**The phylogenetic relationship and cross-infection of nucleopolyhedroviruses between the
invasive winter moth (*Operophtera brumata*) and its native congener,
Bruce spanworm (*O. bruceata*)**

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Abstract

Disease can affect biological invasions by acting as either a synergist or antagonist. Disease-mediated invasions have important implications for understanding the spread of invasive insects, which cost billions of dollars in damages annually. One such non-native, destructive insect is the winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), which causes defoliation and mortality of deciduous trees in its introduced range. In the northeastern United States, winter moth populations overlap with a native congener, Bruce spanworm, *Operophtera bruceata* Hulst. Nucleopolyhedrovirus (NPV), appears to be an important natural enemy in Bruce spanworm and there is some evidence that the NPV infection found in winter moth in the northeastern U.S. may originate from Bruce spanworm. By sequencing two viral genes (the polyhedrin and p74 genes) from field-collected larvae of both species, we found that the winter moth virus (OpbuNPV) is distinct from the virus from Bruce spanworm (OpbrNPV). However, the two viruses do constitute a clade within the Alphabaculovirus Group 2 NPVs, indicating that they are more similar to each other than they are to other lepidopteran viruses, even other geometrid-derived NPVs. As far as we know, this is the first report of sequences from an NPV from Bruce spanworm. Results from cross infection trials suggest that cross infection is uncommon if it occurs at all. Our results show that these two closely related species have distinct viruses and, unlike previous suggestions, Bruce spanworm virus is not mediating the winter moth invasion.

1. Introduction

The role of disease (pathogen or parasite infections) in biological invasions is increasingly of interest because of the multifaceted ways in which disease can mediate invasions. Disease-mediated invasions have recently been reviewed, indicating widespread and far-reaching effects, which can either facilitate or hinder invasions of vertebrate, invertebrate, and plant taxa (Strauss et al. 2012). Whether facilitating or hindering the invasion, the role of disease-mediated invasions has important implications for our understanding and management of invasive species, which damage forests and agricultural systems and incur large ecological and economic costs.

In North America, the European winter moth, *Operophtera brumata* L. (Lepidoptera: Geometridae), is one such non-native insect causing widespread defoliation in rural and urban settings. Winter moth was accidentally introduced to Nova Scotia in the 1950s (Embree 1966) and to the Pacific Northwest in the 1970s (Roland 1986). It was first noted in the northeastern United States in the 1990s, near Boston, MA (Elkinton et al. 2010). Since its introduction to the northeastern United States (New England), winter moth has been in almost continuous outbreak and has spread to coastal New Hampshire, Maine, Rhode Island, Connecticut, and New York (Elkinton et al. 2014). In Massachusetts, outbreak densities of 500 pupae/m² have resulted in 50% defoliation and significant damage to blueberry, cranberry, and apple crops (Elkinton et al. 2015). With repeated defoliation, winter moth has caused growth reduction and mortality of deciduous trees (Embree 1967, Simmons et al. 2014), resulting in far-reaching effects on forest and agricultural landscapes.

The outbreaks by winter moth were initially assumed to be caused by a native geometrid, such as Bruce spanworm (*Operophtera bruceata*, Hulst) (Elkinton et al. 2010). Bruce spanworm was suspected because it is prone to outbreaks (though relatively localized and short-lived outbreaks), is indigenous to the area (Brown 1962), and is difficult to distinguish morphologically from winter moth. Additionally, Bruce spanworm produce and respond to the same pheromone compound as winter moth (Roelofs et al. 1982) and can hybridize with winter moth (Troubridge and Fitzpatrick 1993, Elkinton et al. 2010, Elkinton et al. 2014). The cause of the widespread and persistent defoliation in Massachusetts was suspected to be winter moth because it remained in continuous outbreak levels each year rather than subsiding due to natural enemies as Bruce spanworm population tend to do. Upon close examination of adult females and subsequent DNA analyses, the outbreaks were definitely identified as being caused by winter moth in December of 2003 (Elkinton et al. 2010). The close relationship but differences in population dynamics between these two species can provide insight into possible biological control methods for winter moth.

Baculoviruses, most notably nucleopolyhedroviruses (NPVs), are important natural enemies of many Lepidoptera and appear to be particularly important in species whose populations undergo cycles of outbreak and collapse (Cory 2010, Myers and Cory 2013). They may play a vital role in the suppression of outbreak populations in Bruce spanworm. Bruce spanworm populations rarely exhibit outbreaks, and when a Bruce spanworm outbreak does occur, it is usually localized and only lasts for a few years before the population collapses (Brown 1962, Rose and Lindquist 1997). In Bruce spanworm outbreaks that have been reported, NPVs have been noted. Smirnoff (1964) reported finding NPVs in Bruce spanworm collected in Quebec, Canada in 1961 and

suggested that NPV infection was responsible for the successful control of an outbreak of Bruce spanworm on maple trees. Similarly, a Bruce spanworm outbreak at the same time in Nova Scotia was found to have NPV, and NPV was thought to have caused the decline of this population (Neilson 1965). A decade later in Alberta, Canada, an outbreak of Bruce spanworm was also suppressed by an NPV epizootic. In this outbreak, while trees were sprayed with NPV derived from the caterpillars collected from the Alberta population the previous year, control plots also experienced significant mortality from the virus and had a prevalence of up to 77% percent of larvae (Ives and Cunningham 1980), indicating high levels of NPV naturally occurring at this site.

Winter moth, on the other hand, is known to continuously outbreak in its introduced range (Embree 1965, Roland 1990, Elkinton et al. 2015) and an NPV epizootic has never been noted. Prior to the introduction of parasitoids as biological control agents in Nova Scotia and British Columbia, winter moth persisted at outbreak levels (Embree 1965, Roland 1990), and while the biocontrol agent builds up in New England, populations remain high (Elkinton et al. 2015). Following the introduction of winter moth to Nova Scotia, Neilson (1965) conducted surveys of winter moth in that province from 1955 to 1960 and none were found to be infected with NPV. In 1961, a single larva was found with NPV-like symptoms. Similarly, Cunningham et al. (1981) surveyed winter moth larvae in British Columbia and found low (0 to 1%) incidences of naturally occurring NPV. Further, in the most recent North American invasion of winter moth in Massachusetts, only low levels of NPV have been detected, and no viral epizootic has been noted (Burand et al. 2011, Elkinton et al. 2015).

On the other hand, Neilson (1965) reported substantially more NPV-infected individuals and more mortality in his survey plots in Nova Scotia in the years following the 1960 survey. This decline of winter moth occurred simultaneously and sympatrically with a Bruce spanworm epizootic, which he speculated might have crossed over into winter moth (Neilson 1965, Murdoch et al. 1985). Nielson (1965) found, however, that the occlusion bodies isolated from the two species were different in shape. He called for further research to determine if they were the same virus and if they cross-infect. Gillespie et al. (1978) inadvertently tried cross-infection trials on an outbreak of geometrids was reported on southern Vancouver Island, B.C., that was thought to be Bruce spanworm. They tried to infect caterpillars from this outbreak with Bruce spanworm virus. Infection was not successful and it was later confirmed that the insect outbreaks were of winter moth and not Bruce spanworm. This suggested that cross infection does not occur, but when this work was done, it was not known if the Bruce spanworm virus stock was still infectious.

Identifying the NPV infecting Bruce spanworm, and evaluating the relationship of this virus to winter moth's NPV and others', are necessary steps towards understanding the role that disease plays in the current winter moth invasion as well as the earlier Nova Scotia invasion. We need to know if disease from Bruce spanworm is mediating the winter moth invasion or, alternately, if disease from winter moth can harm Bruce spanworm. Cross infection, as was proposed by Nielson (1965) and piloted inadvertently by Gillespie et al. (1978), warrants careful testing. Additionally, we now have molecular techniques to help elucidate the relationship between the NPV found in Bruce spanworm and that found in winter moth.

We build on these earlier observations to address two questions: 1) What is the phylogenetic relationship between the NPV infecting Bruce spanworm and the NPV infecting winter moth, and 2) in a laboratory setting, can Bruce spanworm-derived NPV infect winter moth and can winter moth-derived NPV infect Bruce spanworm.

2. Materials and methods

2.1 Collecting larvae and rearing

To acquire virus samples and live specimens to rear eggs from, Bruce spanworm and winter moth larvae were collected from the field in late May of 2013 and 2014. The larvae were reared to adulthood, and mated to produce eggs used the following springs (2014 and 2015) for cross infection trials. Any mortality (cadavers) from the collections was noted and inventoried for disease prevalence. In 2013, Bruce spanworm larvae were collected from a small outbreak population near Millinocket, Maine (Township 2, Region 8 NWP). This is 150 km inland of the area where winter moth has been detected in Maine and in an area that only has Bruce spanworm (Elkinton et al. 2010). In 2014, Bruce spanworm larvae were collected in the Mohawk Trail State Forest, Charlemont, MA, which is 60 km from any detected winter moth. Both years, winter moth larvae were collected as fourth and fifth instars in long-term study sites in eastern Massachusetts, where winter moth is abundant and pure winter moth make up almost 100% of the genetic stock (Elkinton et al. 2010; Elkinton et al 2014). Larvae were reared in batches of 500 or fewer in ventilated 20 L (5 gallon) buckets with the foliage from the tree species on which they were found. Larvae collected from different sites were reared in separate buckets; in this

way winter moth larvae were reared separately from Bruce spanworm larvae. The foliage was mounted in Oasis Floral Foam bricks (Smithers-Oasis North America). Foliage was replaced every other day and any cadavers were removed. Larvae were reared for a week or less, which is not enough time for any NPV transmitted within the buckets to spread between individuals (Wigley 1976). Any larval cadavers that were found were placed individually in sterile 1.5 mL microcentrifuge tubes (Fisherbrand) and immediately frozen at -20 °C. When the larvae started to show signs of pupating (thickening body shape and rolling a leaf edge over themselves), sifted peat moss was added to the bottom of the buckets for pupation. The resulting pupae were sifted, sorted by sex, and stored at 10-12°C until adult emergence at the end of October to beginning of November.

To produce eggs for the cross infection trials, adult moths were mated with their conspecifics. Batches of approximately 50 adults were placed together in clear, 350 mL (12-ounce) cups lined with paper, in an incubator (Percival) set to 10-12 °C with a 13.5/10.5 h LD cycle. Females laid eggs on the paper lining of the mating cups. The temperature in the incubator with the eggs was reduced gradually to 2 °C by mid-January.

2.2 DNA amplification and sequencing

Larval cadavers that had been set aside from the rearing process and stored frozen were thawed and homogenized with 200 uL molecular grade water. An aliquot from each individual was screened for NPV using light microscopy. DNA was extracted from potentially NPV-infected

cadavers using 250 uL DNAzol (Invitrogen Life Technologies), 50 uL sample, (cadaver homogenized with 200uL molecular grade water), and an additional 50 uL water.

DNA was amplified from two different loci—the polyhedrin gene region (polh, associated with the envelope protein) and the p74 gene region (a *per os* infectivity factor gene, associated with the virus envelope). To amplify the polyhedrin gene of NPV from Bruce spanworm, the primer set Polyh-81F/Polyh-249R, a generalized NPV polyhedrin primer, was used. Initially, the primer set ObPol1 and ObPol2, designed to detect winter moth NPV (Graham et al. 2004) was tried, but it was not successful in amplifying the Bruce spanworm NPV. For PCR using Polyh-81F/Polhy249R, a reaction of 12.5 uL Taq 2X Master Mix (New England Biolands) with 5.5 uL RNase free water, 1.5 uL MgCl₂, and 0.5 uL of each primer was mixed with 4 uL DNA template for each sample. Reactions were run on an Eppendorf Mastercycler ep. using the following temperature profile: 95°C for 3 min; 36 cycles of 95°C for 30 sec, 49.4°C for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min.

The resulting PCR product was run on an agarose gel, as described in Burand et al (2011), and the resulting NPV DNA fragments from four Bruce spanworm individuals from the 2013 Millinocket, ME collection (sample number #7 and #8) were excised from the gel, purified (Qiagen gel purification kit), and pooled to render enough DNA for sequencing. PCR products were Sanger sequenced at the University of Massachusetts Genomic Resource Laboratory on ABI Model 3130XL and 3100 sequencers yielding clean (>60HQ%) sequences. The sequence pairs (forward and reverse-complement) were aligned and edited using Geneious 8.1.8 (Biomatters Ltd.) to produce a consensus sequence.

For the p74 gene, DNA was extracted and amplified from Bruce spanworm and winter moth cadavers collected in 2013 using generalized p74 primers for Bruce spanworm and winter moth specific p74 primers (Supplementary Table S1) using the following temperature profile: 95°C for 3 min; 36 cycles of 95°C for 30 sec, the primer specific annealing temperature (50.8°C for winter moth and 52.1°C for Bruce spanworm) for 1 min, and 72°C for 1 min; and a final extension of 72°C for 10 min. The resulting PCR product was run on an agarose gel and the resulting DNA fragments were excised from the gel, re-amplified using the p74 PCR procedure described above, and sequenced, as was described for the polyhedrin gene. To extend the Bruce spanworm sequence, a new primer (Supplementary Table S1) was designed using NCBI Primer BLAST to acquire the flanking sequences at the leading and trailing ends of the read. These new primers were used with the same PCR protocol described above to amplify the sequence ends for the Bruce spanworm DNA extraction.

2.3 Polyhedrin and p74 gene comparisons

The resulting polyhedrin sequence for Bruce spanworm and p74 sequences for both species were aligned with published sequences acquired from GenBank (Table 1). The sequence previously submitted by Burand et al. 2011 (accession no. HQ663848.1) was used for the winter moth polyhedrin gene comparison. The sequences were aligned using Geneious 8.1.8 (Kearse et al. 2012), adjusted by eye, and then truncated to the length of the shortest included gene sequence. JModelTest was used in the CIPRES Science Gateway (Miller et al. 2010) to select the best base-pair substitution model for each loci. HKY was selected as the best substitution model for

analysis of the polyhedrin gene fragment and GTR was selected as the best model for p74. To infer the phylogenetic relationships, we conducted neighbor-joining, Maximum Likelihood, and Bayesian analyses. Neighbor-joining analyses were implemented in Geneious (Kearse et al. 2012) using a Jukes-Cantor genetic distance model for both genes and 1000 bootstrap replication. Maximum likelihood analyses were run using PhyML (Guindon et al. 2010) using the designated substitution model for each locus. Support for each node was estimated using 1000 bootstrap replication. Bayesian analyses were run using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001) with the designated substitution model for each locus. A MCMC chain of 1,000,000 was used with a burn in length of 10%. MabrNPV (accession no. JQ798165.1), with the lowest percent pairwise identity with our sequences, was used as the outgroup for both trees. The computed output were visualized using FigTree Version 1.4.2 (Rambaut 2014).

2.4 Cross infection trials

To test for the possibility of cross infection, in spring 2014 and 2015 larvae of both winter moth and Bruce spanworm were exposed to virus from the other species. We ran four different combinations: (1) Bruce spanworm larvae exposed to Bruce spanworm virus, (2) Bruce spanworm exposed to winter moth virus, (3) winter moth exposed to Bruce spanworm virus, and (4) winter moth exposed to winter moth virus. Controls, with the larvae not receiving a virus inoculum, were also run for each larval type. Replicates were not balanced across treatments due to differences in rearing outcomes.

Batches of eggs, still on the paper lining of the mating cups, were surface-sterilized using a 2% bleach solution wash, followed by three rinses with distilled water, then allowed to air dry. The paper with the eggs was stapled to the inside of surface of the diet cup lid. A standard, high wheat germ gypsy moth diet was used (Bell et al. 1981). The diet was made at the USDA Animal and Plant Health Inspection Service lab at Otis ANGB, MA. The eggs and diet were moved from 2 °C storage to a 15°C incubator to temperature-induce hatch. The neonates were allowed to feed for 8-12 days with diet refreshed once during this time. When they reached their third instar (body ~1 cm long, head capsule 2 mm wide), individual larvae were placed singly in 1.5 mL Eppendorf microcentrifuge tubes and starved overnight. After starvation, the caterpillars were given a 0.5 cm³ cube of the same gypsy moth diet formulation treated with 8 µl of dilute NPV inoculum—the maximum volume the diet cube could absorb.

The inoculum was prepared by combining 3-10 NPV-positive larval cadavers with 500 uL dH₂O, crushing with a sterile pestle, and allowing to settle overnight. The top layer was removed so that only the NPV concentrate at the bottom of the tube remained, and an additional 500 uL dH₂O was added. Separate inoculum solutions were made from infected winter moth cadavers and from infected Bruce cadavers. The resulting occlusion body (OB) concentrations were counted using a hemocytometer (American Optical Spencer Bright-Line, 1/10 mm deep) and the resulting concentration was found to be approximately 2.0×10^8 OB/mL for the winter moth inoculum and 1.0×10^8 OB/mL for the Bruce spanworm inoculum. The winter moth inoculum was diluted to 1.0×10^8 OB/mL and stored at 4°C.

Treated larvae were stored at 22°C and monitored daily. If the infected diet was completely consumed, additional non-infected diet was added as needed. Any dead caterpillars were removed and immediately frozen at -20 C. A subset of the resulting larval cadavers were tested for NPV using PCR and gel electrophoresis as described above. Prior to DNA extraction from the larval cadavers, the surface of the larvae were rinsed three times with distilled water to remove any superficial virus particles, such that only internal virus would be detected.

3. Results

3.1 Polyhedrin and p74 gene phylogenetic comparisons

Larval cadavers were recovered from both winter moth and Bruce spanworm field collections with a higher percent mortality evident in both years from Bruce spanworm (Table 2). Using light microscopy on the recovered cadavers, occlusion bodies characteristic of NPV were found in both the winter moth samples as well as the Bruce spanworm. No noticeable difference in the shape of the occlusion body was noted. The polyhedrin gene segment from Bruce spanworm (OpbrNPV_polh) had only an 83.0% sequence similarity to that of the Massachusetts winter moth (OpbuNPV_polh) strain (Supplementary Table S2a). However, the OpbuNPV and OpbrNPV sequences were more similar to each other than to the gene sequences of viruses from the other lepidopteran species included in the gene tree (Fig. 1A). Aligned p74 sequences for winter moth (OpbuNPV_p74) and Bruce spanworm (OpbrNPV_p74) were only 81.2% similar to one another (Supplementary Table S2b), but when considered with other Lepidoptera NPVs, also

separated into their own clade (Fig. 1B). For both loci, OpbuNPV and OpbrNPV fell out with the Group II Alphabaculoviruses.

3.2 Cross infection trials

In the cross infection trials, the Bruce spanworm virus (OpbrNPV) was able to infect Bruce spanworm larvae (26.7% infected), but was unable to infect winter moth larvae (0%; Table 3). Similarly, the winter moth virus (OpbuNPV) was able to infect winter moth larvae (41.1% infection), but was unable to infect Bruce spanworm larvae (0%). In a few cases (2 of 86 tested; 2.3%), when winter moth larvae were exposed to Bruce spanworm virus the larvae appeared to die from winter moth NPV infection, as determined by sequencing the NPV in the resulting cadavers. When Bruce spanworm larvae were inoculated with the same Bruce spanworm virus (OpbrNPV), no winter moth virus (OpbuNPV) was detected in the resulting cadavers. No virus was detected in the controls.

4. Discussion

The winter moth and Bruce spanworm have distinct NPV species, but the NPV are more closely related to each other than to viruses taken from other host species, including other Geometridae. The ObPol1/ObPol2 primer set, designed for winter moth, did not amplify Bruce spanworm virus DNA while the general polyhedrin primer, Polyh-81F/Polyh-249R, amplified Bruce spanworm virus DNA, but not that of winter moth. This was the first indication that the two sequences were distinct. The closest match to the resulting Bruce spanworm NPV polyhedrin

sequence on Genbank was that of Massachusetts winter moth NPV strain (OpbuNPV_polhMA) as published by Burand et al. (2011). When compared to sequences obtained from a United Kingdom winter moth also published in Burand et al. (2011), the match was just slightly less similar. However, when compared to other Lepidoptera NPV sequences, including other NPVs derived from geometrids (*Ectropis obliqua*, *Buzura suppressaria*, and *Lambdina fiscellaria*), OpbuNPV matched OpbrNPV more closely than any other sequence. The overarching phylogenetic relationships of the viruses between the two trees are slightly different, but in both trees the winter moth and Bruce spanworm NPVs formed part of a clade within the Alphabaculovirus Group 2 sequences. Not surprisingly, the relationships are more resolved in the p74 tree than in the polh tree. The polh region has been traditionally used for simple molecular identification and has many published sequences available for comparison; however, its utility in resolving phylogenetic relationships has been questioned (Lange et al. 2004; van Oers et al. 2004). The p74 gene has been shown to be conserved and highly host specific, thus providing a more reliable phylogenetic tree (Haas-Stapleton et al. 2004). Overall, the trees show that the NPVs infecting these two congeners are closely related Group 2 Alphabaculoviruses, but they have distinct viral lineages.

Previous studies examining cross-infectivity have indicated that the host range of NPVs is highly variable. Many tend to have narrow host ranges, confined to a single species or family, but a few (e.g AfMNPV and AcMNPV) have broad host ranges extending across multiple families of Lepidoptera (Hostetter and Puttler 1991, Cory and Myers 2003). Our cross infection trials show that despite the close phylogenetic relationship between Bruce spanworm and winter moth and the parallel relationship between their respective NPVs, the viruses cannot cross-infect and are

specialized on a single host species. We know that the virus inoculum was viable; when Bruce spanworm larvae were given NPV derived from Bruce spanworm, some of the Bruce spanworm cadavers showed detectable levels of Bruce spanworm NPV. Similarly, when winter moth larvae were given winter moth derived NPV, some of the winter moth cadavers showed detectable levels of winter moth NPV. This shows that, contrary to previous suggestions (Neilson 1965), winter moth does not appear to be vulnerable to Bruce spanworm NPV and thus, winter moth's invasion is not likely mediated by Bruce spanworm's virus. This also means that if winter moth virus were to be considered as a microbial control in North America, it is unlikely to affect Bruce spanworm populations.

Interestingly, some winter moth NPV was detected when winter moth was exposed to Bruce spanworm NPV. Covert infections can be triggered and can switch to overt infections (Murillo et al. 2011, Cory 2015). It is possible that when the winter moth larvae were exposed to the Bruce spanworm virus, this stress served as the trigger inducing an overt expression of the previously covert (latent) OpbuNPV infection in the eggs. Similar results have been found in other lepidopteran NPV studies (Jurkovicova 1979, McKinley et al. 1981, Hughes et al. 1993; Cooper et al. 2003); these studies show that a host's covert infection can be activated when exposed to a similar but heterologous NPV. It is also possible that our results reflect contamination by the winter moth virus either in the egg sterilization process, during larval feeding, or in the inoculum; however, this is unlikely as no control samples showed contamination and when Bruce spanworm larvae were given the same inoculum, no winter moth NPV was detected any the resulting cadavers.

To compare the viruses for this study, winter moth and Bruce spanworm larvae were collected from the field. As was found in other studies (Elkinton et al. 2015), winter moth was abundant, easy to find across eastern Massachusetts, and maintained high density populations year to year. On the other hand, Bruce spanworm larvae could only be found in small, isolated populations which varied year to year. The Bruce spanworm collection site in Maine used in 2013 was checked the following year (2014) and Bruce spanworm densities were found to have declined precipitously, likely due to an epizootic (Brown 1962, Rose and Lindquist 1997). Similarly, in our rearing of field-collected larvae, Bruce spanworm experienced more mortality than winter moth. These observations reflect the difference in population dynamics between the two species.

While Bruce spanworm larvae experienced higher mortality rates, there was no notable trend in the percent of cadavers with NPV between the two species. The low rates of NPV infection in our winter moth larvae field collections are consistent with those found in other studies both in the introduced and native range of winter moth. In New England, where the current study was conducted, comparable incidences of larvae with NPV have been noted (Burand et al. 2011, O'Donnell 2015). In prior introductions in Nova Scotia and British Columbia, disease was also not noted as an important source of mortality in winter moth (Cunningham et al. 1981, Roland and Embree 1995). In its native range, winter moth experiences NPV, but historically at low prevalence levels and seemingly without significant effects on its population dynamics (Feeny 1970, Varley et al. 1973, Wigley 1976). NPV caused mortality above 50% only in outbreaks of winter moth on heather in Orkney, U.K. (Graham et al. 2004) likely due to suboptimal food quality, which has been shown to correlate with incidences of NPV (Raymond et al. 2005, Raymond and Hails 2007). For Bruce spanworm, the high larval mortality, but relatively low

NPV infection may be because there is another dominant source of mortality in Bruce spanworm.

Overall, our results do not support the suggestion that previously reported epizootics of NPV in winter moth in Canada originated from Bruce spanworm. Similarly, in Massachusetts, Bruce spanworm virus is likely not mediating the winter moth invasion. However, it appears that Bruce spanworm larvae experience more mortality than winter moth larvae, thus further work should be conducted to study the role other pathogens and parasites on the populations of these two species.

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Figure Legend

Figure 1. Phylogenetic tree of the (A) NPV polyhedrin gene nucleotides and (B) p74 envelope protein gene. The sequences acquired from winter moth (OpbuNPV) are shown in red and those from Bruce spanworm (OpbrNPV) are shown in blue. Node support values are Bayesian posterior probability (probability out of 1) with Neighbor Joining/Maximum likelihood support (support out of 100) below.

Table 1. Lepidoptera species used to construct the NPV phylogenetic estimates

Virus Genus	Virus	Virus Host	Host Common Name	Host Family	p74 Accession No.	Portion from	Polyhedron Accession No.	Portion from
Alphabaculovirus Group I	AgMNPV	<i>Anticarsia gemmatalis</i>	Velvetbean moth	Noctuidae	DQ813662.2	complete genome	KR815463.1	complete genome
Alphabaculovirus Group I	AcMNPV	<i>Autographa californica</i>	Alfalfa looper	Noctuidae	M31301.1	complete cds	JN674785.1	partial cds
Alphabaculovirus Group I	BomaNPV	<i>Bombyx mandarina</i>	Wild silkmoth	Bombycidae	JQ071499.1	complete genome	DQ483053.1	complete cds
Alphabaculovirus Group I	BmNPV	<i>Bombyx mori</i>	Domesticated silkmoth	Bombycidae	L33180.1	complete genome	KR139829.1	complete cds
Alphabaculovirus Group I	CfMNPV	<i>Choristoneura fumiferana</i>	Eastern spruce budworm	Tortricidae	M97904.1	complete cds	AY327402.2	complete cds
Alphabaculovirus Group I	HycuNPV	<i>Hyphantria cunea</i>	Fall webworm	Arctiidae	AP009046.1	complete genome	AF300872.1	partial cds
Alphabaculovirus Group I	PlxyMNPV	<i>Plutella xylostella</i>	Diamondback moth	Plutellidae	DQ457003.1	complete genome	DQ457003.1	complete genome
Alphabaculovirus Group II	AdhoNPV	<i>Adoxophyes honmai</i>	Summer fruit tortrix	Tortricidae	AP006270.1	complete genome	AP006270.1	complete genome
Alphabaculovirus Group II	EcobNPV	<i>Ectropis obliqua</i>	Tea looper	Geometridae	DQ837165.1	complete genome	KC960018.1	complete genome
Alphabaculovirus Group II	BusuNPV	<i>Buzura suppressaria</i>	Tung oil geometrid	Geometridae	KM986882.1	complete genome	KM986882.1	complete genome
Alphabaculovirus Group II	LafiNPV	<i>Lambdina fiscellaria</i>	Hemlock looper	Geometridae	KP752043.1	complete genome	KP752043.1	complete genome
Alphabaculovirus Group II	LdMNPV	<i>Lymantria dispar</i>	Gypsy moth	Erebidae	AF081810.1	complete genome	AF499687.1	complete cds
Alphabaculovirus Group II	LyxyNPV	<i>Lymantria xylina</i>	Casuarina tussock moth	Lymantriidae	GQ202541.1	complete genome	GQ202541.1	complete genome
Alphabaculovirus Group II*	OpbuNPV	<i>Operophtera brumata</i>	Winter moth	Geometridae	KY064006	partial cds	HQ663848	partial cds
Alphabaculovirus Group II*	OpbrNPV	<i>Operophtera bruceata</i>	Bruce spanworm	Geometridae	KY064005	partial cds	KY064007	partial cds

Alphabaculovirus Group II	SeMNPV	<i>Spodoptera exigua</i>	Beet armyworm	Noctuidae	HG425347.2	complete genome	GQ392064.1	partial cds
Alphabaculovirus Group II	SfMNPV	<i>Spodoptera frugiperda</i>	Fall armyworm	Noctuidae	EF035042.2	complete genome	KC845532.1	partial cds
Alphabaculovirus Group II	TnSNPV	<i>Trichoplusia ni</i>	Cabbage looper	Noctuidae	DQ017380.1	complete genome	JN674704.1	partial cds
Alphabaculovirus Group II	MabrNPV	<i>Mamestra brassicae</i>	Cabbage moth	Noctuidae	JQ798165.1	complete genome	AB198073.1	complete cds

*Suggested placement

Table 2. Larval collections and mortality

Species	Year	Collection Site	Number of larvae collected	Larval cadavers recovered	Percent mortality	Number of cadavers with NPV	Percent of cadavers with NPV
Bruce spanworm	2013	Millinocket, ME	433	173	40.0	8	4.6
Winter moth	2013	Across Massachusetts Sites	15677	203	1.3	56	27.6
Bruce spanworm	2014	Mohawk Trail, Charlemont, MA	170	25	14.7	5	20.0
Winter moth	2014	Across Massachusetts Sites	17347	600	3.5	198	33.0

Table 3. Cross-infection trials larvae infection results

Larvae exposed	Treatment	No. Larvae (N)	% shown to have OpbuNPV	% shown to have OpbrNPV
Bruce spanworm	OpbrNPV	15	0.0	26.7
	OpbuNPV	7	0.0	0.0
	Control	27	0.0	0.0
Winter moth	OpbrNPV	245	2.3	0.0
	OpbuNPV	140	41.1	0.0
	Control	8	0.0	0.0

OpbrNPV = virus from Bruce spanworm; OpbuNPV = virus from winter moth

Figure 1

Is **winter moth**, an invasive, affected by a NPV from, its native congener, **Bruce spanworm**?

Larvae exposed	Treatment	No. Larvae (N)	% shown to have	% shown to have
			OpbuNPV	OpbrNPV
Bruce spanworm	OpbrNPV	15	0.0	26.7
	OpbuNPV	7	0.0	0.0
	Control	27	0.0	0.0
Winter moth	OpbrNPV	245	2.3	0.0
	OpbuNPV	140	41.1	0.0
	Control	8	0.0	0.0

The winter moth virus (**OpbuNPV**) is distinct from the virus from Bruce spanworm (**OpbrNPV**), but they share a clade.

Cross infection trials suggest that cross infection is uncommon if it occurs at all.

Results shows that these two closely related species have distinct viruses and, unlike previous suggestions, **Bruce spanworm** virus is not mediating the **winter moth** invasion.