 ml of 0.1% sodium dodecyl sulphate (SDS) and centrifuged for 5 min at 1600 g. These pellets were resus-pended in 0.3 ml of 0.1% SDS and the suspensions carefully placed on 0.5 ml of a 40% sucrose cushion and centrifuged at 23,000 g dur-ing 30 min, resuspended in 1 ml of 0.1% SDS, centrifuged at 4000 g for 5 min and washed twice in distilled water. When necessary, the suspensions were briefly sonicated to break up OB aggregates. Pure OB pellets were finally resuspended in 0.3 ml distilled water and stored at −20 °C for further use. Isolate identity was confirmed after each multiplication by REN digestion and agarose gel electro-phoresis (as described in section 2.4 below). Previously identified strains from Spain (HaSP1 and HaSP2, referred as HearSP1 and HearSP2, respectively, in this paper for consistency with ICTV nomenclature) (Figueiredo et al., 1999) and the HearNPV Chinese strain G4 (HearG4 in this paper), whose genome has been com-pletely sequenced (Chen et al., 2001) and was kindly provided by Dr. van Oers (University of Wageningen, The Netherlands), were also multiplied following identical procedures

2.4. Viral DNA extraction and REN analysis

Purified OB suspensions were incubated with one-third volume of 3× DAS (0.3 M Na_2CO_2 + 0.5 M NaCl + 0.03 M EDTA, pH 10.5) at 37 °C for 5 min to dissolve the polyhedrin matrix. Heavy particles including undissolved OBs were pelleted by 6000 g centrifugation for 5 min. To rupture virion and nucleocapsid envelopes, virion-containing supernatants were transferred to sterile 1.5 ml vials and incubated at 45 °C with 500 μg/ml proteinase K during 2.5 h and a further 30 min with 1% SDS. Viral DNA was extracted once with an equal volume of TE-buffer-saturated phenol and then at least twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) or with chloroform until the interface became completely clean. DNA was precipitated with 10% (v/v) 3 M sodium acetate (pH 5.2) and twice the volume of ice-cold 96% ethanol at 12,000 g for 15 min; then it was washed with 70% cold ethanol and centrifuged for 5 min. DNA pellets were resuspended in 10 mM Tris–HCl pH 8.0 and kept at 4 °C until use.

For REN digestion, 1–2 μg of viral DNA was incubated with 1 U of BglII and PstI according to the supplier's instructions, at 37 °C for 4–12 h. These RENs were chosen as they allowed proper differenti-ation of HearNPV strains in previous studies (Figueiredo et al., 1999). Reaction was stopped by the addition of one-sixth volume of 6× loading buffer (0.25% bromophenol blue; 40% sucrose, w/v). Fragments were separated by electrophoresis carried out in 0.7% TAE agarose gels containing 0.25 μg/ml ethidium bromide at 20–30 V for ca. 14 h and visualized on a Gel Doc Imaging System (Bio-Rad, Alcobendas, Spain). DNA fragment sizes were estimated by comparison to a standard molecular weight marker (bacterio-phage λ genomic DNA digested with HindIII) with the aid of Quan-tity One 4.6 (Bio-Rad). HearSP1 fragments were labelled alphabetically (from A to M with BglII and from A to F for PstI) from higher to lower molecular weight using upper case letters. The

Table 1
Origin of *Helicoverpa armigera* larvae collected, number of larvae collected with signs of patent nucleopolyhedrovirus (HearNPV) disease, number of isolates with presence of occlusion bodies, and number and nomenclature of the different HearNPV strains identified in each location.

Collection sites	No. diseased larvae	No. isolates	No. HearNPV strains (identified strains)
Olivenza (Spain)	7	7	5 (HearSP3, HearSP4, HearSP5, HearSP6, and HearSP7)
Toledo (Spain)	9	3	2 (HearSP5 and HearSP8)
Oeste (Portugal)	33	10	2 (HearPT1, HearPT2)
Total	49	20	8

polymorphic fragments appearing in the rest of the isolates were labelled with lower case letters referring to the closest fragment of HearSP1 of equal or upper molecular weight followed by a sub-index that distinguished polymorphic fragments with the same minor case letter but different molecular weight.

2.5. Cluster analysis

A matrix of presence/absence of RFLP fragments was generated based on BglII and PstI restriction profiles and this was used to calculate a similarity matrix based on the Dice similarity coefficient using SPSS 12.0. This matrix was used in a hierarchical cluster analysis utilizing the UPGMA (unweighed pair group method analysis) to construct a dendrogram using NTsys-PC 2.01b (Rohlf, 1997). The confidence level of this dendrogram was estimated by bootstrap analysis with Winboot (Yap and Nelson, 1996) with 5000 bootstrap replications. These analyses included all the observed submolar bands in the isolates since they usually belong to the most dominant genotype present in the virus population (Muñoz et al., 1998; Muñoz and Caballero, 2000; Simón et al., 2004).

2.6. Bioassays

A total of eight novel Iberian strains were identified by REN: HearNPV-SP3 (HearSP3), HearNPV-SP4 (HearSP4), HearNPV-SP5 (HearSP5), HearNPV-SP6 (HearSP6), HearNPV-SP7 (HearSP7), Hear-NPV-SP8 (HearSP8), HearNPV-PT1 (HearPT1) and HearNPV-PT2 (HearPT2). Viral pathogenicity expressed as 50% lethal concentration (LC_{50}) and MTD were determined for HearSP4, HearSP7, HearSP8, HearPT1 and HearPT2. These strains were selected for testing because they showed the most distinct REN profiles. The HearSP2 strain was included as a reference.

To determine concentration–mortality responses, five different viral concentrations which were found to cause 5–95% mortality in second instars in preliminary assays were used: 1×10^2, 1×10^3, 1×10^4, 1×10^5 and 1×10^6 OBs/ml. OB concentrations were determined with an improved Neubauer hemocytometer under a phase contrast microscope at $400\times$. Newly molted second instars from the laboratory colonies were individually separated to prevent cannibalism and starved for about 12 h at 20 °C. They were then allowed to drink viral suspensions containing 10% sucrose and 0.05% (w/v) of the food dye, Fluorella Blue, by using the droplet-feeding method (Hughes and Wood, 1986). Groups of 25–30 larvae were used per concentration and viral isolate, and inoculated insects were individually transferred to 25-well plates containing maize diet plugs. As controls, larvae were allowed drinking a water solution containing only the blue dye and sucrose at the same concentrations. Larvae were incubated at 25 ± 1 °C, 70 ± 5% relative humidity and 16:8 h day–night photoperiod. Mortality was recorded every 24 h for 10 days. The experiment was replicated four times and about 100 larvae were used in total per concentration and viral isolate.

To determine time–mortality responses, similar bioassays were performed. For these, only the concentration causing the highest mortality was used (1×10^6 OBs/ml). Mortality was recorded at 8 h intervals for 10 days. Larvae that did not die during the assay were not included in the analysis. Similarly, replicates in which 90% mortality was not achieved after applying Abbott's correction for control mortality, were also discarded.

2.7. Statistical analysis

Concentration–mortality data were subjected to Probit analysis (Finney, 1971) using the POLO-PC program (LeOra Software, 1987). The relative potencies were estimated and compared as described by Robertson and Preisler (1992). Time–mortality results of individuals that died due to NPV infection by different isolates were subjected to Weibull analysis (Aitikin et al., 1989). The validity of the Weibull model was determined using the Kaplan macro present in the GLIM program.

3. Results

3.1. Identification of NPV strains present in Iberian isolates

The number of field-collected larvae from the Iberian Peninsula with baculovirus infection symptoms and the number of larvae in which the presence of OBs was detected by microscopic inspection is presented in Table 1.

From the seven field-collected isolates from Olivenza, Spain (Table 1), five different REN profiles were obtained with both BglII and PstI. Similarly, the three isolates from Toledo, Spain and the ten isolates from Oeste, Portugal, each exhibited two distinct REN profiles. In total, eight distinctive strains were obtained by combining the isolates BglII and PstI profiles from all locations (Fig. 1). Seven of these strains (HearSP3, HearSP4, HearSP5, HearSP7, HearSP8, HearPT1, and HearPT2) could be differentiated based on their BglII profiles alone (Fig. 1b). An additional isolate, HearSP6, which showed a BglII profile identical to HearSP5 could be distinguished from all the others with PstI (Fig. 1c). All the different isolates sharing the same HearNPV strain came from the same origin (two isolates from Olivenza consisting of HearSP7; two from Toledo of HearSP8, two from Oeste of HearPT1 and eight from Oeste of HearPT2) except HearSP5, which was found in two isolates from Olivenza and one isolate from Toledo.

All Iberian strains exhibited the BglII fragments A, I, J, K, and M as well as the PstI fragments A, B, C, E and F, whereas the presence of the BglII fragments B, C, D, E, F G, H and L and the PstI fragment D varied among the strains (Fig. 1b, c, and d). The BglII fragments b1, f1, g1, h1, i1, i2, j1, and PstI fragment d1 have strain recognition value (Fig. 1b, c, and d). Strains HearSP1, HearSP2, HearSP3, HearSP4, and HearPT1 presented submolar BglII fragments while HearSP6, displayed a submolar PstI fragment, indicating that they were composed of a mixture of different genotypes (Fig. 1b, c and d). BglII fragment f1, which is only present in HearSP4, seems to be similar to one present in a Russian isolate profile analyzed by Figueiredo et al. (1999).

The Chinese HearG4 genotype displays a BglII profile in which only four out of 13 fragments show the same molecular weight to those of HearSP1 (Fig. 1a) but its PstI profile is identical to that of the Iberian strains (not shown).

3.2. Cluster analysis of the Iberian HearNPV strains

The strains presented some polymorphism (Dice coefficient ⩾0.69). The cophenetic correlation (correlation between the cophenetic value matrix and the similarity matrix upon which the clustering was based) was equal to 0.90 (Mantel t-test = 6.37), revealing a good fit for the cluster analysis. Some of the strains were more closely related to geographically distant strains than to those collected in the same location, as observed by the cluster dendrogram (Fig. 2). In fact, taking into account a similarity threshold of 87%, four groups can be identified: (1) HearSP1, HearSP2, HearPT1 and HearPT2, (2) HearSP3, HearSP5, HearSP6 and HearSP7, (3) HearSP8, and (4) HearSP4. The HearSP4 and HearSP8 strains were the most different and, even at lower estimated similarities (77%), they continued to be separated from all the others.

The bootstrap estimate of the dendrogram (consensus tree) was equal to that obtained by NTsys, excluding the HearPT1 strain, which was placed separated from the cluster formed by HearSP1, HearSP2, and HearPT2 at a Dice similarity coefficient value of 0.87 and within the cluster formed by HearSP3, HearSP5, HearSP6,
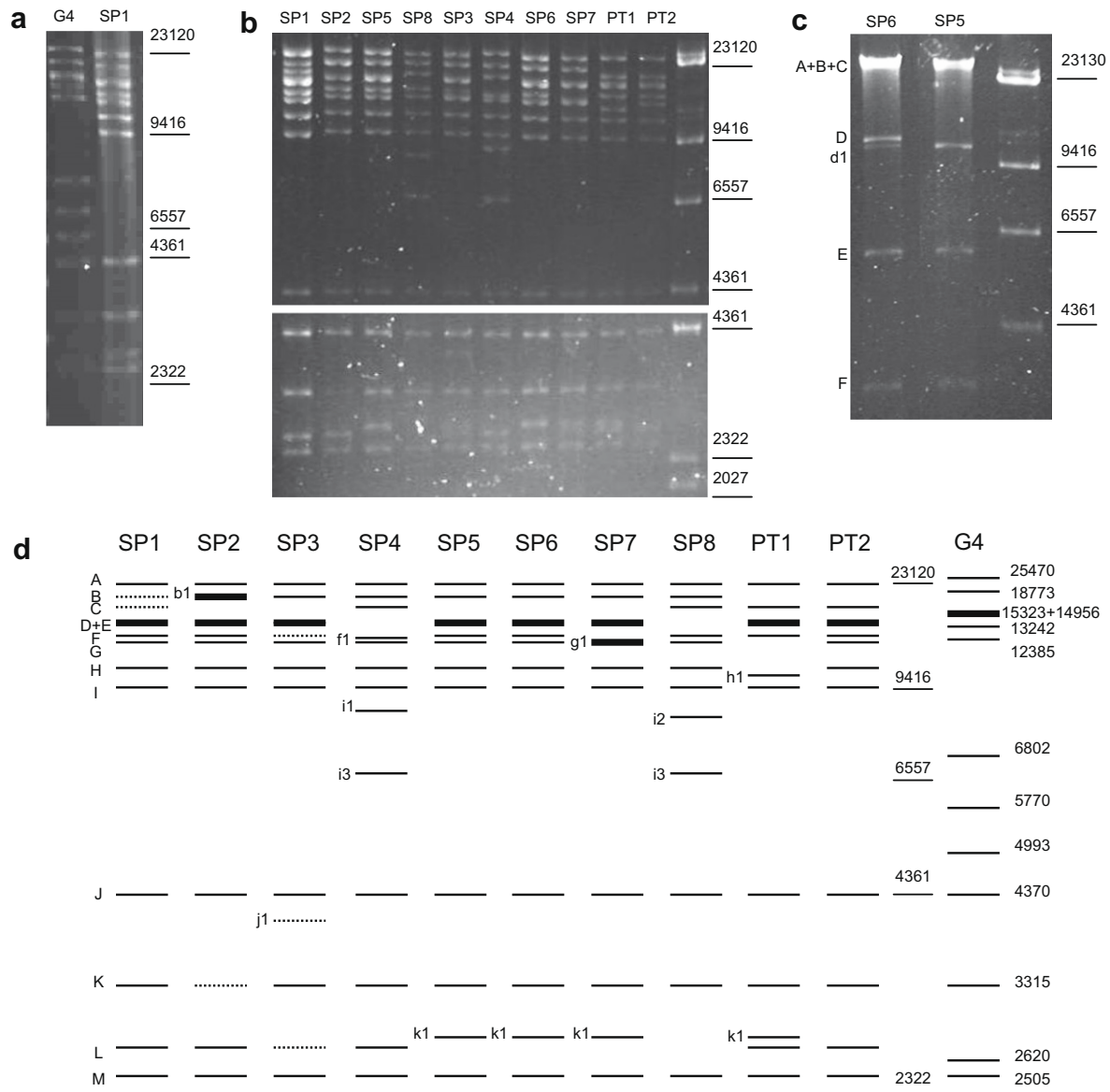
Fig. 1. REN profiles of *Helicoverpa armigera* nucleopolyhedrovirus [HearNPV-G4 (G4)] and of the ten distinct genotypic Iberian strains of HearNPV [HearSP1 (SP1), HearSP2 (SP2), HearSP3 (SP3), HearSP4 (SP4), HearSP5 (SP5), HearSP6 (SP6), HearSP7 (SP7), HearSP8 (SP8), HearPT1 (PT1) and HearPT2 (PT2)] obtained with BglII (a and b) and PstI (c). λ DNA digested with HindIII was used as a molecular weight marker and fragment sizes in base pairs (bp) are displayed above the marker fragments or to the right of the HearG4 fragments. A line diagram of the REN profiles is displayed (d) to show more clearly hypermolar (thicker lines), equimolar or submolar (dotted lines) fragments and to include fragment nomenclature to the left of each profile: capital letters were designated for the Iberian type strain (HearSP1) profile; and small letters followed by a number for the polymorphic fragments appearing in different strains.

and HearSP7 at the same Dice value (Fig. 2). This is also the position at which the UPGMA cluster analysis places HearPT1 when submolar bands are not considered (dendrogram not shown).

3.3. Biological activity of six Iberian HearNPV strains

Regressions lines for all six virus strains fitted the Probit model well ($\chi^2 = 29.21$, $df = 10$; $p = 0.001$) but they could not be fitted in parallel ($\chi^2 = 15.64$; $df = 5$; $p = 0.008$). The LC$_{50}$ ratios indicated that HearSP7 and HearPT2 were 2.6- and 2.8-fold more pathogenic, respectively, than HearSP2 which showed the highest LC$_{50}$ values. The LC$_{50}$ values of HearSP4, HearSP8, and HearPT1 were statistically similar to that of HearSP2 (Table 2).

The different strains killed between 95.5 and 97.7% of the treated second instars and in a time range of 64–160 hpi (hours post-infection), with the exception of four larvae that died later

(176-232 hpi). The MTD values for the different strains ranged from 98.4 hpi (HearPT2) to 115.2 hpi (HearSP8). HearSP4 and HearPT2 both killed larvae significantly faster than HearSP8, whereas HearSP2, HearSP7 and HearPT1 showed intermediate MTD values that were not significantly different from those of HearSP4, HearPT2 or HearSP8 (Fig. 3). The instantaneous risk of death increased over time (shape parameter of the Weibull distribution $\alpha = 3.87$). Time-mortality distribution curves peaked twice: once at 72–80 hpi and again at 112–120 hpi (data not shown).

4. Discussion

The REN profiles of all the strains analyzed in this work were very similar but not identical to those of the HearNPV isolates described by Figueiredo et al. (1999), indicating that they constitute novel HearNPV strains. The novel strains represented 40% of the to-
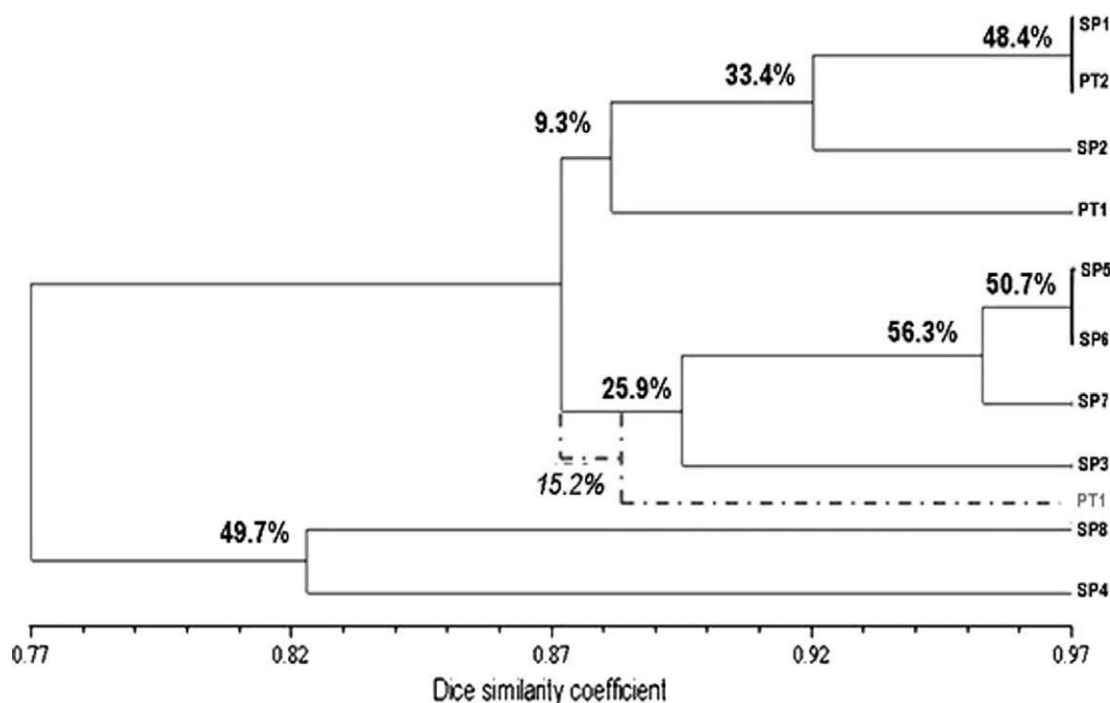
Fig. 2. Dendrogram of genotypic similarity resulting from hierarchical cluster analysis of the Iberian *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) identified strains [HearSP1 (SP1), HearSP2 (SP2), HearSP3 (SP3), HearSP4 (SP4), HearSP5 (SP5), HearSP6 (SP6), HearSP7 (SP7), HearSP8 (SP8), HearPT1 (PT1) and HearPT2 (PT2)] (UPGMA clustering method on proximity matrix based on Dice coefficient calculated from presence/absence of both equimolar and submolar fragments on REN profiles of the strains DNA digested with BglII and PstI) – cophenetic correlation $r = 0.90$. The frequency of each group in all dendrograms constructed by bootstrapping is presented in bold and behind the respective node for the groups common to both the cluster analysis and bootstrap dendrograms, and in italic for the different position of HearPT1 in the bootstrap dendrogram (marked with a dotted line).

Table 2
Concentration–mortality responses for six *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) strains on second instar *H. armigera* larvae.

Strain[a]	Intercept ± SE	Slope ± SE[b]	χ^2	p	LC$_{50}$ (OBs/ml) [c]	Potency[d]	95% Fiducial limits	
							Lower	Upper
HearSP2	−4.87 ± 0.65	1.07 ± 0.13 b	2.32	0.51	34897	1.00 a	—	—
HearSP4	−3.85 ± 0.49	0.87 ± 0.10 ab	2.74	0.43	26149	1.34 a	0.78	2.30
HearSP7	−3.40 ± 0.43	0.82 ± 0.09 ab	2.06	0.56	13399	2.59 b	1.55	4.33
HearSP8	−3.08 ± 0.39	0.71 ± 0.08 a	2.25	0.52	20043	1.74 ab	0.69	4.41
HearPT1	−4.66 ± 0.57	1.09 ± 0.12 b	1.56	0.67	18687	1.86 ab	0.96	3.61
HearPT2	−3.13 ± 0.39	0.77 ± 0.08 ab	2.35	0.53	12327	2.83 b	1.44	5.57

Probit regression (pooled data from four replications).

[a] HearNPV isolates from Spain and Portugal. See text and Table 1 for origin of isolates.

[b] SE, Standard error; slopes followed by the same letter were not different at $p = 0.05$.

[c] OBs, Occlusion bodies.

[d] Potency (i.e., lethal concentration ratios with respect to that of HearSP2) followed by the same letter were not different at $p = 0.05$.

tal number of isolates collected (larval cadavers where OBs were observed), but this figure may not be a realistic representation of field diversity, as no systematic effort at sampling was performed. Two different strains were identified in each of the three geographical regions surveyed, indicating a high variability of variants within each collection site. This is probably because the analysis was performed from individual cadavers, which increases the chances of quantifying the diversity present compared to analyses based on isolates composed of pooled groups of larval cadavers. Also, all isolates sharing the same HearNPV strain came from the same origin, except HearSP5, which was found in two isolates from Olivenza and one isolate from Toledo. It is fairly common to find the same strain in isolates from different origins, especially from sites geographically so close (Laitinen et al., 1996; Cooper et al., 2003; Graham et al., 2004).

Interestingly, intraspecific diversity was detected among the eight HearNPV strains identified. Within strains HearSP3, HearSP4

and HearPT1, a mixture of different genotypic variants was detected, as indicated by the presence of submolar bands in their corresponding REN profiles. These results suggest that insects were not infected by single variants but rather a mixture of them in a single isolate. This is a common phenomenon in baculovirus wild-type isolates, which are usually formed by a mixture of genotypes in distinct proportions (Muñoz et al., 1998; Murillo et al., 2001; Kamiya et al., 2004) even when obtained from single host individuals (Cherry and Summers, 1985; Graham et al., 2004; Cory et al., 2005), as occurred in our study. Furthermore, the absence of submolar bands is not a guarantee of the presence of a single genotype within the isolate (Vickers et al., 1991).

Some strain groups were highly similar with a relatively high bootstrap value. These strains were also placed together using other methods of classification (nearest neighbor, average linkage between groups or within groups – data not shown). It should be noted that the cluster analysis presented here was based on
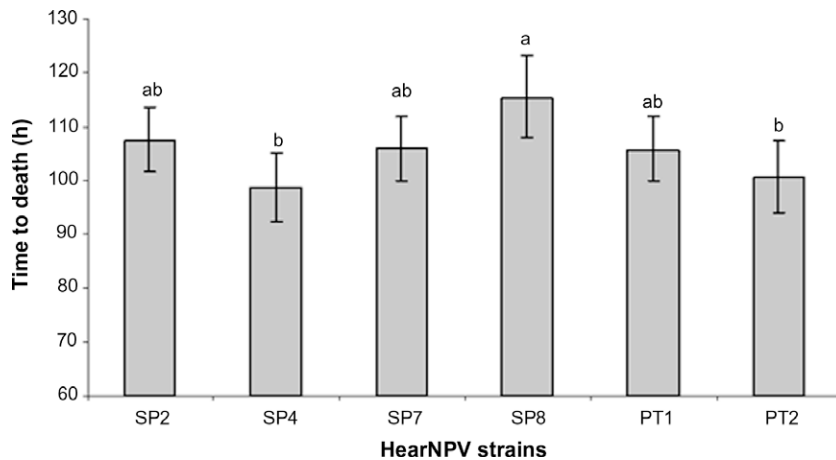
Fig. 3. Mean time to death for *Helicoverpa armigera* second instars inoculated with the six tested *H. armigera* nucleopolyhedrovirus (HearNPV) Iberian strains (HearSP2 or SP2, HearSP4 or SP4, HearSP7 or SP7, HearSP8 or SP8, HearPT1 or PT1 and HearPT2 or PT2) at a ∼95% lethal concentration. Vertical bars indicate SE.

presence/absence of fragments with similar electrophoretic mobility and not in the similarity/divergence of the genome. Therefore, the strain clustering data can only be considered as an approximation of phylogenetic relatedness.

Genotype phylogeny was not found to be associated with geographical origin; the most related genotypes were not always the ones from the same or closely located sites. REN analysis of several Heliothinae NPVs verified that some strains were more closely related to a geographically distant strain than to those originating from a closer location (Gettig and McCarthy, 1982; Figueiredo et al., 1999), which is not unexpected due to the high migration abilities of Heliothinae species (Fitt, 1989). Migrations allow the transport of associated NPVs over long distances and their importance on the distribution patterns of NPVs was previously emphasized by Shapiro et al. (1991), Takatsuka et al. (2003) and Fuxa (1987). Moreover, HearNPV populations may be dispersed by insectivorous birds (Entwistle et al., 1993) and larval parasitoids (Young and Yearian, 1989). Such organisms are very frequent in the Portuguese and Spanish fields and greenhouses as natural enemies of noctuids (Meierrose et al., 1989; Figueiredo and Mexia, 2000). On the other hand, the well known variability of NPV genomes contributes to differentiate closely located genotypes. The high number of variants per site and the near absence of common strains among sites reveal the high genomic plasticity of HearNPV and suggest that, along with NPV strain dispersion, genotypic selection also occurs within individual larvae, as suggested by Graham et al. (2004). Without a doubt, intrapopulation diversity within a single host is ecologically relevant as the larva represents the basic unit of virus inoculum (Cooper et al., 2003). In addition, the maintenance of diversity may be an important mechanism for virus adaptation and survival under different conditions (e.g., host density, host resistance, host plant), and therefore, represent an evolutionary advantage for the virus (Cory and Myers, 2004).

Concentration–mortality assays resulted in significant differences among the Iberian HearNPV strains; LC_{50} values differed by 2.5-fold among strains. This level of heterogeneity in HearNPV was also reported by Zhang et al. (2005) between two Chinese isolates. The estimated LC_{50} values were similar to those reported by other authors for isolates from Africa (Ogembo et al., 2005) and China (Sun et al., 2004).

Differences among strains in the mean speed to kill varied only moderately since the difference between the slowest and fastest virus was only of about 16 h. The MTD values obtained for the Iberian isolates were similar to those reported for the fastest-killing Heliothinae SNPV isolates studied by Hughes et al. (1983), which included

HearNPV strains from South Africa and Australia, a *Helicoverpa zea* SNPV from the US (Elcar®), a *Helicoverpa punctigera* SNPV from Australia, and a *Heliothis virescens* SNPV from the US. In fact, Hughes et al. (1983) verified that the most virulent isolates had lethal time (LT_{50}) values ranging from 72.8 to 79.6 h in neonates. In contrast, the MTD values obtained in our study seemed to be lower than those obtained by Ogembo et al. (2005), who reported MTD values of 6.5 days for second instars inoculated with similar concentrations. MTD values were also lower than those obtained by Teakle et al. (1985), who reported a LT_{50} value of 4.1–4.5 days for second instars. In contrast, our results were more similar to those of Williams and Payne (1984) with neonates and third-instar larvae.

The strategy to control *H. armigera* will probably be based on inundative applications of a microbial insecticide using a highly virulent strain/variant. Therefore, strains HearPT2, HearPT1, and HearSP7 seem to be the best candidates for potential insecticidal formulations, although extensive field evaluation is required.

Finally, these results can contribute to understand how HearNPV varies spatially in natural insect populations. This information is important in understanding baculovirus-insect evolution, design pest control programs, and in assessing the environmental impact of viral releases (Cory et al., 2005). Further analysis of the genotypic composition of these strains and their individual biological activities as well as comparison with strains from other regions of Iberia, North Africa and France, the immediate migration areas for the Iberian *H. armigera* populations, would give a broader picture of the ecology and evolution of this virus.

Acknowledgments

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