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Classification, genetic variation and pathogenicity of *Lymantria dispar* nucleopolyhedrovirus isolates from Asia, Europe, and North America

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ABSTRACT

Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) has been formulated and applied to control outbreaks of the gypsy moth, *L. dispar*. To classify and determine the degree of genetic variation among isolates of *L. dispar* NPVs from different parts of the range of the gypsy moth, partial sequences of the *lef-8*, *lef-9*, and *polh* genes were determined for *Lymantria* spp. virus samples from host populations throughout the world. Sequence analysis confirmed that all *L. dispar* virus samples tested contained isolates of the species *Lymantria dispar multiple nucleopolyhedrovirus* (*Baculoviridae: Alphabaculovirus*). Phylogenetic inference based on the *lef-8* sequences indicated that the LdMNPV isolates formed two groups, one consisting primarily of isolates from Asia, and one consisting primarily of isolates from Europe and North America. The complete genome sequence was determined for an isolate from the Asian group, LdMNPV-2161 (S. Korea). The LdMNPV-2161 genome was 163,138 bp in length, 2092 bp larger than the previously determined genome of LdMNPV isolate 5–6 (CT, USA). The two genome sequences were co-linear, with an overall nucleotide sequence identity of 97.5% and some differences in ORF content. In droplet-feeding bioassays against neonate *L. dispar* larvae, isolates LdMNPV-3029 (Virin-ENSh/Russia) and LdMNPV-Ab-a624 (MA, USA) killed neonate larvae with an LC_{50} values that were 1.8- to 3.2-fold lower than a sample of Gypchek® (CT, USA) and isolates LdMNPV-3041 (Japan) and LdMNPV-2161. This study expands our knowledge about genetic variation among LdMNPV isolates and provides novel information on the distinct groups in which these NPVs occur.

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1. Introduction

The gypsy moth, *Lymantria dispar* (Lepidoptera: Noctuidae: Lymantriinae), is a defoliating pest of trees. Subspecies of the gypsy moth occur in Europe, North Africa, Asia, and North America (Pogue and Schaefer, 2007). The gypsy moth is not indigenous to North America; rather, *L. dispar* was introduced to the United States in the latter 1860s as part of an effort to develop a native silk industry (Liebhold et al., 1989). Escaped moths established a population in Massachusetts that was causing extensive local tree defoliation by 1879. Since then, the gypsy moth has continued to disperse throughout the northeastern US, and currently is established in an area extending from Maine south to Virginia and west to Wisconsin, and also to adjacent parts of Ontario, Quebec, New Brunswick, and Nova Scotia in Canada (USDA/APHIS/PPQ, http://www.aphis.usda.gov/plant_health/plant_pest_info/gypsy_moth/downloads/gypmoth.pdf). Periodic outbreaks of gypsy moth result in high densities of larvae that completely defoliate trees, making them more susceptible to disease and leading to widespread deforestation and shade, fruit, and ornamental tree death. Control efforts against *L. dispar* in North America currently focus on suppressing periodic outbreaks in areas where gypsy moth is established, and on eradicating new colonies of gypsy moth occurring either at the leading edge of the established gypsy moth population or in areas where the gypsy moth is not already established (Hajek and Tobin, 2009). Control approaches consist of mating suppression with the gypsy moth pheromone and/or application of a gypsy moth pathogen (Hajek and Tobin, 2009; Solter and Hajek, 2009). The pathogens used consist primarily of the bacterial pathogen *Bacillus thuringiensis kurstaki* and also a baculovirus, *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV).

LdMNPV originally was identified from gypsy moth larvae dying from "wilt" or "flacherie" disease (Chapman and Glaser, 1916), also known as nuclear polyhedrosis, and was found to cause epizootics within dense populations of gypsy moth larvae (Doane, 1970). Research on the use of this virus for control of *L. dispar* began in the 1950s and 1960s (Lipa, 1998; Rollinson et al., 1965) and led to the development of the products Virin-ENSh in the USSR (Alyoshina, 1980) and Gypchek® in the US (Reardon et al., 2012),

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with an additional product, Disparvirus®, developed from Gypchek® for use in Canada (Moreau and Lucarotti, 2007).

Much of the research conducted on LdMNPV has been carried out either with the LDP-67 strain of this virus from Hamden, Connecticut, USA, which is used to produce Gypchek®; or with *in vivo*- or *in vitro*-derived clonal isolates from Gypchek® (McClintock et al., 1986; Slavicek and Podgwaite, 1992; Slavicek et al., 1995, 1992). In addition to the characteristic nuclear polyhedrosis caused by LdMNPV infection, the ultrastructural features of LdMNPV (Harrap, 1972a,b) and the genome sequence of the LdMNPV plaque isolate 5–6 (Kuzio et al., 1999) collectively indicate that baculovirus isolates from *L. dispar* are group II nucleopolyhedroviruses (NPVs) of genus *Alphabaculovirus* (Herniou et al., 2011).

Many different geographic isolates of LdMNPV have been evaluated for biological activity against gypsy moth (Duan et al., 2012; Lewis et al., 1984; Narang et al., 2001; Shapiro et al., 1984). It has been generally assumed that these isolates are variants of a single species, which is currently listed as *Lymantria dispar multiple nucleopolyhedrovirus* by the International Committee on Taxonomy of Viruses (http://www.ictvonline.org/virusTaxonomy.asp). Comparison of restriction endonuclease fragment patterns generated from the genomic DNA of LdMNPV isolates from France, Korea, and Massachussetts, USA indicate that they are indeed variants of the same virus (Narang et al., 2001). The authors of this study claimed that the fragment pattern profile of these isolates was the same or similar to that of a plaque isolate (LdMNPV 5-7D) derived from Gypchek® (McClintock and Dougherty, 1988). However, restriction endonuclease analysis of DNA from the LdMNPV isolate of Virin-ENSh revealed fragment patterns that differed significantly from that of the Gypchek® LDP-67 isolate (Dougherty, 1983). There are several instances in which distinctly different alphabaculoviruses have been harvested from the same host lepidopteran species (Gettig and McCarthy, 1982; Keathley et al., 2012; Lauzon et al., 2005; Li et al., 2002). The occurrence of genotypic variants within LdMNPV isolates (Slavicek and Hayes-Plazolles, 2003; Zhang et al., 2010) may complicate the interpretation and comparison of restriction endonuclease fragment patterns of different LdMNPV isolates.

To clarify the range of alphabaculoviruses that occur in *L. dispar* and expand on what we know about the genetic variation of *L. dispar* NPV populations, a PCR-based approach was used to identify and examine the relationships and variation of geographic isolates of LdMNPV from Asia, Europe, and North America. Phylogenetic analysis was performed with partial sequences from the highly conserved *lef-8* gene. The genome sequence from a representative Asian LdMNPV isolate from Korea was determined and analyzed. Bioassays against larvae of *L. dispar dispar* were performed to detect differences in the biological activity of representative isolates from the different groups identified from the phylogenetic analysis.

2. Materials and methods

2.1. Virus isolates and insects

The viruses examined in this study consist of NPV samples primarily from *L. dispar*, with some samples from other *Lymantria* spp., that were part of an insect virus collection developed and maintained by researchers at a USDA Agricultural Research laboratory in Beltsville, MD (Table 1). These isolates include a sample of Gypchek® (3049) deposited in the collection in September 1997 and a sample of the Russian *L. dispar* NPV product Virin-ENSh (3029). In addition to the above collection samples, a plaque isolate, LdMNPV-Ab-a624, derived from an Abington, MA strain of LdMNPV was also analyzed, as well as a recently discovered isolate, LdMNPV-HrB, extracted from cadavers of larvae reared from eggs collected near Harbin, China. Aside from LdMNPV-Ab-a624, none of these virus isolates have been plaque-purified or passaged through cell culture. The collection samples consist of a mixture of freeze-dried polyhedra, aqueous suspensions of polyhedra, and larval cadavers stored at 4 °C.

L. dispar larvae used in this study were reared from eggs obtained the USDA APHIS rearing facility in Otis AFB, Massachusetts on *L. dispar*-specific diet from Southland Products (Lake Village, AR) at 28 °C on a 14:10 light:dark cycle.

2.2. PCR and Sanger dideoxy sequencing

Methods used for baculovirus PCR template preparation, PCR amplification, and sequencing were carried out as previously

Table 1
Lymantria spp. nucleopolyhedrovirus samples analyzed in this study.

Isolates	Source/identifier	Host	Date acquired or deposited	*lef-8* GenBank ID	*lef-9* GenBank ID[a]	*polh* GenBank ID[a]
463	Denmark (Sickeborg)/O. Zethner	*L. monacha*	January 1, 1973	KF695070	KF695071	KF695072
1010	Massachusetts (Abington), USA	*L. dispar*	March 16, 1992	KF695055	KF695066	KF695053
2161	South Korea/D.K. Reed	*L. dispar*	September 28, 1993	KF695050[b]	KF695050[b]	KF695050[b]
3006	Unknown	*L. monacha*	Unknown	KF695062	KF695066	KF695053
3029 (Virin-ENSh)	Russia	*L. dispar*	Unknown	KF695056	KF695065	KF695051
3041	Japan	*L. dispar*	Unknown	KF695057	KF695066	ND
3042	Japan (Ibaraki)/K. Katagiri	*L. dispar*	Unknown	KF695055	ND	ND
3049 (Gypchek)	Unknown	*L. dispar*	September 1997	KF695062	KF695066	KF695053
3051	Croatia (Dalmatia)	*L. dispar*	February 3, 1984	KF695062	KF695066	KF695051
3054	Spain	*L. dispar*	November 26, 1980	KF695058	ND	ND
3055	Slovenia	*L. dispar*	January 27, 1981	KF695062	KF695066	KF695051
3056	Serbia	*L. dispar*	January 27, 1981	KF695062		
3057	Romania	*L. dispar*	May 5, 1981	KF695059	KF695066	KF695051
3058	"Asia"	*L. dispar*	January 27, 1981	KF695060	KF695066	KF695051
3060	Ontario (Kaladar), Canada	*L. dispar*	Unknown	KF695062	KF695066	KF695053
3061	Taiwan/P.W. Schaefer	*L. xylina*	June 5, 1996	KF695067	KF695068	KF695069
3063	Japan (Gumma prefecture)	*L. fumida*	Unknown	KF695061	ND	ND
3065	Poland (Trzcianno)	*L. dispar*	1978	KF695062	KF695066	KF695052
3121	Ontario (Pitts Ferry)	*L. dispar*	1983	KF695062	KF695066	KF695053
3152	France (Alsace)	*L. dispar*	Unknown	KF695063	KF695066	KF695053
Ab-a624	Massachussetts (Abington), USA	*L. dispar* (plaque isolate)	–	KF695055	KF695066	ND
HrB	China (Harbin)/M.A. Keena	*L. dispar*	February 28, 2013	KF695064	KF695066	KF695054

[a] ND: Not determined.
[b] Complete genome sequence.

described (Rowley et al., 2010). Briefly, polyhedra from the samples were diluted to a concentration of 10^6 polyhedra/μl, solubilized with 1/10 volume 1 M Na_2CO_3 (pH 11.4) at room temperature (approximately 24 °C) for 30 min, and neutralized with 1 M Tris–HCl pH 7.0. For templates that were recalcitrant to PCR, larger quantities of polyhedra were solubilized as above and DNA was isolated and purified by extraction with 1:1 phenol:chloroform and precipitation with ethanol.

Primers for PCR amplification and sequencing are listed in Supplementary Table 1. In general, highly conserved loci (*lef-8*, *lef-9*, and *polh*) were amplified with the degenerate primers described in Lange et al. (2004). In some cases, species-specific primers were used to amplify and sequence these loci. Reactions were set up in 50 μL PCR reactions with Taq polymerase (Promega) and the equivalent of 1.22×10^6 solubilized polyhedra or a dilution of purified viral DNA. PCR conditions were as described in Rowley et al. (2010) except that the concentrations of *lef-8*, *lef-9*, and *polh* degenerate primers were doubled. PCR amplimers were precipitated away from contaminating primers and deoxynucleotides prior to sequencing as previously described (Harrison and Lynn, 2007). Some of the amplimers were cloned prior to sequencing as described (Harrison and Lynn, 2007; Rowley et al., 2010).

PCR products generated with degenerate primers were sequenced with M13 forward and reverse primers as previously described (Harrison and Lynn, 2007), while amplimers generated with LdMNPV-specific primers were sequenced with the same primers used for PCR.

2.3. Phylogenetic inference

Sequence data from each locus were assembled into contigs and aligned by CLUSTAL W (Thompson et al., 1994) using the Lasergene 10 software suite (DNAStar, Madison, WI), using default parameters. Alignment of *lef-8* sequences also included previously generated sequence data from other *Lymantria* spp. NPV strains and isolates found in Genbank.

Phylogenetic analysis of aligned nucleotide sequences generated from the isolates was carried out using MEGA 5.1 (Tamura et al., 2011) with minimum evolution (ME) and maximum parsimony (MP) methods with bootstrap re-sampling as previously described (Harrison et al., 2008), except the value of the shape parameter for the discrete gamma distribution used for modeling rate differences among sites was estimated from the alignment. The Kimura-2-parameter substitution model was used to estimate pairwise nucleotide distances between the taxa in the alignment.

2.4. Genomic DNA preparation and 454 sequencing

Fifteen mL of 3rd and 4th instar *L. dispar* larval cadavers resulting from LdMNPV-2161 infection were homogenized in 20 mL 0.5% sodium dodecyl sulfate (SDS) using an Ultra-Turrax T25 (IKA Labortechnik) set at 8000 rpm for 30 s. The homogenate was filtered through three layers of cheesecloth, and polyhedra were collected by centrifugation for 10 min at 1328*g*. The polyhedra were washed by resuspension in 25 mL 0.1% SDS and re-pelleted by centrifugation, then washed again in 25 mL 0.5 M NaCl and collected again by centrifugation. Polyhedra were re-suspended in 1 mL distilled deionized H_2O and counted, then diluted to a concentration of 10^9 polyhedra/mL in 0.1 M Na_2CO_3. The polyhedra were incubated for 30 min at room temperature, followed by 15 min at 37 °C. The solubilized polyhedra suspension was then neutralized by addition of 1 M Tris–HCl pH 7.6 to a final concentration of 0.1 M, and insoluble material was removed by centrifugation for 10 min at 1328*g*. Occluded virus from the solubilized polyhedra was collected by centrifugation through a 25% w/w sucrose pad for 75 min at 103,900*g*. Viral DNA was extracted from the occluded virus pellet as described in Harrison and Lynn (2007).

Ethanol-precipitated LdMNPV-2161 DNA was pelleted by microcentrifugation and resuspended in distilled deionized H_2O. After resuspension, DNA was sheared, size fractionated, and a multiplexed Roche GS FLX Titanium library was prepared for sequencing at the Georgia Genomics Facility (https://gsle.ovpr.uga.edu). Initial sequencing was carried out on an in-house Roche 454 GS Junior instrument. Reads were sorted and assembled using the Lasergene SeqMan NGEN V3.0 (DNAStar) assembler program with default parameters. Gaps were closed and sequencing ambiguities were resolved by PCR amplification and dideoxy sequencing of the corresponding genomic regions from viral DNA. The SeqManPro V9 sequence editor was used to prepare the finalized contig, which was 163,138 bp in length with an average coverage of 19.34X. This relatively low degree of coverage for 454 sequencing was due in part to the inclusion of three NPVs in addition to LdMNPV-2161 in the same sequencing run.

Open reading frames (ORFs) of at least 50 codons that did not overlap adjacent ORFs by more than 75 bp were selected for annotation, BLASTp queries and further characterization using DNAStar GeneQuest. If two ORFs overlapped by more than 75 bp, the larger ORF was selected for annotation. ORFs that had annotated homologs in other baculovirus genomes, including the LdMNPV 5–6 genome, were also selected for annotation in the LdMNPV-2161 genome.

2.5. Bioassays

Polyhedra stocks for bioassays were prepared as described above from virus-killed 3rd- and 4th-instar larvae, and resuspended in deionized distilled H_2O. For bioassays, neonate *L. dispar* larvae were infected *per os* by the droplet feeding method (Hughes and Wood, 1981) with five concentrations of polyhedra (0.3×10^4, 1×10^4, 3×10^4, 1×10^5, and 3×10^5 polyhedra/mL) containing green food coloring. Thirty-two larvae per concentration were used. Bioassays were incubated at 20 °C and scored at 28 days post-infection. Four replicate bioassays were carried out. The LC_{50} (concentration of polyhedra required to kill 50% of the test larvae) for each virus was calculated using PoloPlus 2.0 (Robertson et al., 2007), and hypotheses concerning the parallelism and equality of probit dose–response lines were tested using the same program.

3. Results

3.1. Identification and relationships of Lymantria spp. NPV samples

Partial sequencing and BLAST analysis of the *lef-8*, *lef-9*, and *polh* genes of virus samples from *Lymantria* spp. revealed that all the samples analyzed contained isolates of NPVs previously described from *L. dispar*, *Lymantria monacha*, and *Lymantria xylina*. Alignment of the *L. dispar* NPV sequences with the corresponding sequences in the LdMNPV 5–6 genome showed a relatively small degree of variation in the *lef-9* and *polh* loci, with only synonymous substitutions observed. In the *lef-9* locus, a GTC → GTT substitution occurs in isolate 3029 in the codon encoding valine-231, while a TAC → TAT substitution occurs in isolate 2161 in the tyrosine-240 codon. In the *polh* locus, the HrB isolate contains a GGG → GGC substitution in the glycine-63 codon. Isolate 3065 has a GCC → GCT substation in the alanine-141 codon. Isolates HrB, 2161, 3029, 3051, 3057, and 3058 contained a ATT → ATC substitution in the isoleucine-169 codon. Furthermore, 2161 contained silent substitutions in *polh* codons threonine-188, isoleucine-220, and valine-224. The *lef-8* locus exhibited a greater degree of variation among

the LdMNPV isolates, with polymorphic sites, indels, and both synonymous and non-synonymous substitutions in a total of 16 codon sites, some of which are shown in Fig. 1.

Because of the relatively low degree of variation among the *lef-9* and *polh* sequences, phylogenetic inference was carried out solely with an alignment of the *lef-8* sequences generated in this study and including previously generated *lef-8* sequences from other *Lymantria* spp. NPVs. This analysis placed the isolates into three main clades (Fig. 2A): one consisting of isolates from *L. monacha*, including isolate 463; one consisting of isolates from *L. xylina*, including isolate 3061; and one consisting mostly of isolates from *L. dispar*. The *L. dispar* NPV clade also contained isolate 3006 from *L. monacha* and isolate 3063 from *Lymantria fumida*.

In the *L. dispar* NPV clade, the isolates were separated into two groups (Fig. 2B), one containing mostly isolates from North America and Europe, and the other consisting of a large proportion of isolates from Asia. Neither group enjoyed strong bootstrap support at its base, but some nodes within the Asian group were relatively well-supported in both ME and MP trees, particularly a node containing LdMNPV isolates from Japan, S. Korea, and China. LdMNPV A24-6 was not placed in either group, possibly due to a high number of polymorphic nucleotide sites in its *lef-8* sequence. Pairwise nucleotide distances among the LdMNPV *lef-8* sequences calculated with the Kimura-2-parameter model ranged from 0 to 0.014 substitutions/site, indicating that the isolates were all variants of the same baculovirus species according to the criterion for baculovirus species demarcation proposed by Jehle et al. (2006). The isolates in the Asian group were more divergent, at least at the *lef-8* locus, than those in the European/North American group. This was evident both in the longer branch lengths separating the taxa of the Asian group as well as in the pairwise nucleotide distances, which measured only 0.000–0.003 substitutions/site among the European/North American group isolates.

3.2. Genome sequence of isolate LdMNPV-2161 from S. Korea

To obtain a more comprehensive view of the genetic variation separating the two groups of LdMNPV isolates in Fig. 2B, the complete genome sequence of isolate LdMNPV-2161 from the Asian group was determined. A comparison of the LdMNPV-2161 genome with the genome of LdMNPV 5–6 (Table 2) showed that the LdMNPV-2161 sequence is 2092 bp larger than that of LdMNPV 5–6, but otherwise the two genomes were very similar, with an overall nucleotide sequence identity of 97.5%. Although 568 gaps were required to obtain an optimal alignment of the two genome sequences, the sequences were largely co-linear, with all the homologous repeat regions (*hrs*) and most of the ORFs of isolate 5–6 also found in 2161 (Supplementary Table 2). Excluding *baculovirus repeated ORF* (*bro*) genes, the two genomes had 145 orthologous ORFs in common, 19 of which shared 100% predicted amino acid sequence identity when translated. The 37 baculovirus core genes and 27 common alphabaculovirus genes (Garavaglia et al., 2012) were found in both genomes, with an average predicted amino acid sequence identity of 98.0%. The least conserved shared ORF was *chaB1* (listed as ORF 58 in LdMNPV-2161 and ORF 61 in LdMNPV 5–6), which exhibited a 61.2% amino acid sequence identity between the two isolates. This gene is a homolog of ORF *ac58/59* in *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). The encoded ChaB-like protein was found in both budded and occluded virions of AcMNPV by mass spectrometry proteomic methods (Braunagel et al., 2003; Wang et al., 2010), but insertional mutation of the *Bombyx mori* nucleopolyhedrovirus homolog of this gene had no apparent effect on infectivity or replication (Ono et al., 2012).

There were some differences in the ORF content of LdMNPV 5–6 and LdMNPV-2161. The LdMNPV 5–6 ORFs 6, 8, 10, 13, 31, 66, 121, 133, and 134 were not found in the LdMNPV-2161 genome. The

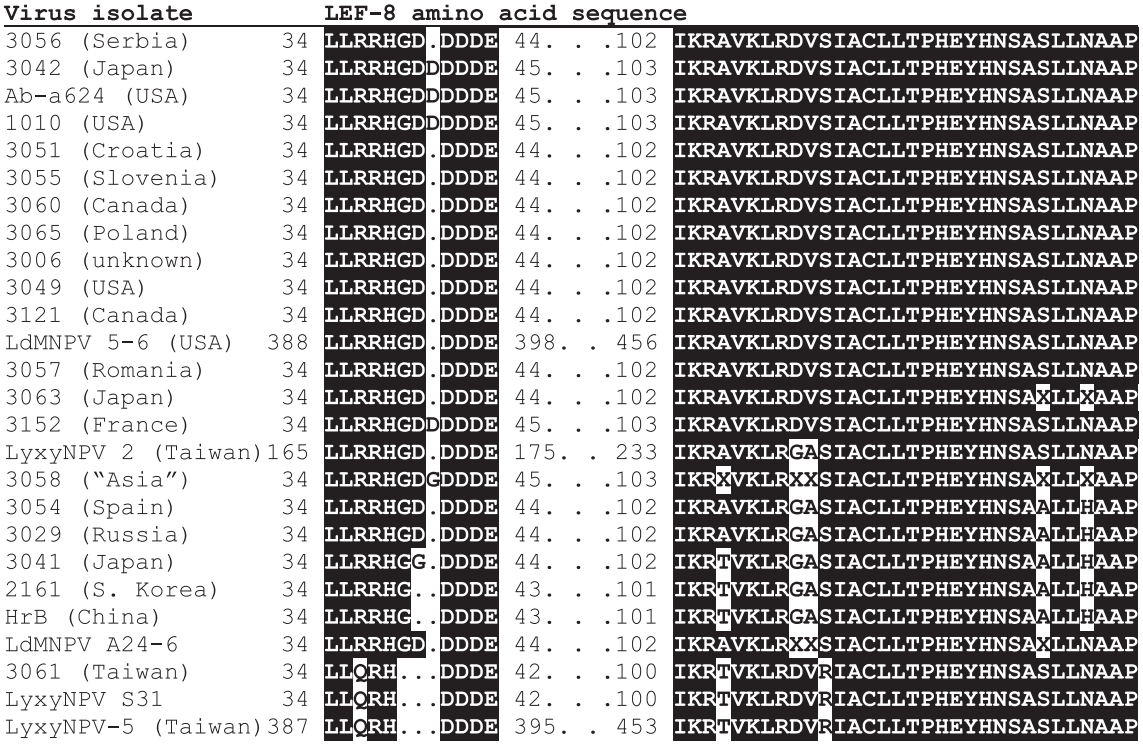

Fig. 1. Two sections of an alignment of predicted LEF-8 amino acid sequences from *Lymantria* spp. NPVs. Sequences from most of the isolates listed in Table 1 were aligned with the published corresponding sequences from *Lymantria dispar* MNPV 5–6 (LdMNPV 5–6; Kuzio et al., 1999), *Lymantria xylina* MNPV 2 (LyxyNPV 2, otherwise known as LdMNPV-like virus; Nai et al., 2009), *Lymantria dispar* MNPV A24-6 (LdMNPV A24-6; Jehle et al., 2006), *Lymantria xylina* MNPV S31 (LyxyMNPV S31; Jehle et al., 2006), and *Lymantria xylina* MNPV-5 (LyxyMNPV-5; Nai et al., 2010). Amino acid positions of the sections from the conceptual translations of the nucleotide sequences are indicated. Amino acids listed as "X" correspond to codons with polymorphic nucleotide sites.

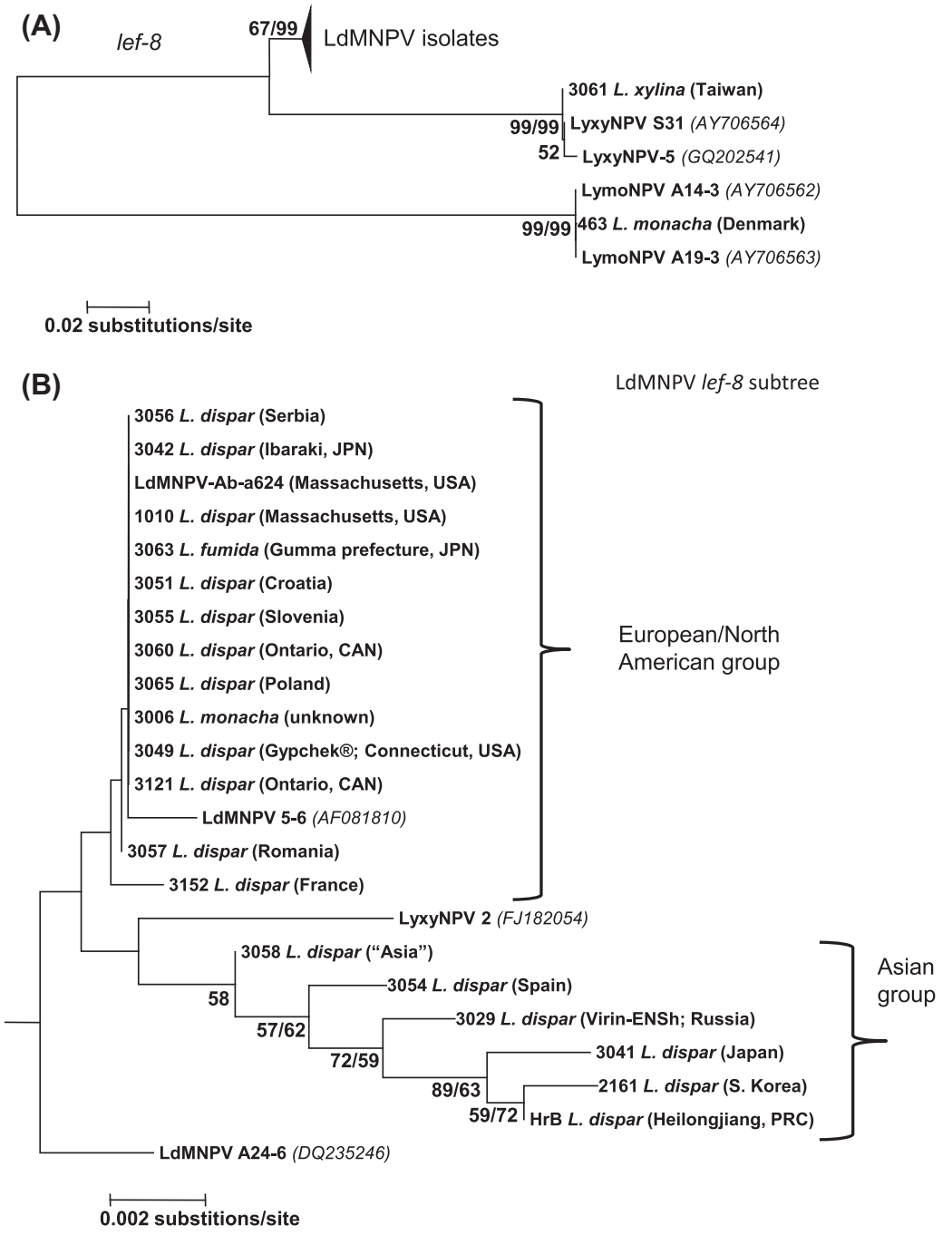

Fig. 2. Phylogenetic analysis of partial *lef-8* nucleotide sequences showing relationships of all the *Lymantria* spp. NPVs examined (A) and the relationships of isolates of *Lymantria dispar multiple nucleopolyhedrovirus* (B). Minimum evolution (ME) phylograms inferred from the alignment are shown with bootstrap values ⩾50% for ME and maximum parsimony (MP) trees at each node where available (ME/MP). The host species from which the samples were derived (*L. dispar*, *L. fumida*, *L. monacha*, and *L. xylina*) are indicated after the sample number, and the sources of the samples are indicated in parentheses. Taxa in the tree representing previously published *Lymantria* spp. sequences are followed by Genbank accession numbers and include the viruses listed in Fig. 1 legend as well as *Lymantria monacha* NPV isolates A14-3 and A19-3 (Jehle et al., 2006).

Table 2
Characteristics of LdMNPV Cl5–6 and LdMNPV-2161(S. Korea) genome sequences.

LdMNPV isolate	Size (bp)	% GC	# ORFs	# hrs	# bro genes
Cl5–6	161,046	57.5	163	13	16
2161	163,138	57.3	174	13	20

absence of these ORFs from the LdMNPV-2161 genome was due to insertions and deletions that either removed some or all of the ORF sequence or resulted in a frameshift. Seven of these ORFs (6, 8, 10, 13, 31, 121, 133, and 134) were unique to LdMNPV 5–6, with no homologs in other NPVs. ORF 66, also listed as *ctl-2* (Kuzio et al., 1999), encodes a peptide with sequence similarity to snail conotoxins (Becker and Terlau, 2008). The LdMNPV 5–6 sequence has two *ctl* genes, *ctl-1* and *ctl-2*, of which only one (*ctl-1*) is present in isolate 2161. Conversely, there are a number of ORFs in the LdMNPV-2161 genome sequence that either are not present or not listed in LdMNPV 5–6 (Table 3). LdMNPV-2161 ORFs 137 and 138 are missing from the 5–6 genome due to a deletion of the sequence containing all of 137 and half of 138 (Slavicek and

Table 3
LdMNPV-2161 ORFs not listed or not present in the LdMNPV 5–6 genome sequence.

2161 ORF	Position	aa	Promoter motifs[a]	Present in 5–6?	% identity (range)	Notes
34	37880 → 38125	81	–	No	–	Indel + frameshift in Cl5–6
52	53673 ← 53909	78	L	Yes; 55a	96.2% (75/78)	Homologs in other NPVs
74	70990 ← 71715	241	–	Yes; 74a	90.3% (84/93)	
123	118597 → 118767	56	–	No	–	Indel + frameshift in 5–6
124	118997 ← 119203	68	C	Yes	100% (67/67)	Entirely contained within 5–6 ORF121, which is missing from 2161
135	131670 → 132026	118	C	No	–	Indel + frameshift in 5–6
137	132186 → 132506	106	C, T	No	–	Homologs in other LdMNPV isolates and other NPVs
138 (*p24*)	132522 → 133193	223	L	No	–	Homologs in other LdMNPV isolates and other NPVs
141	134669 → 134938	89	L	Yes, 136a	92.1% (82/89)	
144	136653 → 136814	53	–	No	–	Indel + frameshift in 5–6
151	140942 ← 141292	116	–	No	–	Substitution creating stop codon in 5–6
157	146786 ← 146965	59	–	No	–	Indel + frameshift in 5–6
160	148165 ← 149001	278	C	Yes; 151a	98.8% (166/168)	Smaller version of ORF in 5–6
162	149125 → 149391	88	C	Yes; 152a	91.3% (63/69)	
166	153645 → 153866	73	C, L, T	Yes; 155a	100% (73/73)	Homologs in other NPVs

[a] Presence of following promoter motifs within 120 bp of the start codon: CA(G/T)T (C; cap site/initiator), TAAG (L; late gene promoter motif), TATAA (T; TATA box).

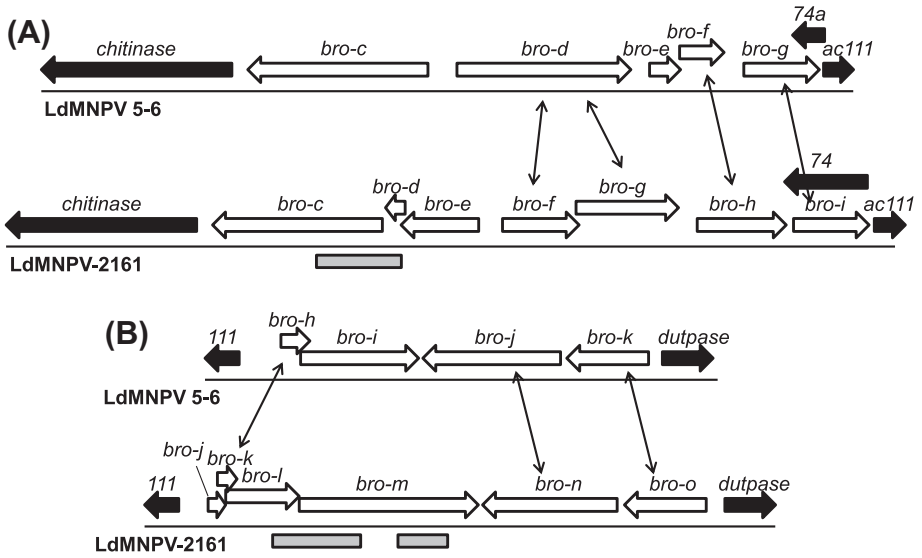

Fig. 3. Structure of the two regions in LdMNPV, bound by the *chitinase* and *ac111* homolog genes (A) and the LdMNPV ORF-111 and *dutpase* genes (B), which contain clusters of *baculovirus repeated ORF* genes. The positions and orientations of *bro* genes (open arrows) in the LdMNPV 5–6 and -2161 genome sequences are indicated. Lines with arrowheads at both ends indicate *bro* genes in the two genomes that appear to be orthologues. Gray boxes indicate the positions of large insertions in the LdMNPV-2161 genome relative to the LdMNPV 5–6 genome.

Hayes-Plazolles, 2003). This deletion accounts in part for the difference in the genome sizes of the two isolates. Homologs for ORF 137 occur in several group II alphabaculoviruses, while ORF 138, which encodes the capsid-associated protein P24 (Wolgamot et al., 1993), occurs in all alpha- and betabaculovirus genomes described to date (Garavaglia et al., 2012). Ten other LdMNPV isolates derived from Gypchek were also found to contain these two ORFs, indicating that their absence is unique to LdMNPV 5–6 (Slavicek and Hayes-Plazolles, 2003). LdMNPV-2161 ORFs 52, 74, 141, 160, 162, and 166 are also present in LdMNPV 5–6, but are not listed in the 5–6 ORF annotation. Two of these ORFs (52 and 166) have homologs in other NPVs.

The substitutions and indels that distinguish the genomes of variant genotypes and isolates of NPVs are often clustered around *hrs* and members of the *baculovirus repeated ORF* (*bro*) gene family (van Oers and Vlak, 2007). This was also found to be true for LdMNPV 5–6 and -2161 genomes. Although the two LdMNPV genome sequences possess *hrs* in homologous positions on their respective genomes, the numbers of unit repeats vary between the two isolates (Supplementary Table 2). Both LdMNPV 5–6 and -2161 have two clusters of *bro* genes, one cluster between the *chitinase* and ORF 75 (*ac111*) genes and the other between the ORF 111 and *dutpase* genes (Fig. 3). In the first cluster, there is an extra 760 bp (nt 66876–67635) in the LdMNPV-2161 genome that occurs within a region corresponding to LdMNPV 5–6 *bro-c* and contains part or all of three novel *bro* genes (*-c*, *-d*, and *-e*) in 2161 (Fig. 3A). This insert shares 87.1% nucleotide sequence identity with nt 64375–64939 of the *Lymantria xylina* nucleopolyhedrovirus-5 (LyxyNPV-5) genome sequence, which contains the 5′ end and upstream region of LyxyNPV-5 ORF 68, *bro-d* (Nai et al., 2010). Additional smaller indels and many substitutions also occur in this region. A similar array of indels and substitutions, including insertions of 789 bp (nt 110322–111110) and 441 bp (nt 111439–111879) in isolate 2161, resulted in a similar rearrangement of reading frames in the second cluster of LdMNPV *bro* genes (Fig. 3B). The first insertion in the second *bro* cluster shares 63.9% sequence identity with LdMNPV 5–6 nt 67630–68298, containing the 5′ end and upstream region of *bro-c* in the 5–6 genome. The second insertion shares 84.3% with LdMNPV 5–6 nt 66844–67172 and 78.9% with LyxyNPV-5 nt 63642–64507, containing part of LdMNPV 5–6 *bro-c* and parts of LyxyNPV-5 *bro-c* and *bro-d*, respectively.

Table 4
Dose–mortality response (polyhedra/mL) of neonate *L. dispar* larvae infected with LdMNPV isolates.

Isolate	LC_{50}[a] × 10^4 (95% CL)	Slope ± SEM	Heterogeneity (χ^2/n)
2161 (S. Korea)	7.99 (4.22–18.7)[a]	1.201 ± 0.119	2.17
3029 (Russia/Virin ENSh	2.72 (2.13–3.42)[b]	1.401 ± 0.123	0.66
3041 (Japan)	4.4 (2.54–7.84)[c]	1.191 ± 0.114	1.53
3049 (USA/Gypchek®)	6.55 (5.16–8.47)[a]	1.339 ± 0.125	0.38
Ab-a624 (USA)	2.47 (1.08–4.89)[b]	1.050 ± 0.107	1.97

[a] Values with different letters are significantly different as assessed by comparison of 95% confidence levels of lethal dose ratios (Robertson et al., 2007).

3.3. Biological activity of LdMNPV isolates against L. dispar larvae

Many of the virus samples caused mortality in neonate larvae of the *L. dispar* New Jersey Standard Strain when the samples were pipetted onto the surface of artificial diet where larvae were feeding. Second-, third-, and fourth-instar larvae infected with extracts from neonate cadavers died with symptoms of nuclear polyhedrosis, including flaccidity and rupturing of the cuticle. Infected third- and fourth-instar larvae almost invariably climbed to the top of the cups in which they were incubated and attached themselves to the paper lids prior to death.

In droplet feeding bioassays against neonate larvae, representative isolates from the European/North American and Asian groups killed larvae with LC_{50} values ranging from 2.47 × 10^4 polyhedra/mL to 7.99 × 10^4 polyhedra/mL (Table 4). Isolates Ab-a624 and 3029 (Virin-ENSh) killed larvae at significantly lower concentrations than the other isolates. Isolate 3029 also appeared to kill larvae more rapidly than the other isolates (data not shown). Isolates 2161 (S. Korea) and 3049 (Gypchek®) produced LC_{50} values at the high end of the observed range, while isolate 3041 (Japan) killed larvae with an intermediate LC_{50}.

4. Discussion

The virus isolate used to prepare Virin-ENSh was reportedly isolated from, and grown in, not larvae of *L. dispar*, but of the satin moth, *Leucoma salicis* (Lipa, 1998). This observation, along with the distinct DNA fragment patterns generated by restriction endonuclease digest of Virin-ENSh genomic DNA (Dougherty, 1983), raised the question of whether the Virin-ENSh virus isolate was a completely different virus and not a variant of the Hamden isolate of LdMNPV. In this study, sequence analysis of three loci from several *Lymantria* spp. virus samples produced evidence that the Virin-ENSh virus, as well as other NPVs from different regions of the world that infect *L. dispar*, are indeed variants of the same virus corresponding to the alphabaculovirus species *Lymantria dispar multiple nucleopolyhedrovirus*. The phylogenetic analysis based on a *lef-8* sequence alignment suggested that LdMNPV isolates from Europe and North America are closely related. This is consistent with a European origin for both the North American gypsy moth and NPVs from North American populations, although European and North American host gypsy moth populations can be distinguished on the basis of several genetic markers (deWaard et al., 2010; Keena et al., 2008). Isolates from different regions of Asia were found to be more divergent. The Asian isolates were likely extracted from populations of the *L. dispar asiatica* and *L. dispar japonica* subspecies of *L. dispar* (Pogue and Schaefer, 2007), which may account for their greater degree of divergence from the European/North American isolates and from each other. There were some geographically incongruous placements of a few of the isolates examined, including the occurrence of an isolate from Spain among the Asian isolates, and two isolates from Japan among the European/North American isolates. This observation is similar to a previous observation of *Cydia pomonella* granulovirus genotypes from one geographic region being discovered in larvae from a completely different geographic region (Eberle et al., 2009). While the *lef-8* locus exhibited sequence variation among the LdMNPV isolates examined, additional sequences from a less-conserved locus such as the mucin-like ORF (Supplementary Table 2) would likely provide a greater degree of information on the relationships among LdMNPV isolates.

Determination of the complete genomic sequence of LdMNPV-2161, a representative Asian isolate, revealed additional genetic variation. Despite a high degree of nucleotide sequence conservation, the genome sequences of isolates 2161 and 5–6 could be distinguished by numerous substitutions and indels that resulted in differences in ORF content, the most striking of which occurred in two clusters of *bro* genes (Fig. 3). In the first cluster of *bro* genes, the extra sequence occurring in the 2161 genome appears also to be present in LyxyNPV-5, suggesting that this sequence has been lost from the corresponding region of LdMNPV 5–6. In the second cluster of *bro* genes, the extra sequences appear to be a result of duplication of sequences originating from LdMNPV 5–6 *bro-c*. *Bro* genes are widespread among invertebrate viruses (Bideshi et al., 2003) and while characterization of the properties of some individual BRO proteins has been published (e.g. Kang et al., 2006, 2003), it is unclear what function they perform during baculovirus infection. Duplication and loss of *bro* genes among genotypic variants of baculoviruses appears to be commonplace (Harrison et al., 2012; Zhou et al., 2012).

In the original report of the LdMNPV 5–6 genome sequence in 1999, ORFs were selected for annotation only if their codon usage matched the codon usage of other large ORFs in LdMNPV 5–6 that were conserved among the small number of baculovirus genomes that had been sequenced at that time (Kuzio et al., 1999). The broader criterion used in this study identified additional ORFs, including a couple of ORFs present in other baculovirus genomes as well as a number of ORFs that appear to be unique to LdMNPV (Table 3). All reports of baculovirus genome sequences contain annotated ORFs that do not appear to be present in any other genome sequence, and the expression and biological significance of such ORFs are unknown. A recent comprehensive deep-sequencing study of transcripts produced during AcMNPV infection of insect cells in culture revealed that all but one annotated AcMNPV ORF was transcribed (Chen et al., 2013). Chen and co-workers also found a previously unknown degree of mRNA splicing and antisense mRNA synthesis during AcMNPV infection, suggesting the potential for additional coding capacity in NPV genomes.

In previous bioassays comparing the activities of Gypchek® and Virin-ENSh against *L. dispar* larvae, the virus isolates from these products killed *L. dispar* larvae with equivalent or slightly different LC_{50}s (Podgwaite et al., 2006; Shapiro, 1983). The Virin-ENSh isolate in our droplet-feeding bioassays killed neonate *L. dispar* larvae with an LC_{50} that was almost 3× lower than that of the Gypchek® isolate, a difference that was found to be statistically significant by lethal dose ratio analysis (Table 4). A Japanese isolate of LdMNPV (3041) also killed *L. dispar* larvae with a lower LC_{50} value than Gypchek®. In other studies, LdMNPV isolates from Japan tended to exhibit less pathogenicity against *L. dispar* larvae than other LdMNPV isolates, including isolates derived from Gypchek® (Duan et al., 2011; Ebling et al., 2004; Rollinson and Lewis, 1973; Shapiro, 1983). Finally, LdMNPV-2161 killed larvae with a significantly higher LC_{50} compared to LdMNPV-Ab-a624. A previous study comparing activities of a Korean isolate with Ab-a624 reported a similar trend (Narang et al., 2001). Sequence analysis of selected genes in different stocks of Gypchek® revealed the occurrence of non-synonymous substitutions in stocks from 1982, 1985, and

2005 (Zhang et al., 2010). The accrual of substitutions in different Gypchek® stocks and other isolates may have an impact on the relative performance of isolates from one bioassay to the next, and may explain differences in trends observed in bioassays involving Gypchek®. Differences in bioassay techniques, host strains, and non-Gypchek® LdMNPV isolates may also account for some of the differences in trends reported in past literature.

LdMNPV is an important tool in the control of gypsy moth outbreaks. This study adds to our knowledge of genetic diversity among isolates of LdMNPV and provides data that can be used for the evaluation and improvement of LdMNPV as a biological control agent. Genome sequencing of additional isolates examined in this study is expected to provide an even more detailed and comprehensive view of the genetic diversity among isolates of LdMNPV.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2013.12.005.

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