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Short Communication

Characterisation of a nucleopolyhedrovirus and *Spiroplasma* sp. bacterium associated with outbreaking populations of the Antler moth *Cerapteryx graminis*

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ABSTRACT

A broad survey was undertaken to characterise microbes associated with larval outbreaks of the Antler moth *Cerapteryx graminis* in Cumbria, United Kingdom. A nucleopolyhedrovirus present in all sampled populations at ⩽5% prevalence, was characterised via restriction fragment length polymorphism and partial sequencing the *Polyhedrin*, *Lef-8* and *Lef-9* genes; indicating a previously uncharacterised species most closely related to *Agrotis ipsilon* NPV. A survey of the host-associated bacterial community detected a species phylogenetically related to *Spiroplasma* sp., a male-killing phenotype previously isolated from Lepidoptera and Coleoptera, present at <63% prevalence in larvae. The implications of these associated microbes for host population dynamics are discussed.

1. Introduction

The Antler moth *Cerapteryx graminis* (Lepidoptera: Noctuidae) is an agricultural outbreak pest that can cause the devastation of upland grassland (Heath et al., 1979). It is widely distributed throughout Europe and North America, being abundant throughout much of the United Kingdom with a southern range limit of 45°N (Entwistle and Rivers, 1972; Morris, 1979; Mõttus et al., 1998). It inhabits meadows, open ground, hills and acidic moorland above 200 m with its larvae feeding on gramineous species (Entwistle and Rivers, 1972; Danell and Ericson, 1990). Previous studies have shown that it has the potential to change the composition of meadow pasture directly, through grazing and fertilisation by their faeces, and indirectly increasing herb growth due to the reduction in dense grass swards (i.e. through competitive release; Danell and Ericson, 1990).

Long-term datasets on Antler moth abundance in the United Kingdom (the Rothamsted Insect Survey; Woiwod and Harrington, 1994) indicate that UK populations exhibit 7-year cycles. In this study, as part of a broader investigation into the factors contributing to the population dynamics of Antler moths, we conducted an investigation into the microorganisms associated with the larval stages. The majority of previous research has been undertaken on cyclic populations of forest dwelling Lepidoptera and associated microorganisms (Dwyer and Elkinton, 1993; Dwyer et al., 2000; Myers, 1988; Tanada and Fuxa, 1987). Baculovirus epidemics have often been reported following host outbreaks (Entwistle and Evans, 1985; Tanada and Fuxa, 1987), and are thought to have potential in regulating cyclic host populations (Anderson and May, 1980, 1981; Briggs and Godfray, 1996; Liebhold et al., 2000; Myers, 1988). We detected a novel nucleopolyhedrovirus (NPV) and a *Spiroplasma* sp. endosymbiont, believed to be a male-killing phenotype. Here, we characterise each microbe via molecular methods and explore the possible biological associations between the host, bacteria and virus.

2. Methods and materials

During June 2008, there were large outbreaks of Antler moth larvae in the English Lake District National Park (northern England). Larvae were sampled at three outbreak sites in June 2008 (Helvellyn, Matterdale and Kentmere). Quadrat counts were used to determine larval densities along altitudinal transects, using three randomly positioned 0.5 m × 0.5 m quadrats at each 10 m point along the transect, commencing where larvae were first observed. Transects were over 100 m long, with a minimum of 30 quadrat counts per population. Random collections of live larvae and NPV cadavers were made over a wide spatial area at each site, to ensure a representative sample for each population. Live larvae were reared individually under laboratory conditions until adult emergence.

Field-collected NPV was purified from individual cadavers, a method modified from Hunter-Fujita et al. (1998). Briefly, each infected cadaver was homogenised in sterile 1.5 ml microtubes with 400 μl of 0.1% sodium dodecyl sulphate (SDS). The virus was pelleted at 8000 g for 3 min and resuspended in 750 μl sterile

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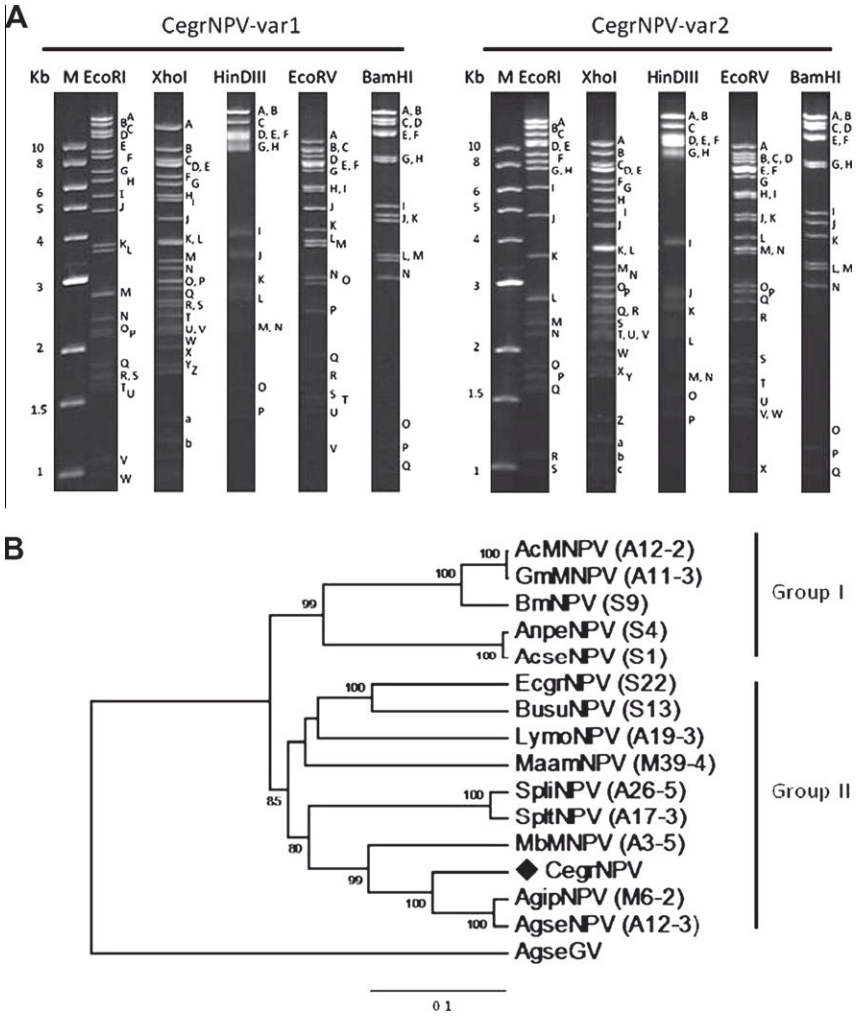

Fig. 1. (A) Restriction endonuclease fragment profiles of CegrNPV-var1 and CegrNPV-var2 digested with *EcoRI, XhoI, HinDIII, EcoRV* and *BamHI*, and electrophoresed on a 0.6% agarose gel. M indicates 1 Kb DNA ladder (Invitrogen), which was used as a size standard. (B) UPGMA distance tree based on concatenated amino acid (aa) sequences of the partial *Polyhedrin/Granulin*, *Lef-8* and *Lef-9* genes of 16 baculoviruses. AgseGV of the beta-baculoviruses is used as an outgroup. The scale bar represents a 10% estimated difference in aa sequence. Numbers given at each node correspond to the percentage bootstrap values (for 1000 repetitions). Replicate numbers of <70% were not included in the figure. ♦ indicates where CegrNPV fits into Group II of the alpha-baculoviruses.

Milli-Q water. Viral polyhedra were dissolved by adding 3 µl Proteinase K (20 mg/ml), 10 µl 1 M Na_2CO_3 and 15 µl 10% SDS. The nucleic acids from this mixture were phenol/chloroform extracted twice, dialysed and stored at 4 °C until use. Purified viral DNA was digested with the endonucleases *EcoRI*, *XhoI*, *HinDIII*, *EcoRV and BamHI* (New England Biolabs), fractionated in 0.6% agarose gels, and band sizing undertaken using Quantity One software (BioRad Inc., USA).

Polymerase Chain Reaction (PCR) was used to amplify a portion of the NPV *Polyhedrin*, *Lef-8* and *Lef-9* genes, following the standard protocol of Jehle et al. (2006a). Concatenated sequences of baculovirus *Lef-8*, *Lef-9* and *Polyhedrin/Granulin* genes are suitable markers to mirror viral speciation (Jehle et al., 2006a). Reaction mixtures (50 µl) contained PCR buffers (10 mM Tris–HCl pH 8.3 at 25 °C; 50 mM KCl; 1.5 mM MgCl; 0.001% gelatin), 5 µM each of dATP, dTTP, dCTP and dGTP, 10 µM of the relevant primers (Jehle et al., 2006a), 1 unit *Taq* polymerase (Qiagen) and approximately 5 ng DNA template. Reactions were carried out in a Techne TC-512 thermal cycler (Bibby Scientific Ltd., Stone, UK), using the following reaction conditions: (i) 94 °C for 5 min, 1 cycle; (ii) 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, 35 cycles; and (iii) 72 °C for 5 min, 1 cycle. PCR amplicons were purified (PCR Purification Kit, Qiagen), and sequenced (Source Bioscience, UK). Concatenated sequence alignment was undertaken using ClustalW (Thompson et al., 1994), and phylogenetic trees constructed using MEGA4 (Tamura et al., 2007). The evolutionary distances were computed using UPGMA distance analyses, and resultant tree topologies evaluated in bootstrap analyses based on 1000 re-samplings.

The bacterial communities of Antler moths were surveyed using the 16S rRNA gene primers p515F-GC and p806R (Relman, 1993) to amplify region V4. Reaction mixtures (50 ml) contained PCR buffer (10 mM/L Tris–HCl (pH 8.3) at 25 °C, 50 mM/L KCl, 1.5 mM/L MgCl, and 0.001% gelatin), 10 mM/L each of dATP, dTTP, dCTP, and dGTP, 0.1 mM/L of each primer, 1 unit *Taq* polymerase (Qiagen), and approximately 10 ng of insect genomic DNA template. PCRs were done with the settings (i) 94 °C for 5 min, 1 cycle; (ii) 94 °C for 20 s, 52 °C for 20 s, 72 °C for 30 s, 40 cycles; and (iii) 72 °C for 5 min, 1 cycle. PCR products were separated by Denaturing Gradient Gel Electrophoresis (DGGE) using the TV400-DGGE system (Progen Scientific Ltd., London, UK). Gels consisted of 1 mm thick 6% polyacrylamide with a denaturing gradient of 30–70% (100% denaturant corresponds to 7 mol/L urea and 40% v/v deionized formamide) and 1 × TAE buffer (40 mM/L Tris–acetate (pH 8.0) and 2 mM/L EDTA). Electrophoresis was performed at 60 °C and 80 V in 1 × TAE running

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Table 1
Diversity and phylotype of partial 16S rRNA bacterial sequences isolated from Antler moth samples.

No.	Accession No.	Isolate ID	Genus	Clade	BlastN match	nt% identity
1	HQ603185	AM1	*Spiroplasma* sp.	Mollicutes	AB542741	99
2	HQ603186	AM2	*Pseudomonas* sp.	γ-Proteobacteria	HQ003435.1	100
3	HQ603187	AM3	*Caulobacter* sp.	α-Proteobacteria	FN566954	100
4	HQ603188	AM4	Uncultured bacterium	β-Proteobacteria	GU734088.1	100
5	HQ603189	AM5	Uncultured bacterium	β-Proteobacteria	HM747232.1	100

buffer for 16 h, and gels were stained with SYBR Gold nucleic acid stain (Invitrogen). The DNA bands were excised with a sterile razor-blade and placed in 100 μl of sterile distiled water at 94 °C for 5 min to elute DNA for direct sequencing.

3. Results and discussion

Surveys indicated that larvae were confined within a fairly narrow altitudinal band, between 630–810 m, 610–760 m and 700–770 m at Helvellyn, Matterdale and Kentmere, respectively. Mean abundances reached 172 larvae per m^2 at Helvellyn and Matterdale (range = 0–340 larvae per m^2, quadrat number = 54), and 60 larvae per m^2 at Kentmere (range = 0–76 larvae per m^2, quadrat number = 30).

NPV mortality was recorded in all three Antler moth populations, with virus prevalence in the field being 0.74% at Matterdale and 5.08% at Kentmere (accurate NPV mortality was not recorded at Helvellyn). NPV mortality was spatially widespread throughout the populations, found in 4 of 54 quadrats at Matterdale and 7 of 30 quadrats at Kentmere (accurate NPV surveys were not recorded at Helvellyn). NPV infection was confirmed using standard Giemsa staining and standard microscopy methods (Hunter-Fujita et al., 1998), with the virus designated as *C. graminis* NPV, CegrNPV. Viral DNA was isolated individually from 11, 17 and 10 cadavers from Helvellyn, Matterdale and Kentmere, respectively. Investigation of the NPV populations indicated low genotypic variation, with only two variants being found in all populations, assigned CegrNPV-var1 and CegrNPV-var2. *EcoRI*, *XhoI*, *HinDIII*, *EcoRV and BamHI* were used to digest the genome of both CegrNPV variants (Fig. 1a). Digested band sizing estimates an overall mean genome size of 119 424 bp (CegrNPV-var1) and 117 461 bp (CegrNPV-var2). The ratios (CegrNPV-var1: CegrNPV-var2) in each population were 7:4, 7:10 and 6:4, suggesting no dominance by a single variant. Concatenated sequence data for *Polyhedrin*, *Lef-8* and *Lef-9* was identical for both variants, and placed the virus in Group II of the alpha-baculoviruses (Jehle et al., 2006b), most closely related to the NPV isolated from the noctuid *Agrotis ipsilon* (Fig. 1b). The sequences for these three genes have been deposited in Genbank (Accession Nos. HQ603182–HQ603184).

To screen the microbial community, PCR and DGGE analysis was undertaken for six healthy larvae, six healthy adults and six larval cadavers collected from Matterdale; and six healthy larvae, six healthy adults and six larval cadavers collected from Kentmere. Sequence analysis of gel bands identified five bacterial rRNA isolates, assigned AM1, AM2, AM3, AM4 and AM5 (Table 1; Genbank Accession Nos. HQ603185–HQ603189). All five gel bands were present in the two populations.

Of most interest was the discovery of a new *Spiroplasma* sp. sequence, closely phylogenetically allied to the male-killing phenotypes of this genus (Tinsley and Majerus, 2006; Table 1). Further analysis using *Spiroplasma* genus-specific primers (SP1 and SP2; Alexeeva et al., 2006) detected *Spiroplasma* sp. widespread in both Matterdale (58.8%; $n = 17$) and Kentmere (62.5%; $n = 16$) populations. Of the larvae collected and reared to adult emergence in the laboratory (12 from Matterdale and 11 from Kentmere), all emerged as female. Nineteen (82.6%; $n = 23$) were subsequently found to be infected with *Spiroplasma*. This suggests a sex-ratio distortion, and we speculate that this could possibly be due to the presence of the *Spiroplasma* infection with a male-killing phenotype; although it should be noted that we cannot be entirely certain of the phenotype at this stage of our research. The male-killing trait can occur if the agent is vertically transmitted or is a mix of vertical and horizontal transmission (Hurst and Jiggins, 2000). The features of host biology and ecology that increase the benefit to the bacterium of killing male embryos are sibling egg consumption (females eat their dead brothers) antagonistic interactions between siblings (male-killing may reduce both cannibalism of females and the intensity of competition between siblings), and deleterious inbreeding (Hurst and Jiggins, 2000); all of which could be important factors in high-density populations of the Antler moth. However, a high prevalence of male-killers may increase the proportion of female hosts that fail to mate (Jiggins et al., 2000), potentially reducing the population size of the host, and causing a population crash. The findings from this study provide evidence that microbial pathogens could play a role in the population dynamics of this agricultural outbreak pest. In the UK, populations of Antler moth exhibit stable limit cycles, outbreaking for 1–2 years approximately every 7 years. Overt NPV was spatially widespread in the three populations studied, causing up to 5% larval mortality. It is possible that the NPV and skewed sex-ratio (conceivably caused by a male-killing *Spiroplasma* sp. endosymbiont) contribute to the population cycles of this species, via their effects on larval mortality and the availability of male mates.

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