Journal of Invertebrate Pathology 114 (2013) 258–267

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Pseudoplusia includens single nucleopolyhedrovirus: Genetic diversity, phylogeny and hypervariability of the *pif-2* gene

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ARTICLE INFO

Article history:
Received 8 April 2013
Accepted 22 August 2013
Available online 5 September 2013

Keywords:
Pseudoplusia includens SNPV
Genotypic variants
pif-2 Gene polymorphisms
Phylogeny

ABSTRACT

The soybean looper (*Pseudoplusia includens* Walker, 1857) has become a major pest of soybean crops in Brazil. In order to determine the genetic diversity and phylogeny of variants of *Pseudoplusia includens single nucleopolyhedrovirus* (PsinSNPV-IA to -IG), partial sequences of the genes *lef-8*, *lef-9*, *pif-2*, *phr* and *polh* were obtained following degenerate PCR and phylogenetic trees constructed using maximum parsimony and Bayesian methods. The aligned sequences showed polymorphisms among the isolates, where the *pif-2* gene was by far the most variable and is predicted to be under positive selection. Furthermore, some of the *pif-2* DNA sequence mutations are predicted to result in significant amino acid substitutions, possibly leading to changes in oral infectivity of this baculovirus. Cladistic analysis revealed two closely related monophyletic groups, one containing PsinNPV isolates IB, IC and ID and another containing isolates IA, IE, IF and IG. The phylogeny of PsinSNPV in relation to 56 other baculoviruses was also determined from the concatenated partial LEF-8, LEF-9, PIF-2 and POLH/GRAN deduced amino acid sequences, using maximum-parsimony and Bayesian methods. This analysis clearly places PsinSNPV with the Group II *Alphabaculovirus*, where PsinSNPV is most closely related to *Chrysodeixis chalcites* NPV and *Trichoplusia ni* SNPV.

1. Introduction

Pseudoplusia includens single nucleopolyhedrovirus (PsinSNPV) is a baculovirus pathogenic to the soybean looper, *Pseudoplusia includens* (Lepidoptera: *Noctuidae*) (Walker, 1857) (synonym: *Chrysodeixis includens*). The *P. includens* caterpillar is an emerging pest of soybeans, and also of various other crops of economic importance in Brazil, such as cotton, beans, potatoes, tomatoes, tobacco, sunflower and other vegetables (Bueno et al., 2009). Seven PsinSNPV isolates (IA to IG) were obtained from *P. includens* larvae collected in soybean and cotton crops in Brazil and Guatemala. Using restriction endonuclease analysis (REN) and virulence bioassay, these seven isolates showed different restriction patterns with the presence of sub-molar bands and possessed varying pathogenicity, where isolates IA, IE and IF were more virulent than others isolates (Alexandre et al., 2010).

Baculoviruses are a large group of arthropod-specific DNA viruses that infect mostly the larval stages of insects in the orders Lepidoptera, Hymenoptera and Diptera (Herniou et al., 2012; Jehle et al., 2006a). Occlusion bodies (OBs) are viral particles that permit virus survival in the environment. OBs are composed of a crystalline matrix of protein (polyhedrin in nucleopolyhedroviruses – NPVs and granulin in granuloviruses – GVs) that occludes the enveloped virions (Adams and McClintock, 1991; Bilimoria, 1991; Boucias and Pendland, 1998). The nucleopolyhedroviruses exhibit OBs called polyhedra, composed of several virions, which may contain single (SNPV) or multiple (MNPV) nucleocapsids per envelope. The granuloviruses (GVs) exhibit OBs in the form of granules, containing one or, uncommonly, two or three virions per granule (Friesen and Miller, 2001; Herniou et al., 2012). The *Baculoviridae* family is divided into four genera: *Alphabaculovirus* – lepidopteran-specific NPVs, subdivided into Group I and Group II based on the type of budded virus (BV) fusogenic protein; *Betabaculovirus* – lepidopteran-specific GVs; *Gammabaculovirus* – hymenopteran-specific NPVs; and *Deltabaculovirus* – dipteran-specific NPVs (Carstens and Ball, 2009; Herniou et al., 2012; Jehle et al., 2006a).

Baculoviruses are widely and successfully used as biocontrol agents of insect pests of agricultural, forest and vegetable production systems (Copping and Menn, 2000; Erlandson, 2008; Moscardi et al., 2011; Souza et al., 2007; Szewczyk et al., 2006, 2009, 2011). High virulence, specific host range, stability and low environmental impact are considered to be advantages of using baculovirus insecticides. These viruses are also used as biotechnological tools for the production of recombinant proteins in insect cells and for

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0022-2011/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jip.2013.08.005

gene delivery into mammalian cells for scientific and medical purposes (Hu et al., 2006; van Oers and Vlak, 2008).

Baculovirus genomes are covalently closed, circular, double-stranded DNA, ranging from 80 to 180 kb, which are packaged in rod-shaped virions with a single (S) or multiple (M) nucleocapsids per envelope (Friesen and Miller, 2001; Herniou et al., 2012). Genomic comparisons of members of the *Baculoviridae* family, containing approximately 90–181 genes, have revealed a set of about 31 genes common to all baculoviruses, which are regarded as baculovirus ancestral genes and are referred to as core genes (Miele et al., 2011; Rohrmann, 2011). Recently, it has been shown that the *ac53*, *ac78*, *ac93*, *ac94*, *ac101* (*p40*), and *ac103* (*p48*) genes have orthologs in all baculovirus genomes and should also be considered core genes (Yuan et al., 2011; Garavaglia et al., 2012). Among these conserved genes, late expression factor 8 (*lef-8*), late expression factor 9 (*lef-9*), *per os* infectivity factor 2 (*pif*-2), polyhedrin/granulin (*polh/gran*) genes have been widely used in phylogenetic analyses, studies on genotypic diversity and baculovirus evolution (Coutinho et al., 2012; Eberle et al., 2009; Herniou et al., 2004; Jakubowska et al., 2007; Jehle et al., 2006b; Lange et al., 2004; Liang et al., 2012; Rowley et al., 2010; van Houte et al., 2012). The *lef-8* and *lef-9*) genes are essential for expression of late and very late promoters and encode the viral RNA polymerase subunit (Li et al., 1999; Lu and Miller, 1994, 1995; Passarelli et al., 1994; Titterington et al., 2003; Todd et al., 1995). The *pif*-2 gene, a member of the PIF family, is expressed in the late phase, and is essential factor for oral infectivity, the main route of baculovirus infections in insect larvae (Braunagel et al., 2003; Fang et al., 2006; Kikhno et al., 2002; Pijlman et al., 2003). This gene is thought to be one of the most conserved baculovirus ancestral sequences and, along with the *lef-9* gene, is less variable than other core genes, where the *desmoplakin* gene and *helicase* gene (*p143*) have been reported to be the most variable (Miele et al., 2011). The *polh/gran* gene is highly conserved in lepidopteron-specific baculovirus and encodes the main protein of occlusion body. These four genes and another gene, CPD photolyase (*phr*), which encodes an enzyme, acting on cyclobutane pyrimidine dimers, for repairing UV-radiation-induced DNA damage, were used in the present study. Homologues of this gene (*phr*) have been reported for *Trichoplusiani* SNPV (Willis et al., 2005), *Spodoptera litura* GV (Wang et al., 2008), *Thysanoplusia orichalcea* NPV (Xu et al., 2008), *Clanis bilineata* NPV (Zhu et al., 2009), and *Apocheima cinerium* NPV (Biernat et al., 2011). Studies showed that *phr* genes are conserved in group II *Alphabaculoviruses* that infect insects of the subfamily *Plusiinae* of the Noctuidae (Xu et al., 2008).

The objective of this study was to determine the genetic variability of seven PsinSNPV (IA to IG) isolates and the phylogenetic position of the PsinSNPV virus in relation to other members of the family *Baculoviridae* based on *lef-8*, *lef-9*, *pif-2*, *polh*, and *phr* gene sequences.

2. Materials and methods

2.1. Insects and virus

Pseudoplusia includens SNPV isolates IA, IB, IC, ID, IE, IF and IG were obtained from infected *P. includens* larvae. These larvae were collected on soybean and cotton farms from differents localities in Brazil and Guatemala (Alexandre et al., 2010) and donated by Dr. Flávio Moscardi, Embrapa Soja, Londrina-PR, Brazil.

2.2. Purification of viral occlusion bodies (OBs)

Occlusion bodies (OBs) from *P. includens* larvae infected with each of the seven PsinSNPV isolates were purified according to procedures described by Maruniak (1986). Infected *P. includens* larvae were macerated in homogenization buffer (1% ascorbic acid, 2% sodium dodecyl sulphate-SDS, 10 mM Tris–HCl, pH 7.8 and 1 mM EDTA) and filtered through six layers of gauze. The filter was centrifuged at 10,000*g* for 15 min at 4 °C, and the pellet washed with 0.5% SDS, 0.5 M NaCl. The OB pellet was resuspended in sterile distilled water, and further purified using 40–65% continuous sucrose gradient centrifugation (100,000*g* for 40 min at 4 °C). The OB band was collected, diluted to three times its original volume with TE buffer (10 mM Tris–HCl and 1 mM EDTA pH 8.0) and centrifuged 14,000*g* for 30 min at 4 °C. The OBs were resuspended in sterile distilled water and stored at −20 °C.

2.3. Viral DNA amplification by PCR

Viral DNA from the seven PsinSNPV isolates was extracted from purified OB suspensions as described by O'Reilly et al. (1992) and viral DNA was amplified using degenerate primers for *lef-8*, *lef-9*, *pif-2*, *phr* and *polh/gran* genes (Table 1). DNA of the seven PsinSNPV isolates was used as template. The PCR reactions consisted of 40 ng of viral DNA, 1x PCR buffer, 0.4 μM of forward and reverse primers, 100 μM of each dNTP, 1 U of Taq polymerase in a total volume of 25 μl. Amplification steps were: 95 °C/3 min, followed by 36 cycles at 95 °C/30 s, primer-pair-specific annealing temperature/30 s (Table 1) and 72 °C/30 s, with a final extension at 72 °C/10 min. PCR products were separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide and visualized and photographed under UV light.

2.4. PCR product sequencing

The products resulting from PCR amplification of *lef-8*, *lef-9* and *phr* genes were purified using the *ExoSAP-IT PCR Clean-up Kit* (GE Healthcare, Little Chalfont, UK) and PCR products resulting from *pif-2* and *polh* gene amplification were purified using the GFX PCR DNA & Gel Band Purification Kit, according to the conditions recommended by the manufacturer (GE Healthcare, Little Chalfont, UK). Both strands of the PCR products were sequenced using the amplification primers, respectively, with the BigDye Terminator Cycle Sequencing kit Version 3.1, according to the manufacture's protocol (Applied Biosystems, Foster City, CA, USA). The sequencing reaction products were resolved using an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. Phylogenetic analyses

The PsinSNPV-IE (selected as representative) partial DNA sequences of the *lef-8*, *lef-9* and *pif-2* genes were translated, and the deduced amino acid sequences aligned in MUSCLE version 3.5 (Edgar, 2004), along with corresponding sequences from 56 completely sequenced baculoviruses available from the GenBank database (Table 3). The alignments were trimmed to the size of the partial PsinSNPV sequences, and the alignments of each gene were concatenated in BioEdit version 7.0.9 (Hall, 1999).

Maximum-parsimony (MP) analyses was performed in PAUP* version 4.0b10 (Swofford, 2003). The trees were built by 1,000 replicates of stepwise addition, and tree bisection reconnection branch swapping was performed to find the best MP trees at each replication. The robustness of the tree topologies was evaluated by bootstrap analysis with 1000 pseudoreplicates.

Bayesian phylogenetic analyses were conducted with MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001), where each alignment was previously analyzed using a statistical model-fitting approach implemented in ProtTest (Abascal et al., 2005), to select the optimum amino-acid substitution models. Five Markov chains were run for 5 million generations, and the maximum-likelihood