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Molecular Identification of a Densovirus in Healthy and Diseased *Zophobas morio* (Coleoptera, Tenebrionidae)

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Tenebrionidae · Large scale production · Insect culture · Densovirus · Pathology · Epizootology

Abstract
Zophobas morio is a tropical darkling beetle which is widely exploited for commercial large-scale insect growing. Outbreaks of a disease may occur causing total devastation of cultures. In the present paper, samples of diseased *Z. morio* were obtained and used for establishment of a laboratory model as they were found infective to the larvae of the same insect species from another source. It took about 1 month to develop symptoms of acute disease in mid-age larvae and about twice as much when younger larvae were used for infection. Affected larvae perished quickly, and within several days up to 90–100% of the colony could perish. Both in healthy and diseased larvae a virus was detected using PCR with degenerate primers specific for a gene coding for a non-structural protein (ORF3). The sequenced gene fragment (Genbank accession #MN732869) confirmed allocation of the virus to Densoviridae, with maximal similarity of 97.2% to *Blatella germanica* densovirus-like virus (#JQ320376) and 66.2% to *B. germanica* densovirus (#AY189948). Genomic DNA samples of *Z. morio* larvae from an independent colony devoid of symptoms of a disease were also positive for this virus with a slightly different (99.7% sequence similarity to the former sequence of the *Z. morio* densovirus) genotype (#MN732870).

Introduction

Zophobas morio (Fabricius, 1775) Kraatz, 1880 is a tropical species of darkling beetles beetle which is widely exploited for commercial large-scale insect growing for feed and food [1–3] as well as for other industries constituting a growing market of insect protein, such as cosmetics, pharmacy etc. [4]. Maintenance of healthy colonies is inevitable for the constant rearing and stable production of insects in culture. Meanwhile, outbreaks of a disease happen quite often and this may incur remarkable damage as total devastation of cultures is frequently observed. Moreover, presence of infectious agents in invertebrate cultures may have an impact on vertebrates fed on the insects and humans either working with or consuming the diseased specimens. For example, certain species of

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Fig. 1. Dried cadavers of *Z. morio* larvae without (**a**) and with symptoms of a devastating disease (**b**).

viruses and microsporidia have been reported to switch between invertebrate and vertebrate hosts [5–7], including the parasites from crickets and locusts [8–10] which are widely used as insect culture both in laboratory and industry. A substantial body of research devoted to diseases of reared darkling beetles is virtually absent. The only publication concerning *Z. morio* infection is a short report of and epizooty in Hungary and an incomplete description of a virus infection associated with the insect herein [11]. In the present paper, we performed molecular detection of a virus in *Z. morio* maintained in culture in Russia.

Materials and Methods

A culture of *Z. morio* was maintained at the Moscow Zoo since mid-1980s using a starting colony from Germany. Beetles from other sources have been added later on to avoid inbreeding. A disease causing mass insect death was observed since early 2015. Samples of diseased larvae were transferred to the laboratory at St. Petersburg for the experiments. Alive larvae were maintained under ambient conditions. Insect mortality and motility were estimated. In particular, changes in locomotive behavior were screened by placing individual larvae on a horizontal plastic surface (see Results sections). Perished insects were assayed for light microscopy analysis, but no apparent pathogens were revealed. Cadavers were dried and stored at room temperature from several weeks to 18 months prior to the experiments.

An independent laboratory larval colony of the same insect species from another source (reared separately in another region of Russia) was used to model the infection. Insects were routinely reared under laboratory conditions on wheat bran as the main fodder and substrate and carrot slices as natural source of vitamins, water etc. For the infection experiment, larvae were assayed at two different developmental stages: (a) young larvae of IV–V larval instar, and (b) mid-aged larvae of VI–VII larval instar. Insects of both ages were divided into groups of 100 specimens, maintained in 1 L plastic containers. Dried samples were added to the containers as whole or ground cadavers. Untreated larvae were used as controls, additionally fed with dried gammarus.

Statistical significance of mortality rate differences between experimental groups was estimated using *t* test in Statistica 7.0 software.

Alive and perished larvae were dissected and inner tissues were processed for molecular genetic analysis. Genomic DNA was extracted as described previously [12]. Degenerate primers BgDNVfor3 (5′-GWMAGGGWGACGAGCAACGTTC-3′) and BgDNVrev3 (5′-RTTTCCCTWGGTTCRTARTTWGGTTC-3′) were designed to span a fragment, ~800 bp long, corresponding to the non-structural protein within the open reading frame 3 (ORF3) closer to its 3′-end, in the genomes of two densoviruses presented by Genbank accession #AY189948 and #JQ320376. The densovirus genome sequences were aligned in BioEdit [13], the primer regions were picked manually and checked for compatibility with PerlPrimer [14], and consensus degenerate sequences were obtained using Genefisher 2 software [15]. The PCR was run with a standard protocol using DreamTaq polymerase (Thermo Fisher Scientific) as a ready-to-use mixture [16]. The amplicons were gel purified and checked for quality by electrophoresis. Selected samples were sequenced in both directions using the core facilities at All-Russian Institute of Agricultural Microbiology (ARRIAM, St. Petersburg, Russia). The obtained nucleotide and deduced amino acid sequences were matched against the Genbank records at the NCBI server using the built-in BLAST utility, *blastn* search algorithm for "somewhat similar" nucleotide sequences and *blastp* algorithm for protein-protein search. Homologous sequences of interest were further imported and aligned in BioEdit and compared using the built-in Sequence Identity Matrix utility.

The evolutionary history was inferred from the final dataset of 768 positions using the Maximum Likelihood method and Tamura-Nei model [17] with 1,000 replicates in MEGA X software [18].

Table 1. Sequence similarity of *Zophobas morio* densovirus and allied species

Virus species, isolate	Genbank accession number	Nucleotide (right-lower cells) and amino acid (left-upper cells) sequence similarity of the virus isolates					
		BgDNV1	BgDNVLV	PmDNV-JL	ZmDNV-MZ12	ZmDNV-GER9	CDenV/Madera-2
Blatella germanica densovirus 1, BgDNV1	AY189948	ID	0.538	0.533	0.546	0.546	0.555
Blattella germanica densovirus-like virus, BgDNVLV	JQ320376	0.651	ID	0.982	0.961	0.961	0.842
Parus major densovirus, PmDNV-JL	KU727766	0.652	0.992	ID	0.953	0.953	0.834
Zophobas morio densovirus, ZmDNV-MZ12	MN732869	0.645	0.973	0.968	ID	0.991	0.846
Zophobas morio densovirus, ZmDNV-GER9	MN732870	0.646	0.973	0.968	0.997	ID	0.842
Culex densovirus, CDenV/Madera-2	MH188043	0.624	0.807	0.802	0.802	0.802	ID

Results

Larvae arrived from the Moscow Zoo to the laboratory in St. Petersburg showed evident absence of any morphological changes. Conversely, their locomotion was remarkably affected. The main symptom of the disease was swirling (swaying anterior and posterior body parts from side to side) and rolling (rotating around the central axis of symmetry) of mid-age and older larvae, as well as slow chaotic wandering on an even horizontal surface. Conversely, healthy larvae were running quickly in a certain direction to escape open space unless a shelter is found. Moreover, after death the cadavers blackened quickly and were more intensively stained (Fig. 1b) as compared to cadavers of larvae died from other reasons (Fig. 1a). Their inner content of blackened cadavers was partially liquefied though the dead larvae tended to dry rather than completely macerate.

When an independent colony was experimentally contaminated with whole or ground cadavers, it took about 1 month to develop symptoms of acute disease in mid-age larvae. In a similar assay where younger larvae were used, the colony collapse typically developed after 2 months. Affected larvae perished very quickly, and within several days, up to 90–100% of the colony were dead. A few survived insects, however, successfully transited to pupae and adults. Meanwhile, the control colonies from an independent stock, maintained under identical conditions, displayed 0% mortality at larval stage, 95–98% pupation rate and above 90% adult emergence, that is, below 10% mortality at larvae to adult transition stage. Mortality rates of infected and control groups were significantly different at $p < 0.01$.

To determine if the cadavers remain infective after prolonged storage, the infective material (perished larvae samples) was stored for several weeks and for 18 months at RT for another experimental infection assay as above. The larval mortality in 2 experimental groups infected using "fresh" and "old" cadavers reached 91 and 97%, respectively, with no significant difference, as opposed to control with 0% larval mortality, significantly different from both experimental groups at $p < 0.001$.

The specific PCR products using the degenerate primers were obtained using the samples of genomic DNA extracted from perished larvae from the Moscow Zoo. The sequenced amplicon (Genbank accession #MN732869) showed maximal similarity of 97.2% to the homologous sequence of *Blatella germanica* densovirus-like virus BgDNVLV (#JQ320376) as well as 96.8% to a similar virus, isolate PmDNV-JL (#KU727766) from the great tit *Parus major* (Passeriformes: Paridae). Meanwhile, the nucleotide sequence similarity to *B. germanica* densovirus 1 (#AY189948) was 66.2%. When a protein BLAST search was performed, it has shown another closely related sequence of the *Culex* densovirus. Its nucleotide (#MH188043) and amino acid sequences (#AXQ04855) were 76.9 and 84.2% similar to the *Z. morio* densovirus, respectively (Table 1). A phylogenetic reconstruction on the basis of these sequences has shown basal position of the most dissimilar *B. germanica* densovirus 1, followed by the *Culex* densovirus, while the cluster of the 2 nearly identical *Z. morio* densovirus isolates was in a sister position to another closely related group of BgDNVLV and PmDNV-JL (Fig. 2).

The genomic DNA samples of the survived larvae were positive when assayed for the PCR analysis. Moreover, *Z. morio* larvae from the independent colony (which served as the insect source for experiments in the present study) were also assayed for PCR analysis and found positive. The sequence obtained (#MN732870) showed 99.7% sequence similarity to the former sequence of the *Z. morio* densovirus.

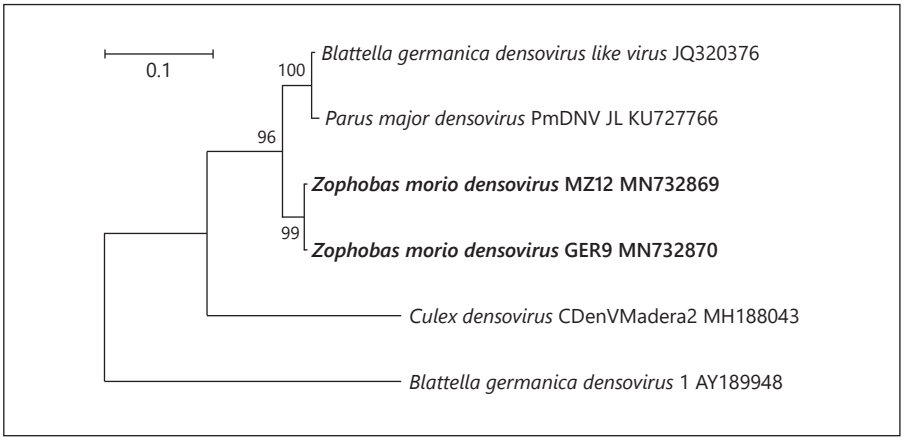

Fig. 2. The phylogenetic reconstruction inferred from an alignment of partial ORF3 nucleotide sequences of *Z. morio* densovirus isolates found in the present study (in bold) and allied species, containing 768 positions. Maximum Likelihood method and Tamura-Nei model with 1,000 replicates were used to build the tree in MEGA X (see Materials and Methods). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Scale bar = 0.1 expected nucleotide changes per site.

Discussion

Little information is available concerning the infectious diseases in the super mealworm. The only published work known refers to a case of a colony collapse of *Z. morio* in Hungary [11]. In that paper, authors described the disease similar to that observed in the present work. The authors also reported of isolating a densovirus similar to *B. germanica*-like densovirus. And because no further information was provided, such as publicly available genome sequence of the virus, the primers were designed targeting potential biodiversity of the densoviruses associated with *B. germanica*.

Positive PCR result suggested presence of a densovirus and sequencing confirmed allocation of the virus to Densoviridae. The canonical *B. germanica* densovirus was similar, but not as close as the other isolates. The closest relatives found in Genbank were the isolates from the bat feces and from the tit's lung. The former might have been present as a symbiont of insects consumed by bats [19] but the latter has demonstrated an apparent ability to develop within the vertebrate tissue [20]. Thus, the hazard of vertebrate host infection with insect densoviruses cannot be excluded. Besides infection via consumption of virus-positive insects, other pathways of pathogen transmission are possible, taking into account presence of such viruses in blood-sucking invertebrates, exemplified by the *Culex* densovirus [21].

Genomic DNA samples of *Z. morio* larvae from an independent colony devoid of symptoms of a disease were also positive for this virus. This indicates that the virus can be widespread. Slight dissimilarity in the genotypes indicates that different isolates of the same species exist in colonies either suffering from the disease or not affected. Similarly, in Hungary the viral infection was found both in healthy and diseased larvae [11] though the insects were from the same colony. Unfortunately, we cannot directly compare the data but it is highly likely that Hungarian researchers have dealt with an isolate of the same (or very similar) virus species. It cannot be ruled out from the dataset obtained whether the virus is the primary cause of the disease. Moreover, it is still unknown which factors may trigger the onset of the disease (be it caused by the virus of some other agent): environmental conditions, genetics of parasite or host, etc. Further research is needed to elucidate this question.

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Statement of Ethics

Not applicable as only samples of diseased *Z. morio* insect larvae were used for experiments at our laboratory in St. Petersburg.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Y.S.T. supervised the research, analyzed literature, designed primers, and wrote the manuscript. S.M.M. performed molecular genetic and phylogenetic analysis. Y.V.V., A.V.G. and M.V.B. performed insect rearing and experimental infection bioassays. All authors read and approved the final manuscript.

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