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Short Communication

High levels of genetic diversity in *Spodoptera exempta* NPV from Tanzania

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ABSTRACT

The African armyworm, *Spodoptera exempta*, is a major pest in sub-Saharan Africa. A nucleopolyhedrovirus (NPV) is often recorded in later population outbreaks and can cause very high levels of mortality. Research has been addressing whether this NPV can be developed into a strategic biological control agent. As part of this study, the variation in natural populations of NPV is being studied. An isolate of *S. exempta* NPV was cloned *in vivo* and found to contain at least 17 genetically-distinct genotypes. These genotypes varied in size from approximately 115 to 153 kb.

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1. Introduction

The African Armyworm, *Spodoptera exempta* (Walker) (Lepidoptera: Noctuidae) is an episodic migratory pest of the Old World tropics but is most prevalent in sub-Saharan Africa, especially on the eastern half of the continent. During almost annual population outbreaks, *S. exempta* larvae can devastate large areas of rangeland and graminaceous crops (Haggis, 1987) and are capable of achieving densities of 200–1000 larvae per m^2 (Rose et al., 2000; Grzywacz et al., 2008). The limited availability and prohibitive cost of effective chemical control measures means that subsistence farmers can do little to counter this rampant pest in outbreak years (Njuki et al., 2004), although larvae at the end of the outbreak season are often killed in large numbers by an NPV (Rose et al., 2000). The development of *S. exempta* NPV (*Spex*NPV) into a biological control agent may offer a viable control option. *Spex*NPV is a specific and extremely pathogenic natural mortality agent of *S. exempta*, which has shown considerable potential in field spray-trials carried out in northern Tanzania (Grzywacz et al., 2008). To support this work, a basic understanding of the diversity and genetic composition of *Spex*NPV is required. Briefly, we report on the isolation of individual *Spex*NPV genotypes and their genetic characterisation using Restriction Fragment Length Polymorphism (RFLP) profiling. The approximate size of individual genomes is estimated and the phylogenetic relationship between genotypes is also investigated.

2. Materials and methods

The *Spex*NPV isolate was collected in 1972 from *S. exempta* populations in Tanzania, amplified *in vivo* and stored at −20 °C. Restriction endonuclease (REN) analysis of its DNA suggested the presence of multiple genotypes. *Spex*NPV is a multiple nucleopolyhedrovirus (MNPV) that can routinely package numerous genotypes within a single occlusion body (OB). *In vivo* cloning was undertaken to isolate the individual genotypes and was chosen over *in vitro* methodologies to avoid the introduction of artificial selection pressures. Smith and Crook (1988) developed *in vivo* cloning as a simple technique to isolate genotypes from mixed populations of baculoviruses. Slight modifications to their original technique have allowed the successful *in vivo* cloning of genotypes from *Spodoptera exigua* NPV (*Se*NPV) (Muñoz and Caballero, 2000) and *Panolis flammea* NPV (Cory et al., 2005) populations. *In vivo* cloning involves the serial infection of larvae using low viral doses until mortality is assumed to have initiated from a single virus genotype. Individual genetically-distinct isolates are provisionally identified by a lack of sub-molar bands in their REN profiles. The purity of suspected single-genotype isolates can be confirmed through the stability of REN patterns through additional rounds of infection. *In vivo* cloning involved the infection of 600 newly-

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Table 1
Mean size (kb) of EcoRV, BamHI and XhoI fragments of *S. exempta* NPV estimated from a minimum number of three independently run agarose gels.

REN fragments	EcoRV	BamHI	XhoI
A	18.56	17.87	22.50
B	16.01	16.54	21.4
C	14.38	15.2	18.74
D	10.53	14.05	16.21
E	9.57	12.82	7.3
F	8.4	11.53	5.47
G	8.25	10.18	5.08
H	7.96	9.20	4.63
I	7.00	8.53	4.26
J	5.80	8.1	3.43
K	5.25	7.56	3.25
L	5.12	6.58	3.01
M	4.78	5.63	2.96
N	4.34	4.46	2.46
O	3.96	2.98	1.94
P	3.6	2.31	1.83
Q	2.5	1.68	1.03
R	2.36	1.55	0.94
S	2.32		
T	2.20		
U	2.2		
V	2.1		
W	2.07		
X	1.86		
Y	1.67		
Z	1.66		
a	1.33		
b	0.84		
c	0.61		

moulted 3rd instar *S. exempta* larvae in the first round and batches of 35 larvae in subsequent rounds, with a viral dose of 1200 OBs (LD_{10} estimated from Reeson et al., 1998), using the diet-plug contamination method. The *S. exempta* larvae used for cloning came from a culture maintained on a wheatgerm-based semi-synthetic diet at the NERC Centre for Ecology and Hydrology, Oxford. The insects were originally collected from Tanzania in 1996 (Vilaplana et al., 2010). A total mortality of 18% was achieved from the initial round of infection from which 37% of the cadavers were characterised with EcoRV. After two rounds of cloning, 18 genetically distinct single-genotype isolates had been identified, which remained stable through a third *in vivo* passage. The profile of one of the isolates produced sub-molar bands when BamHI and XhoI were introduced into the screen at this point and was therefore discarded. A fourth and final *in vivo* passage of the remaining 17 single genotypes confirmed their stability. The *S. exempta* culture used for *in vivo* cloning was known to support a high level of covert infection (Vilaplana et al., 2010); however, the profiles of the *Spex*NPV clones did not change during passage, indicating that expression of the covert virus was not an issue. A minimum number of three gels per enzyme (XhoI, BamHI and EcoRV) were used to estimate fragment sizes and total genome size (Table 1).

In order to investigate the phylogeny of baculovirus species with no existing sequence information, one approach that has proved successful is to use a concatenated sequence from just a few phylogenetically informative genes (Herniou et al., 2004; Lange et al., 2004; Jehle et al., 2006). For this study, four different genes were selected for their proven phylogenetic potential: (1) the highly-conserved *polh* gene, encoding the OB protein (Zanotto

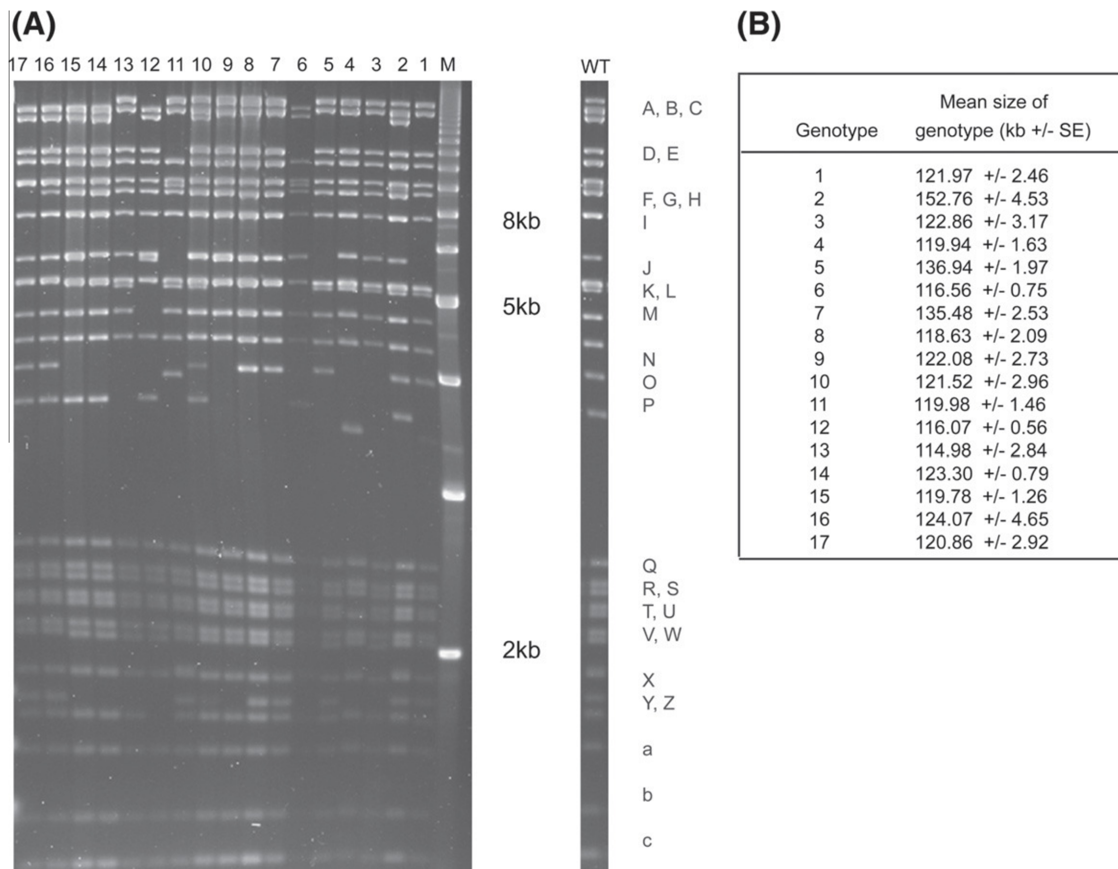


Fig. 1. Restriction endonuclease (REN) analysis used for the estimation of the genome size of *in vivo* cloned *S. exempta* NPV genotypes. (A) EcoRV profiles of 17 genetically distinct, *in vivo* cloned, *Spex*NPV genotypes and wild-type *Spex*NPV fragments, resulting from digestion with EcoRV, named alphabetically, largest to smallest (Vlak and Smith, 1982); (B) mean genome size estimates for the 17 genotypes (± 1 SE) resulting from REN analysis with three different enzymes namely EcoRV, BamHI and XhoI. All fragments sized from a minimum of three separate agarose gels.

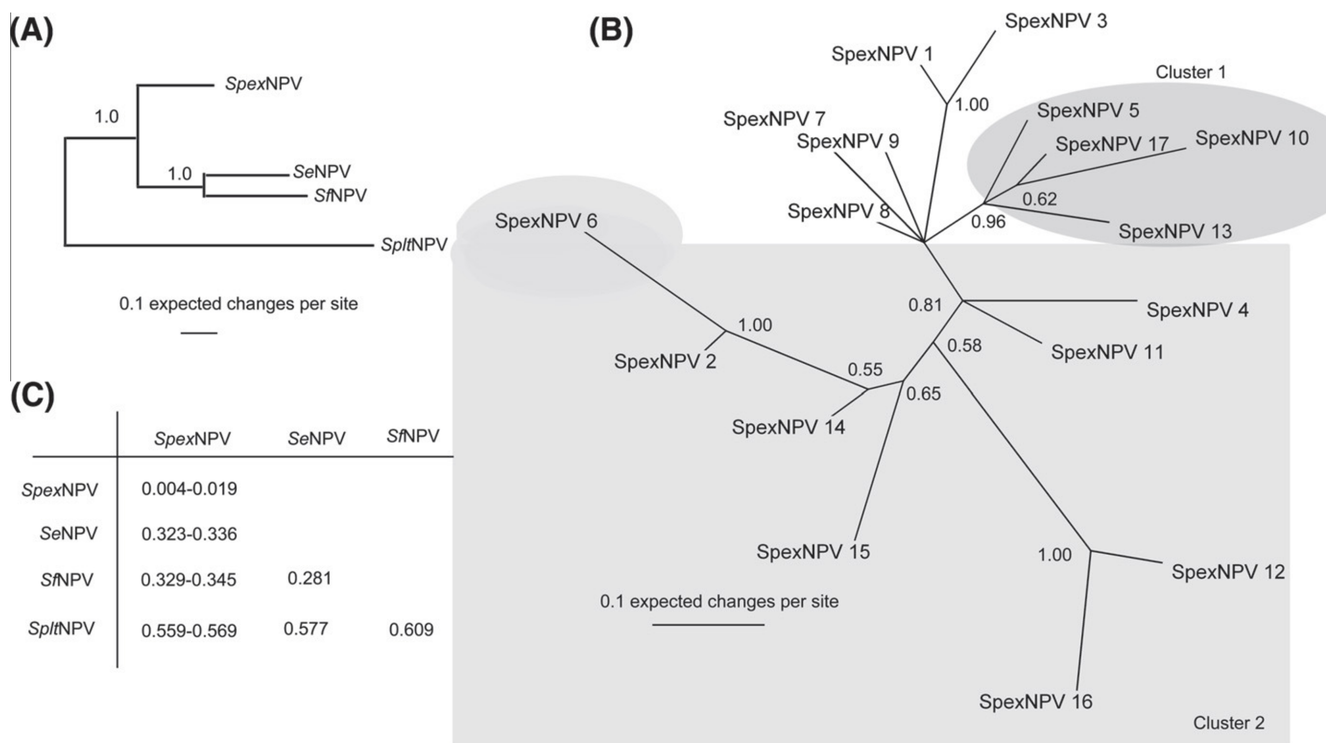
et al., 1993); (2) *lef-8*, an essential late-expressed gene involved in transcription (Herniou et al., 2004); (3) *egt*, an auxiliary gene that interferes with insect moulting (Clarke et al., 1996); and (4) chitinase (*chiA*), another auxiliary gene, important for horizontal transmission (Kang et al., 1998). For the investigation of the phylogeny of the 17 *Spex*NPV genotypes, total DNA was extracted and used as template for the specific amplification of partial regions of each of the genes (*polh*, F:AGCGGCAAAGAGTTTCTCAG, R:GGTGTACTCGGAATGCAGGT; *lef-8*, F:CATGGTGAAATGACTGTGGC, R:GGCGAACATTGAAAGATGGT; *chiA*, F:TCGCATGTGTTGTATGGATTC, R:GACGGCTATTTTATCGTTTCC; *egt*, F:ATCCGGTTTTCGACAACAATC, R:AAGTGTACCAAACTGCCTTG). PCR reaction parameters have been published previously (Vilaplana et al., 2008). The PCR products were directly sequenced in triplicate and multiple alignments of gene sequence were produced with *ClustalX* (Thompson et al., 1997). Bayesian inference of nucleotide substitution parameters and topology was performed in *MrBayes v3.1* (Ronquist and Huelsenbeck, 2003) using a partitioned model. A separate general time-reversible model with an inverse gamma distribution of rate variation across sites was estimated for each gene partition, allowing for potential variability of overall evolutionary rate between genes. The MCMC chain was run for 100,000 iterations (sampled every 10 iterations) and its convergence was determined from the average standard deviation of split frequencies.

3. Results and discussion

The identification of 17 different genotypes from a natural isolate revealed a considerable level of genetic diversity and, with only a third of the viral cadavers analysed, it is likely that other genotypes remain as yet unidentified (Fig. 1A). Restriction fragment length polymorphisms have been reported for a number of species, suggesting that genome variation is common among many baculoviruses (Croizier and Ribeiro, 1992; Muñoz et al., 1999; Cooper et al., 2003; Graham et al., 2004; Cory et al., 2005; Li et al., 2005). For MNPVs, the possibility of the co-occlusion of different genotypes within the same OB means that it is premature to define these, single-genotype isolates as “clones” until their purity has been validated.

Although the genome size estimates of the individual *Spex*NPV genotypes varied considerably from approximately 115 kb to 153 kb (Fig. 1B) they were all within the range of other Lepidopteran-specific NPVs (alphabaculoviruses). Thirty-six of the 50 completely sequenced baculovirus genomes published to date (deposited in genbank: December 2009) are alphabaculoviruses whose genome size varies from 111.7 kb (*Adoxophyes orana* NPV) to 168.0 kb (*Leucania separata* NPV). The nature and causes of the genome size differential of the *Spex*NPV genotypes has yet to be resolved, but could represent the presence of large insertions and deletions, as has been identified in other species. Investigation of the intra-specific genome size variation identified in *Spli*NPV was successfully mapped to large genomic deletions (4.5 kb) of the *pif* gene (Kikhno et al., 2002).

An earlier phylogeny based on the *polh* gene showed an Egyptian *Spex*NPV isolate to be most closely related to *S. exigua* NPV (*Se*NPV), followed by, *Spodoptera litura* NPV (*Splt*NPV) and *Spodoptera frugiperda* NPV (*Sf*NPV) (Herniou and Jehle, 2007). We used four partial gene sequences to confirm that *Spex*NPV was indeed most closely related to *Se*NPV (Fig. 2A). The phylogeny of *Spex*NPV genotypes shows a number of genotypes are very closely related to each other. High posterior probability values (PP) support the close relationships of genotype 1 with genotype 3, genotype 2 with genotype 6 and genotype 12 with genotype 16. There is also statistical support for the formation of two distinct clusters of genotypes



	*Spex*NPV	*Se*NPV	*Sf*NPV
*Spex*NPV	0.004-0.019		
*Se*NPV	0.323-0.336		
*Sf*NPV	0.329-0.345	0.281	
*Splt*NPV	0.559-0.569	0.577	0.609

Fig. 2. Consensus phylogram (50% majority rule) of consensus *Spex*NPV sequence and closely related species (A) and *Spex*NPV genotypes (B). Both figures result from Bayesian analysis of partial *polh*, *lef-8*, *ChiA* and *egt* sequence alignment using MrBayes v3.1 (Ronquist and Huelsenbeck, 2003). Analysis carried out using a partitioned model and a general time-reversible model with an inverse gamma distribution of rate variation across sites (GTR + I + Γ). Values at nodes represent posterior probabilities. Shaded areas represent clusters with statistical support. Estimates of evolutionary divergence between sequences also measured as the number of base substitutions per site. Analyses were conducted using the Kimura 2-parameter method in MEGA4 (Tamura et al., 2007) based on the pairwise analysis of 20 sequences. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1628 positions in the final dataset (C).

(Fig. 2B, grey shading). The first cluster (cluster 1) consists of the relatively closely related genotypes 5, 10, 13 and 17 (PP = 0.96) and cluster 2 is made up of more phylogenetically divergent genotypes 4, 11, 12, 16, 15, 14, 2 and 6 (PP = 0.81). Re-running the analysis without the *polh* sequence data significantly alters this clustering pattern. Cluster 1 is lost completely and genotypes 4 and 11 are lost from cluster 2, whose posterior probability is reduced to 0.67 (data not shown). Such a discrepancy between phylograms caused by the inclusion of the *polh* gene may be evidence of the mosaic nature of this gene, as noted in *Ac*MNPV (Jehle, 2004). For the combined gene sequence, the intra-specific sequence divergence was no more than 0.019, representing just a tiny proportion of the total sequence divergence observed between *Spex*NPV and other closely related species (Fig. 2C). *Spex*NPV is currently being sequenced and should allow for a much more detailed examination of its genetics and phylogenetics.

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