Genetic and biological variation among nucleopolyhedrovirus isolates from the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract A PCR-based method was used to identify and distinguish among 40 uncharacterized nucleopolyhedrovirus (NPV) isolates from larvae of the moth *Spodoptera frugiperda* that were part of an insect virus collection. Phylogenetic analysis was carried out with sequences amplified from two strongly conserved loci (*polh* and *lef-8*) from the 40 isolates in the collection and from eight previously studied *S. frugiperda* NPV (SfMNPV) isolates. To further distinguish these isolates, analysis was also carried out with sequences from two less-conserved loci, *hr4* and *hr5*. Phylogenetic inference from the sequence data could distinguish among several of the individual isolates and between different groups of isolates from Georgia (USA) and Colombia, South America. A stronger degree of bootstrap support for the phylogenetic trees was obtained with the *hr4* and *hr5* homologous repeat sequences. Sequencing and phylogenetic analysis detected a relatively high degree of larva-to-larva sequence divergence occurring among isolates of SfMNPV collected from the same field in Missouri, USA. Restriction endonuclease analysis of viral DNA from larvae infected with five isolates from Georgia, Missouri, Louisiana, Florida (USA), and Colombia allowed for further comparison with other previously reported isolates of SfMNPV. Bioassays with these five geographically distinct isolates detected minor differences in virulence. This study highlights the use of PCR to rapidly distinguish and characterize large numbers of historical baculovirus isolates from the same host using minimal quantities of material, and the use of sequences from homologous repeat regions to distinguish closely related isolates of the same NPV species.

Keywords Baculovirus · Nucleopolyhedrovirus · *Spodoptera frugiperda* · Fall armyworm

Introduction

Baculoviruses are a group of large DNA viruses isolated exclusively from arthropods, particularly larvae of moths (Lepidoptera), mosquitoes (Diptera), and sawflies (Hymenoptera) [1]. All baculoviruses are members of a single family, the Baculoviridae, which is divided into four genera [2]. Two of these genera, *Alphabaculovirus* and *Betabaculovirus*, include the lepidopteran nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), respectively. The study of baculoviruses, particularly those of Lepidoptera, has been driven by their applications as potential biopesticides for use against insect pests and as recombinant protein expression vectors [3–6]. As a consequence, a tremendous amount of information on the pathology, genetics, and molecular biology of baculoviruses has accumulated over the years.

Many NPVs have been isolated from the fall armyworm, *Spodoptera frugiperda* [7–12]. The *S. frugiperda* NPVs isolated to date appear to be variants of the same virus species, *S. frugiperda multiple nucleopolyhedrovirus* (SfMNPV). Isolates of SfMNPV have been evaluated as

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biopesticides to control infestations of *S. frugiperda* on maize [13–20], and studies on NPV ecology have used SfMNPV as a model virus [21].

Genetic variation among SfMNPV isolates has been previously assessed by restriction endonuclease digestion [10–12, 22]. A PCR-based method has been developed for the easy and rapid identification of baculovirus isolates [23, 24]. This method can allow for the study of genetic variation among isolates even when only small amounts of material or badly preserved samples are available. The goal of this study was to adopt this method to identify 40 uncharacterized NPV isolates from *S. frugiperda* that are part of a USDA insect virus collection and to compare them to each other and other SfMNPV isolates for which DNA sequence data exist. Besides sequencing highly conserved loci used previously to distinguish different species of baculoviruses, we also examined sequences from less-conserved homologous repeat (*hr*) regions to see if such loci could allow for a greater degree of resolution of isolates of the same species from different populations. Genetic variation among five of these isolates representing different geographic areas was further characterized by restriction endonuclease digestion, and the restriction endonuclease fragment patterns from this analysis were compared to those previously reported for other isolates of SfMNPV. Biological variation of these five isolates was assessed by bioassay against *S. frugiperda* larvae.

Methods

Virus isolates and insects

The viruses examined in this study included six previously characterized SfMNPV field isolates originally collected in Missouri [25] and 40 uncharacterized NPV isolates from *S. frugiperda* (Table 1), none of which have been plaque-purified or passaged through cell culture. The 40 uncharacterized isolates were part of an insect virus collection developed and maintained by Dr. Jean Adams and other researchers at the formerly named Insect Biocontrol Laboratory (USDA-ARS) in Beltsville, MD. The isolates consisted of a mixture of freeze-dried polyhedra stored at room temperature, aqueous suspensions of polyhedra stored at either room temperature or 4°C, and for one isolate (2570), a larval cadaver stored at 4°C. Aliquots of polyhedra from these samples were used directly for PCR amplification, or to infect *S. frugiperda* larvae to prepare larger stocks of virus for restriction endonuclease analysis and bioassays.

Eggs of *S. frugiperda* were obtained from Bio-Serv (Frenchtown, NJ). Larvae were incubated at 28° C (or 27° C for dose-response bioassays) and a 14:10 light:dark cycle

on a species-specific diet obtained from Southland Products (Lake Village, AR).

PCR and sequencing

Polyhedra from the collection isolates were diluted to a concentration of 10^6 polyhedra/µl and solubilized by adding 1/10 volume 1 M Na_2CO_3 (pH 11.4) and incubating at room temperature for 30 min. Samples were neutralized by addition of 1/10 volume 1 M Tris–HCl pH 7.0. Quantities of solubilized polyhedra solution equivalent to 1.22×10^6 polyhedra were used in 50 µl PCR reactions containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5–2.0 mM $MgCl_2$, 0.2–0.5 mM dNTPs, 0.5 nM primers, and Taq DNA polymerase. We have also found that it was necessary to add 0.05 mg/ml RNAse A in order to obtain amplimers from some of the samples; as a consequence, we routinely added this concentration of RNAse A to all our PCRs. Annealing temperatures were determined empirically for each primer pair, and an extension time of 1 min was used. For the Missouri field isolates, approximately 40 pg of previously isolated DNA [25] was used for PCR.

The sequences used in phylogenetic analysis were amplified from two strongly conserved baculovirus genes, *lef-8* and *polyhedrin* (*polh*), and two SfMNPV-specific loci containing homologous regions *hr4* and *hr5*. Primer pairs used consisted of prL8-2 (5′- CAGGAAACAGCTATGAC CAYRTASGGRTCYTCSGC-3′) and prL8-1B (5′-TAATA CGACTCACTATAGGGCAYGGHGARATGAC-3′) [23], which amplify a portion of *lef-8* including nt 106253–106977 in the SfMNPV-19 genomic sequence [26]; primers Sfpolh1m4 (5′-GAAAGGTACGTTGTCGC-3′) and Sfpolh2m2 (5′-GCGTCAGGAGCAAACT-3′), which amplify a portion of *polh* including nt 154–715 in the SfMNPV-19 sequence; primers Sf40 (5′-CTTGGGCGA TTGAAGCGTC-3′) and Sf42 (5′-ATTTTCAACAAACAA CGAGTG-3′), which amplify a region including nt 38634–39391 in SfMNPV-19 containing all of *hr4*; and primers Sf38 (5′-TCGTCGAAAACTACACCAG-3′) and Sf39 (5′-AATCGACAGGCGTGTAAAG-3′), which amplify a region including nt 48687–49233 in the SfMNPV-3AP2 genomic sequence [25] containing all of *hr5*. PCR was also carried out to amplify and sequence part of *lef-9*, using primers SfL9-1 (5′-TAATGGCGACGACGATAGTAGT A-3′) and SfL9-2 (5′-CGATGAGCGAATTGGGTTTG-3′).

Prior to sequencing, amplimers were precipitated away from excess primers and deoxynucleotides as previously described [27]. In some cases, amplimers were purified by agarose gel electrophoresis or cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Cloned amplimers were extracted directly from bacterial colonies as described [27], then PCR-amplified using primers TOPO9 (5′-CACCCCAGGCTTTACACTTTATG-3′) and TOPO10

Table 1 Nucleopolyhedrovirus isolates from *S. frugiperda* analyzed in this study

Isolates	Source[a]	Date acquired or deposited	Accession no. *lef-8*	Accession no. *polh*	Accession no. *hr4*	Accession no. *hr5*
1–6	Columbia, Missouri, USA (H. J. R. Popham)	February 2007	GQ923694–GQ923699	GQ923749–GQ923754	GQ923708–GQ923713	GQ923735–GQ923740
281	Tifton, Georgia, USA (J. J. Hamm)	May 24, 1966 (freeze-dried)	GQ923700	b	GQ923714,	GQ923741
377–382	Tifton, Georgia, USA (J. J. Hamm)	June 18, 1969 (freeze-dried)	b	b	GQ923715 (380), GQ923716 (382)[b]	b
383–386	Tifton, Georgia, USA (J. J. Hamm)	June 13, 1969 (freeze-dried)	b	b	GQ923717 (383), GQ923718 (384)[b]	b
387–392	Tifton, Georgia, USA (J. J. Hamm)	March 3, 1969 (freeze-dried)	b	b	GQ923719–GQ923723 (388–392)[b]	b
393–394	Tifton, Georgia, USA (J. J. Hamm)	November 2, 1967 (freeze-dried)	b	b	GQ923724 (394)[b]	b
459	Medellin, Colombia, South America (A. E. Bustillo)	June 1, 1978 (refrigerated)	GQ923701	GQ923756	GQ923728	GQ923742
635	Medellin, Colombia, South America (J. R. Fuxa)	Unknown (room-temperature aqueous suspension)	GQ923702	GQ923757	GQ923729	GQ923743
636	Espinal, Colombia, South America (J. R. Fuxa)	Unknown (room-temperature aqueous suspension)	GQ923703	GQ923758	GQ923730	GQ923744
637	Hammond, Louisiana, USA (J. R. Fuxa)	January 1, 1983 (room-temperature aqueous suspension)	GQ923704	GQ923759	GQ923731	GQ923745
638	Florida, USA (J. R. Fuxa)	April 23, 1984 (room-temperature aqueous suspension)	GQ923705	GQ923760	GQ923732	GQ923746
651–655, 666–667	Tifton, Georgia, USA (J. J. Hamm)	Unknown (refrigerated)	b	b	GQ923725 (653), GQ923726 (667)[b]	b
1135	Unknown	November 1968 (refrigerated)	b	b	b	b
1197	Tifton, Georgia, USA (J. J. Hamm)	March 8, 1967 (refrigerated)	b	b	b	b
2403	Unknown	February 11, 1975 (refrigerated)	b	b	b	b
2570	Unknown	April 1969 (refrigerated; larval cadaver)	b	b	b	b
2705	Unknown	Unknown (refrigerated)	GQ923706	GQ923761	GQ923733	GQ923747
3093	Unknown	June 18, 1969 (refrigerated)	b	b	b	b
3101	Unknown	February 14, 1967 (refrigerated)	b	b	GQ923727	b
3105	Unknown	Unknown (refrigerated)	b	b	b	b
3146	Unknown	June 9, 2004 (refrigerated)	GQ923707	GQ923762	GQ923734	GQ923748

a With the exception of the isolates 1–6 from Missouri, all isolates were obtained from the insect virus collection housed at the Invasive Insect Biocontrol and Behavior Laboratory (USDA-ARS) in Beltsville, MD

b All of the *lef-8*, *polh*, and *hr5* sequences for the Georgia isolates and for unknown isolates 1135, 2403, 2570, 3093, 3101, and 3105 were identical to the corresponding sequences for Georgia isolate 281 sequence; accession numbers for the *hr4* sequences of these isolates are indicated. The *hr4* sequences for a subset of these isolates differed from the Georgia isolate 281 *hr4* sequence. The *hr4* sequences of these isolates are indicated

(5′-CGGGCCTCTTCGCTATTAC-3′) and precipitated as above. Amplimers were sequenced using an Applied Biosystems BigDye Terminator Cycle Sequencing kit and an Applied Biosystems 3130xl Genetic Analyzer as previously described [27], and sequence data were assembled into contigs using the Lasergene 8 software suite (DNA-Star, Madison, WI).

Phylogenetic analysis

Nucleotide sequences generated from the isolates were aligned along with the corresponding sequences from SfMNPV-19 [26], an isolate from Brazil, and SfMNPV-3AP2 [25], a plaque-purified isolate derived from Missouri field isolate #3, with CLUSTAL W [28] using the Meg-Align program of Lasergene 8 (DNASTAR). The *lef-8* and *polh* alignments were concatenated with BioEdit [29]. Minimum evolution (ME) and maximum parsimony (MP) phylogenetic relationships were inferred from the *lef-8/ polh*, *hr4*, and *hr5* alignments using MEGA version 4.0 [30], with parameters as described in Harrison et al. [25] except the Kimura-2-parameter substitution model was used to estimate nucleotide distances for the ME trees. Reliability of the trees was tested with bootstrap re-sampling using the MEGA software's default number of replicates (1,000 for ME, 500 for MP).

Restriction endonuclease digest

Isolates 3, 281, 459, 637, and 638, which originate from distinct geographic regions, were selected for restriction endonuclease analysis and bioassay. To obtain sufficient viral DNA for restriction endonuclease digestion, polyhedra from isolates in the collection were deposited on the surface of artificial diet in 30 ml (1-oz.) cups. First- and second-instar *S. frugiperda* larvae were reared on the virus-contaminated diet. Homogenates from the larvae that died from NPV infection were then used to infect third- to fourth-instar *S. frugiperda* larvae per os, allowing larvae to consume droplets of homogenate directly or pipetting homogenate on a small diet cube, to obtain larger yields of polyhedra. Polyhedra were isolated from the resulting virus-killed cadavers as previously described [31]. Approximately 6×10^9 polyhedra/isolate were solubilized with Na_2CO_3 as described above in a concentration of approximately 2×10^8 polyhedra/ml. Undissolved material was pelleted by centrifugation at $1,328 \times g$, and the supernatants were neutralized with 1 M Tris–HCl pH 7.0. Alkali-liberated occlusion-derived virus was pelleted by ultracentrifugation through a 25% w/w sucrose cushion and DNA was extracted from the pelleted virus as previously described [27]. The viral DNAs were analyzed by restriction endonuclease digestion with *Bam*H I, *Eco*R I, and *Pst* I. Restriction fragments were separated by agarose gel electrophoresis.

Bioassays

For lethal concentration bioassays, artificial *S. frugiperda* diet was prepared and poured into the cells of plastic bioassay trays (Bio-BA128©, Bio-Serv, Frenchtown, NJ). Serial dilutions of each SfMNPV isolate were prepared in deionized water from stocks of polyhedra isolated as described above (see "Restriction endonuclease digest"). Virus suspensions were pipetted onto the diet in a volume of 25 μl per cell (200 mm² surface area). Final concentrations of virus were 0.13, 0.40, 1.25, 3.95, 12.50, and 39.50 polyhedra/mm². A control treatment of water only was included in each replicate. Sixteen cells were prepared for each treatment. Treated diet was allowed to dry under a fan until no liquid remained on the surface. One neonate larva was placed in each cell, and the cells were closed with transparent ventilated covers (Bio-CV-16©). Larvae were held at 27°C, and mortality was recorded at 10 days post-infection. Three replicates were included.

Concentrations of SfMNPV were transformed logarithmically prior to statistical analysis. Median lethal concentrations (LC_{50}) for each strain were calculated using the PROC PROBIT procedure of SAS 9.2 (SAS Institute, Cary, NC). In addition, for treatments of 3.95 polyhedra/mm² and lower concentrations, proportion mortality was calculated for each treatment, normalized by arcsine $\sqrt{\%}$ transformation, and analyzed by analysis of variance (ANOVA) for effects of virus strain and concentration (PROC GLM, SAS 9.2). Virus strains were then separated by the least significant difference (LSD) test.

Survival time bioassays were carried out as previously described [32] using a dose of 10^7 polyhedra/ml and 35 larvae for each virus tested. Data from three trials were used to calculate Kaplan–Meier survival curves and mean and median survival times with XLSTAT (Addinsoft, New York, NY). Significant differences between survival curves were evaluated by pairwise log-rank test.

Results

Sequence and phylogenetic analysis

A PCR method for baculovirus identification developed by Lange et al. [23] utilized amplification, sequencing, and phylogenetic inference from partial sequences of the highly conserved baculovirus genes *lef-8*, *lef-9*, and *polh*. To determine the identity of the *S. frugiperda* NPV isolates in our insect virus collection, these three loci were amplified and sequenced. BLAST analyses of the sequences

confirmed that all NPV isolates tested were variants of previously described SfMNPV isolates. Two single-nt polymorphisms (SNPs) were detected in the *polh* sequence of isolate 3146. One of these SNPs could specify either a Leu (wild-type) or an Ile amino acid residue at position 227 of the polyhedrin amino acid sequence. Missouri isolate 3 had six SNPs in *lef-8*, one of which could specify either Asp or Asn at position 554 of the LEF-8 amino acid sequence. Isolate 637 had one silent SNP in *lef-8*. None of the *polh* nucleotide substitutions among the isolates change the polyhedrin amino acid sequence, while substitutions in the *lef-8* sequences change the encoded amino acids at positions 477 (Met → Ile) and 554 (Asp → Asn) with respect to the SfMNPV-3AP2 LEF-8 amino acid sequence. In particular, SfMNPV-19 and the Florida isolate (637) sequences specify Asn at position 554, while sequences of SfMNPV-3AP2 and all the other isolates specify Asp at this position.

Since there was no difference among any of the isolates in the region of the partial *lef-9* sequence, phylogenetic inference was carried out only with concatenated alignments of *lef-8* and *polh* (Fig. 1). The SfMNPV isolates from Georgia grouped with each other, as did the isolates from Colombia. The Missouri isolates, in contrast, did not form a discrete clade. Bootstrap support exceeded 80% for only a couple of clades in the tree, which may have been a consequence of the low nucleotide distances among the isolates (0–0.013 substitutions/site) at these loci. Many of the isolates for which no description was available (1135, 2403, 2570, 3093, 3101, and 3105) grouped with the Georgia isolates. Isolates 2705 and 3146 were distinct from each other and from the other SfMNPV isolates.

To further confirm the relationships suggested by the *polh-lef8* tree and to see if the different SfMNPV isolates could be further distinguished from each other, sequencing and phylogenetic analysis was carried with the SfMNPV homologous regions *hr4* and *hr5*. The *hr*s are intergenic regions in baculovirus genomes consisting of repeated sequences that act as *cis*-acting enhancers of baculovirus gene transcription [33, 34] and as origins of viral DNA replication [35, 36]. A high degree of sequence divergence, including insertion/deletion polymorphisms (indels), has been observed among the *hr*s of NPV variants and closely related baculoviruses [27, 37–40].

In the process of sequencing *hr4* amplimers, two deletion genotypes were discovered among the Georgia isolates (Deletion 1 and Deletion 2; Fig. 2a). The endpoints of Deletion 1 were located 2 nt within the 2nd *hr4* repeat and 2 nt within the 4th repeat, while Deletion 2 endpoints occurred immediately after the 1st and 4th repeats. Similar indels were not found among the *hr4* sequences of the other isolates. SNPs were detected at different positions in several of the *hr4* sequences. The *hr4* regions of the

Colombian isolates 459, 635, and 636 had nine, seven, and five SNPs, respectively, while the Louisiana isolate (637) had two SNPs. Among the full-length *hr4* sequences, Georgia isolates 390, 391, and 653 had two SNPs each, while isolates 380, 382, 383, 384, 388, 389, 391, 394, and 3101 had a single SNP each.

Phylogenetic inference with the *hr4* sequences produced more groups with strong bootstrap support in both ME and MP trees than the *lef-8/polh* phylogram (Fig. 2b). The Colombian isolates grouped with each other, and were also part of a strongly supported clade with the Florida isolate (638). The Georgia isolates also grouped together, but sufficient diversity existed within their *hr4* sequences to distinguish among some of the individual isolates within this clade. The Missouri isolates once again failed to form a single clade.

In general, the *hr5* sequences exhibited a lower degree of sequence divergence among the isolates than the *hr4* sequences. SfMNPV-19 contains a deletion which removes part of *hr5* and the adjacent *dUTPase* ORF relative to SfMNPV-3AP2 [26]. This deletion was not detected in any of the isolates examined in this study. The Colombian isolates 635 and 636 had very polymorphic *hr5* sequences that respectively contained 18 and 31 SNPs and 4 and 6 indels ranging from 1 to 8 nt. The 3146 isolate also contained 8 SNPs and two indels of 4 and 5 nt, while isolate 637 had two SNPs. Phylogenetic inference of the *hr5* sequences (Fig. 3) grouped the Colombian and Georgia isolates, but not the Missouri isolates, into distinct clades. The Florida isolate (638) did not group with the Colombian isolates in this tree.

Restriction endonuclease analysis

Genetic variation among a selection of five of the isolates from Missouri (3), Georgia (281), Lousiana (637), Florida (638), and Colombia (459) was also examined by restriction endonuclease digest and agarose gel electrophoresis. Restriction endonuclease digest with *Bam*H I, *Eco*R I, and *Pst* I (Fig. 4) yielded restriction fragment patterns that were almost identical to those of previously characterized SfMNPV isolates. Isolate 3 contains an approximately 5.1 kbp *Bam*H I fragment present in the Nicaraguan isolate SfMNPV-NIC and in SfMNPV isolates from Argentina and Ohio (USA), but missing from isolates 281, 459, 637, and 638 as well as SfMNPV-3AP2, SfMNPV-2 (a plaque-purified isolate derived from a Georgia isolate), and a Louisiana field isolate of SfMNPV [12, 25, 41, 42]. Isolates 459, 637, and 638 contained a 6.1-kbp *Eco*R I fragment that was also present in SfMNPV-NIC but missing from isolates 3 and 281 as well as SfMNPV-3AP2 and SfMNPV-2 [42]. These isolates also exhibited an approximately 20 kbp

Fig. 1 Phylogenetic analysis of concatenated *polh* and *lef-8* nucleotide sequence alignments. An ME phylogram is shown with bootstrap values >50% for ME and MP trees inferred from the alignments at each node where available (ME/MP); single bootstrap values derive from the ME analysis. Isolate 3AP2 indicates SfMNPV-3AP2 [25], while Brazil 19 refers to SfMNPV-19 [26]. Genbank accession numbers for the SfMNPV-3AP2 and SfMNPV-19 sequences are indicated. Other isolates are as described in Table 1, with abbreviations for Georgia (GA), Missouri (MO), Florida (FL), and Louisiana (LA)
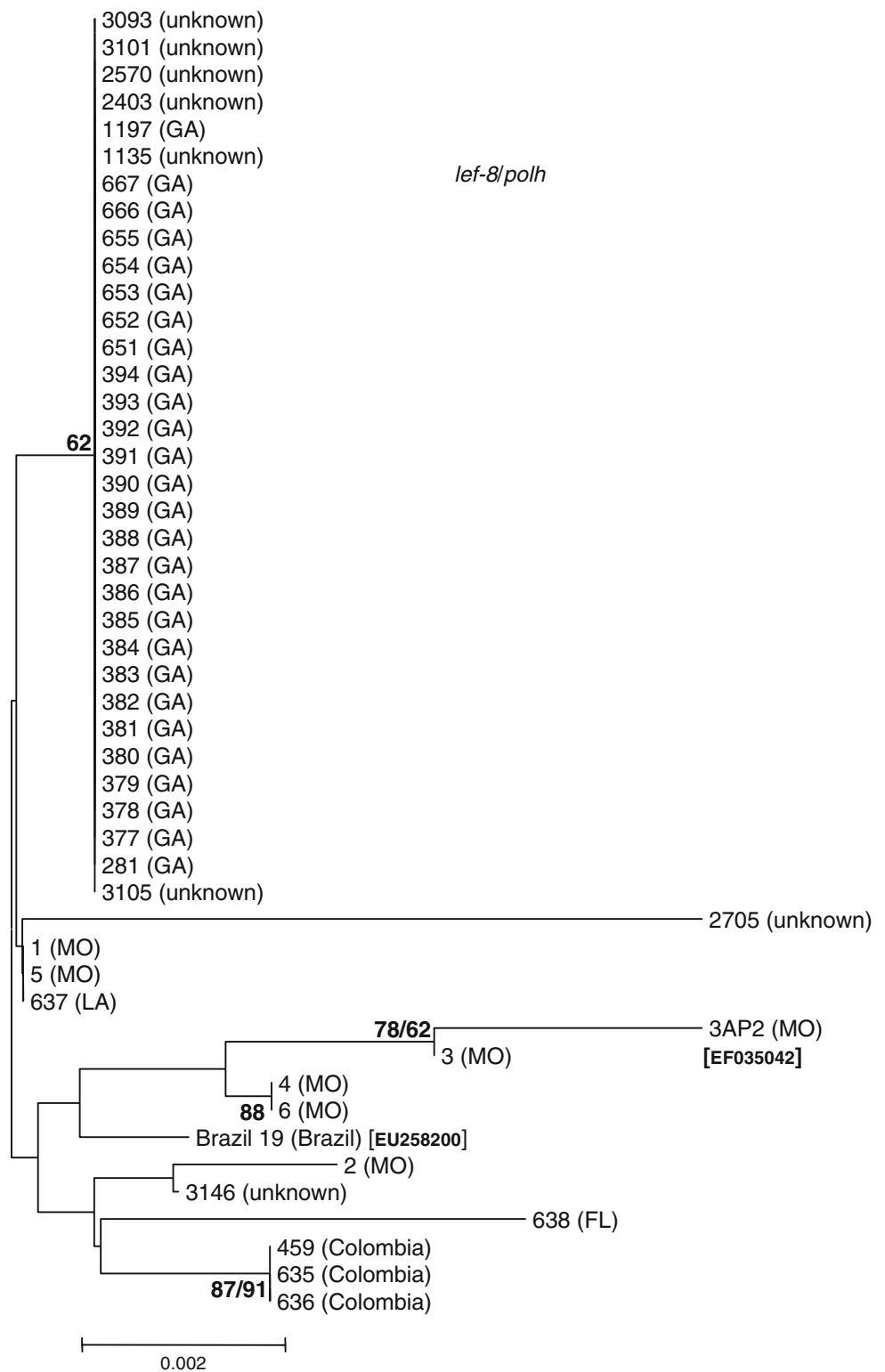
EcoR I fragment not found in the other two isolates. Although a 20-kbp *EcoR* I fragment also was reported for a Mississippi, USA isolate [12], it is uncertain if this fragment in isolates 459, 637, and 638 is an artifact and/or is present in sub-molar quantities. The 459 isolate was distinguished from the other isolates by additional 5.5 kbp *EcoR* I and 4.4 kbp *Pst* I fragments and the absence of a 1.2 kbp *EcoR* I fragment present in the other isolates. The 459 and 637 isolates also appeared to contain other sub-molar fragments in the *BamH* I and *EcoR* I digests.

Fig. 2 a Insertion/deletion polymorphisms in the *hr4* sequence in different genotypes of SfMNPV isolates from Georgia, USA. The locations of deletions in the *hr4* sequences in two different genotypes (Deletion 1 and Deletion 2) are shown, with *shaded boxes* representing the 43-bp imperfect inverted repeats within *hr4*. **b** Phylogenetic analysis of full-length (undeleted) *hr4* nucleotide sequence alignments, showing an ME phylogram. Bootstrap values and isolate designations are as for Fig. 1. Full-length *hr4* sequences were not obtained for isolates 378 and 386
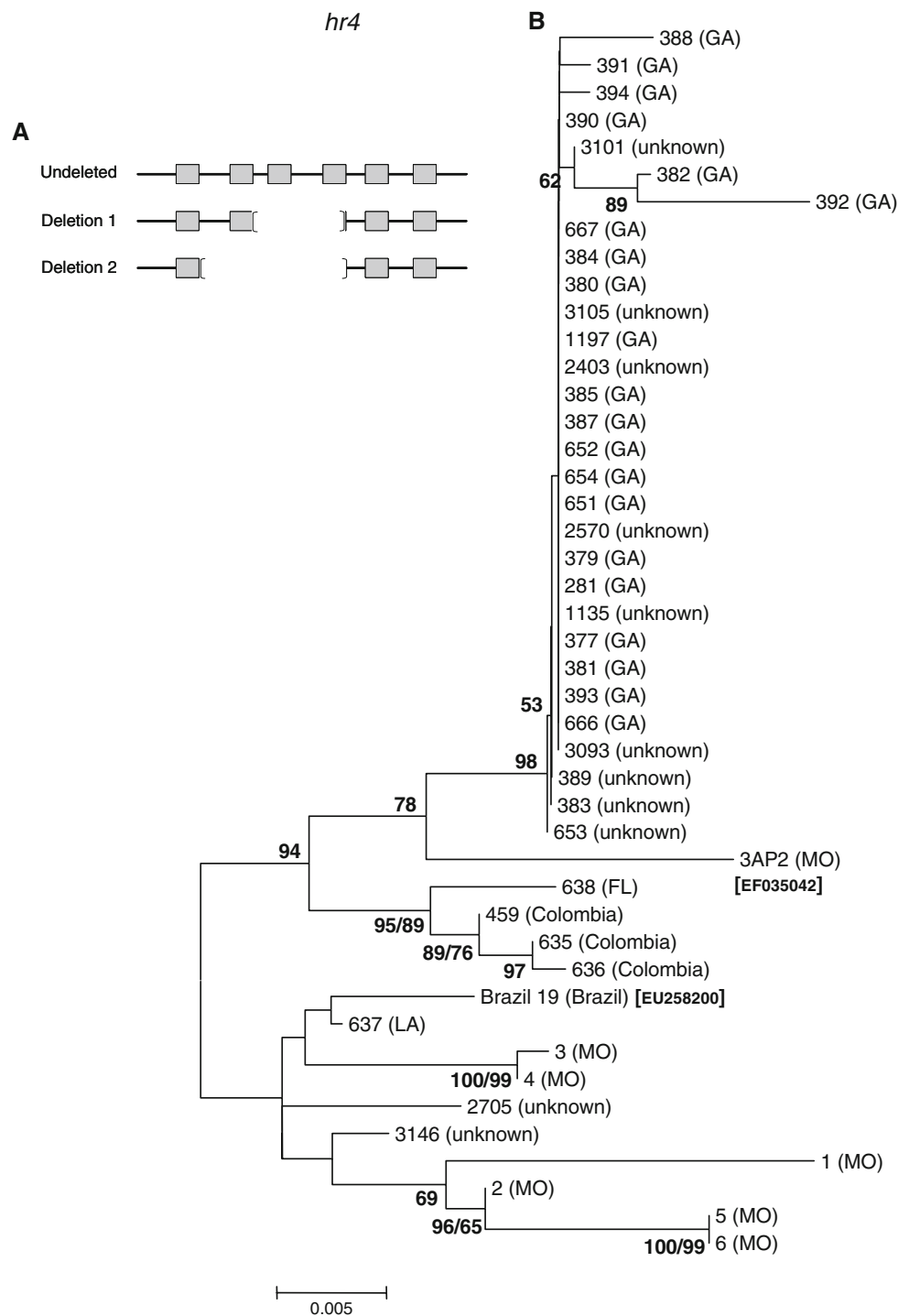

All five isolates generated a 2.9-kbp *Pst*I fragment that contains the *egt* gene (Fig. 4). This fragment is not present in SfMNPV-3AP2 or in eight of nine variant genotypes characterized from SfNIC which have partial or complete deletions of the *egt* gene [25, 43]. PCR of the DNA used in restriction endonuclease analysis confirmed that isolates 3, 281, 459, 637, and 638 all carried intact, non-deleted *egt* genes (data not shown).

Biological activity

Although the polyhedra for many of these isolates were two, three, or even four decades old (Table 1), these isolates were still infectious toward *S. frugiperda* larvae, and it was possible to grow new stocks of these viruses for use in bioassays. The virus isolates exhibited phenotypes typical for nucleopolyhedroviruses, with infections ending in

Fig. 3 Phylogenetic analysis of *hr5* nucleotide sequence alignments, showing an ME phylogram. Bootstrap values and isolate designations are as for Fig. 1. SfMNPV-19 was excluded from the analysis as it contains an extensive deletion removing approximately half of *hr5*
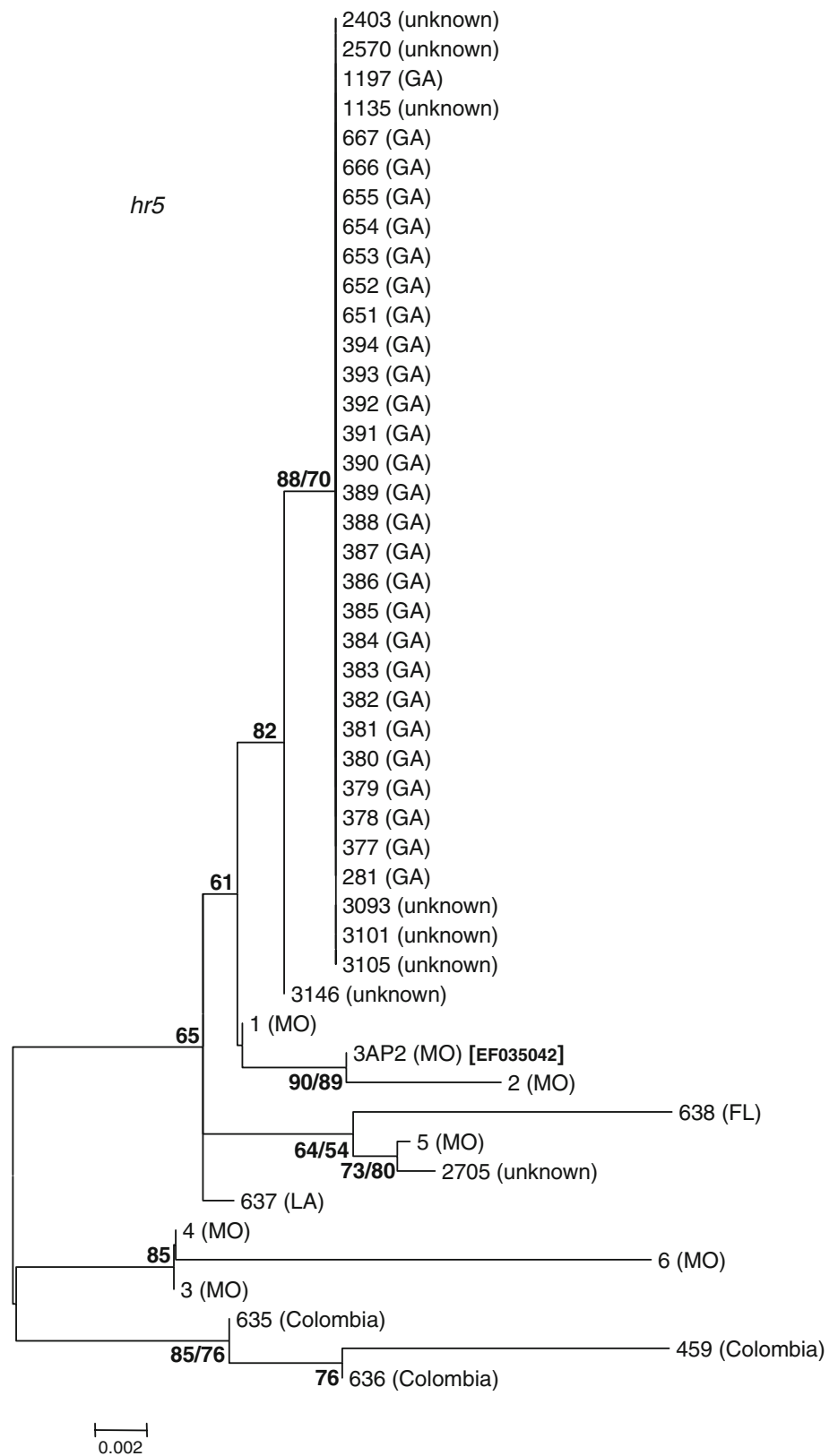
mortality characterized by melanization, fragility of the cuticle, and liquefaction or loss of integrity of the internal anatomy. The restriction endonuclease analyses of DNA

from stocks of selected isolates (see previous sections) confirmed that the NPVs recovered from infections with the isolates retained their identities as isolates of SfMNPV.

Fig. 4 Restriction endonuclease digest analysis of DNA from 5 isolates of SfMNPV with *Bam*H I, *Eco*R I, and *Pst* I. Isolate designations are as in Table 1. Benchtop 1 kb ladder (1 kb; Promega) and *Hin*d III-digested λ DNA (λ HIII) size standards are shown, with fragment sizes in kbp indicated on the sides of the gel
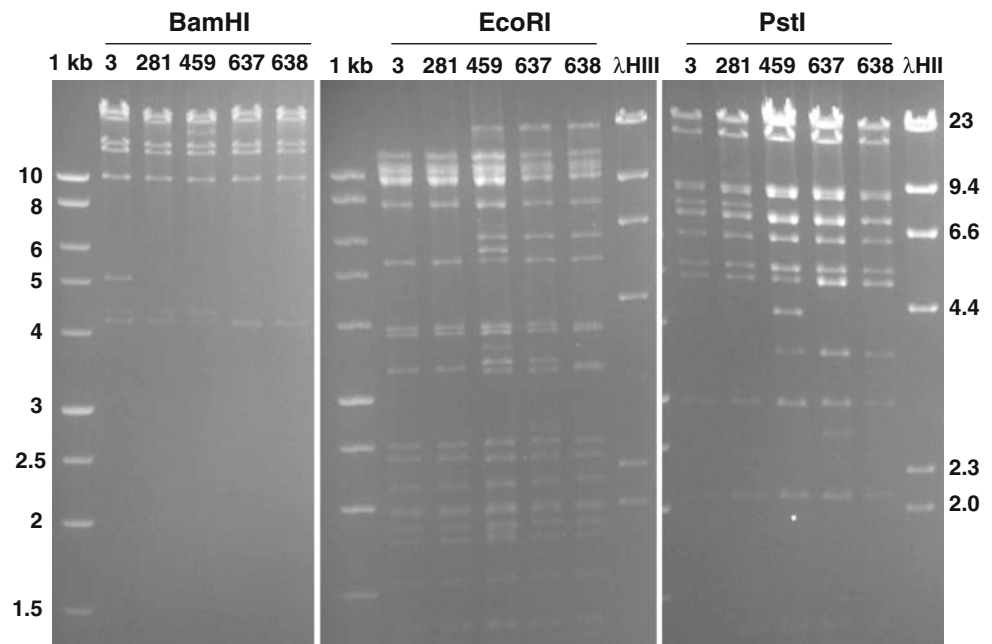
LC$_{50}$ values for the Missouri (3), Georgia (281), Lousiana (637), Florida (638), and Colombia (459) isolates tested by bioassay ranged from 0.48 to 0.75 polyhedra/mm^2 (Table 2). The 95% fiducial limits of all isolates overlapped. However, the ANOVA showed some statistically significant differences in mortality among isolates ($F_{4, 49} = 3.38$, $P = 0.0161$) as well as among virus concentrations ($F_{1, 49} = 452.55$, $P = 0.0001$). The LSD test showed that strains 281 and 637 were significantly more virulent than strain 459, but that other differences among strains were non-significant. In survival time bioassays, slight differences were observed in the survival times of larvae infected with the different isolates (Table 3). Log-rank tests indicated that isolate 459 killed neonate *S. frugiperda* larvae faster than all the other isolates, while isolate 281 killed larvae faster than isolates 3, 637, and 638.

Discussion

Genetic variation among baculovirus isolates is often assessed by restriction endonuclease digestion to detect

Table 2 Dose-mortality response of neonate *S. frugiperda* larvae infected with SfMNPV isolates

Isolate	LC$_{50}$, polyhedra/mm^2	95% fiducial limits
3 (MO)	0.63	0.48–0.81
281 (GA)	0.48	0.35–0.61
459 (Colombia)	0.75	0.57–0.97
637 (LA)	0.51	0.38–0.67
638 (FL)	0.66	0.49–0.85

Table 3 Time-mortality response of neonate *S. frugiperda* larvae infected with SfMNPV isolates

Isolate	Mean survival time (95% confidence limits)	Median survival time (95% confidence limits)
3 (MO)	73.1 (71.75–74.41)	71.0 (68.5–71.0)
281 (GA)	69.9 (68.48–71.25)	68.5 (0.0–88.0)
459 (Colombia)	66.9 (65.57–68.28)	67.5 (66.0–68.0)
637 (LA)	73.8 (72.04–75.51)	73.0 (71.5–74.5)
638 (FL)	70.8 (69.22–72.34)	71.5 (67.5–71.5)

restriction fragment length polymorphisms [44]. We used the PCR approach developed by Lange et al. [23] to characterize genetic variation in *S. frugiperda* NPV isolates from our collection. Only a minimal amount of material from each isolate was required to produce sequence data, and we were able to identify and distinguish a large number of different isolates more rapidly than would have been possible by restriction endonuclease analysis. An attempt to amplify sequences longer than 2 kbp resulted in anomalously short amplification products from many of the isolates, particularly the older freeze-dried isolates (data not shown). It is possible that DNA damage accrued in some of these isolates during storage that prevented the amplification of longer sequences.

The Colombian isolates, which included two separate isolates from Medellin and an isolate from Espinal, formed a clade distinct from the North American isolates. Phylogenetic inference (Figs. 1, 2b) and restriction endonuclease fragment patterns (Fig. 4) indicated that the Florida isolate

(638) was also relatively closely related to the Colombian isolates. The failure of the Florida isolate to group with the Colombian isolates in the *hr5* phylogram may be due to the very polymorphic nature of the isolate 635 and 636 *hr5* sequences. Phylogenetic inference of *hr5* sequences without these two isolates grouped the Florida isolate with Colombian isolate 459 with strong bootstrap support (data not shown). The Georgia isolates also grouped together, and the complete identity of their sequences at three of the four loci examined suggests that they all may have been derived from an NPV first harvested from a laboratory colony of *S. frugiperda* at the USDA-ARS laboratory in Tifton, GA in 1963 [7, 22]. Some genetic variation was still detectable among the Georgia isolates at the *hr4* region, indicating that sequence divergence can occur rapidly at this locus.

The six Missouri isolates did not group together in a single clade with any of the three data sets analyzed. These isolates were collected at the same time from the same population of *S. frugiperda* larvae in a field in Midway, MO, but each isolate was originally purified from separate individual larval cadavers [25]. Hence, the failure of the Missouri isolates to group together in phylogenetic trees suggests a relatively high level of larva-to-larva sequence divergence among NPVs in the individual larvae collected from that field. These results contrast with results from an examination of genotype distribution within and among populations of *Malacosoma californicum pluviale* NPV, which found that genotypic variants within a population were more likely to be identical than variants from geographically separated populations [45]. The degree of larva-to-larva variability may be due to infection of each larva in the Missouri population with a relatively low dose of virus containing one or a few genotypes, leading to the amplification of divergent genotypes within each larva by a stochastic process.

An additional mystery is the large genetic distance evident between the sequences of isolate 3 and SfMNPV-3AP2, which is a plaque isolate derived from isolate 3 (Figs. 2b, 3; [25]). The six SNPs present in the *lef-8* sequence of isolate 3 suggest that it contains a greater degree of sequence diversity than the other Missouri isolates. Given that variant genotypes with deletions in the *egt* region are over-represented among plaque isolates from SfMNPV field populations [25, 43], it is possible that plaque isolate 3AP2 is a minor genotypic variant that was selected for in tissue culture.

Bioassays were carried out with SfMNPV isolates representative of the Georgia and Colombia clades and the Missouri isolates, along with the individual Louisiana and Florida isolates. Although there was genetic variation among geographically distant SfMNPV isolates that was observed by both DNA sequence variation and restriction fragment polymorphism, there was little biological variation among the five representatives of these isolates that were assayed. Some differences were observed in LC_{50} values against *S. frugiperda* larvae (Tables 2, 3), but the magnitudes of the differences were small. This result is consistent with SfMNPV bioassay data reported by Berretta et al. [8] and Harrison et al. [25], but not with the study of Escribano et al. [10] which reported LC_{50} values ranging over two orders of magnitude among four isolates. SfMNPV-2, the isolate with the largest LC_{50} in the Escribano et al. study, was a cell culture-derived plaque isolate and may have contained "few polyhedra" mutants [46], which reduce oral infectivity [47]. The survival times reported for the isolates in Table 3 are rather low compared to other published bioassay data [10, 25]. This pattern may be a consequence of the high dose (10^7 polyhedra/ml) used in the survival time bioassays, as survival time is influenced by dose in NPV bioassays [48, 49].

In conclusion, phylogenetic analysis of PCR-amplified sequences from selected loci was used successfully to identify and distinguish several isolates from a baculovirus collection, with the use of sequences from the less-conserved *hr* regions allowing for the resolution of isolates of the same baculovirus species with a greater degree of bootstrap support. Further examinations of other groups of isolates with a similar PCR-based approach should reveal the extent to which the larva-to-larva variability observed among the Missouri SfMNPV isolates occurs in populations of other NPVs.

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