Journal of Insect Science OPEN ACCESS

RESEARCH

First Report of *Oryctes rhinoceros nudivirus* (Coleoptera: Scarabaeidae) Causing Severe Disease in *Allomyrina dichotoma* in Korea

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Subject Editor: Michael Strand

J. Insect Sci. 15(26): 2015; DOI: 10.1093/jisesa/iev002

ABSTRACT. *Oryctes rhinoceros nudivirus* (*OrNV*) has been known to cause severe disease in coconut palm rhinoceros beetle, *Oryctes rhinoceros*, in Southeastern Asia and is used as a biological control to reduce the pest population. Here, we report for the first time that the *OrNV* may have landed on Korea and may be the major pathogen for diseased larvae of Korean horn beetle, *Allomyrina dichotoma*. After peroral inoculation, over 60% of infected larvae perished in 6 wk. This viral disease spreads very fast in several locations throughout Korea. This threat not only makes economic loss of local farms rearing *A. dichotoma* larvae but also may disturb the ecosystem by transmitting to wild *A. dichotoma*.

Key Words: Coleoptera, Scarabaeidae, diagnostics, virology, insect rearing

In global agriculture, the value of insect resources has been increased immensely, and the insect industry is now considered as a big market. Besides the traditional apiculture and sericulture industry, other insects are used for many purposes including pollinating activity, pet or educational purpose, animal feedstuffs, development of pharmaceuticals or cosmetics, natural enemy of harmful insects, and environmental cleanup. In Korea, the market size of this insect industry was estimated at 160 million dollars in 2012, and it is expected to increase up to 40 billion dollars by 2020. Among them, *Allomyrina dichotoma* is one of the strongest candidates for insect industry as medicinal purpose. *A. dichotoma* belongs to the order Coleoptera, family Scarabeidae, and genus *Allomyrina*. The adult beetles range from 40 to 85 mm and suck out the sap of oak tree, whereas the larvae have three instar stages and feeds on rotting oak tree sawdust. Historically, Korean horn beetle has been used for traditional oriental medicine for various liver diseases and diabetes mellitus in Korea, and there are many reports that *A. dichotoma* larvae also have antineoplastic, antibacterial, and antioxidant effects (Taketa et al. 1986, Jeune et al. 2001, Sagisaka et al. 2001, Yamada et al. 2004, Choi et al. 2006, Kim et al. 2007, Lee and Lee 2009, Suh et al. 2010). Recently, it was reported that extracts from *A. dichotoma* larvae have also antiobesity anti-Alzheimer activity (Chung et al. 2014, Kim et al. 2014).

Oryctes rhinoceros nudivirus (*OrNV*) is a double-stranded DNA virus having an enveloped rod-shaped virion, which is about 200–235 nm in length and 100–120 nm in width (Huger 2005, Wang et al. 2011). *OrNV* causes severe disease in coconut palm rhinoceros beetle, *Oryctes rhinoceros*, in Southeastern Asia (Huger 2005, Ramle et al. 2005). The coconut palm rhinoceros beetle is a serious pest of coconut oil palm industry, and *OrNV* has been used as a biological control agent for the beetle. It was known that *OrNV* can also infect in other members of *Oryctes* genus, including *Oryctes monoceros* in Africa (Bedford 2013). The virus multiplies in the midgut, and fat body of infected larvae and the virus are released from the dead larvae on the breeding site.

In 2012, an incident was reported that *A. dichotoma* larvae being farmed died en masse in Cheongwon County, Korea. The appearances of diseased larvae were not likely as the symptom caused by infection of bacterial or fungal pathogens and the cause of death were suspected by viral disease. However, the viral pathogen was not identified. Since then, the disease has been reported from time to time, but in 2014, suddenly, several cases of the similar symptoms were reported nationwide. Here, we report for the first time that a virus, which seems to be *OrNV*, was identified in diseased larvae of *A. dichotoma*, and this viral disease spreads fast in several locations throughout Korea. Because unlike the coconut palm rhinoceros beetle, Korean horn beetle is not a pest but a good candidate for insect industry in Korea, this viral disease may become a serious threat for Korean farmers and insect industry.

Materials and Methods

Virus Collection and DNA Isolation. The diseased larvae were collected from several places throughout Korea including Cheongwon County, Youngdong County, Pocheon City, Yuseong District, and Gyeongsan City in 2014. The hemolymph was extracted through wound on a leg of a diseased larvae, and the virus was purified with PEG virus precipitation kit (BioVision, Milpitas, CA). First, the hemolymph was centrifuged at $2,000 \times g$ for 15 min at 4°C to remove cell debris, and the supernatant was passed through a cellulose nitrate membrane with pore size of 0.45 μm. Next, 2.5 ml of PEG solution A was added to 10 ml of the supernatant and refrigerated overnight. The virus-PEG mixture was centrifuged at $10,000 \times g$ for 30 min at 4°C, and the viral pellet was dissolved in 20–100 μl of virus resuspension solution. For DNA isolation, hemolymph and midgut of diseased larva were homogenized and centrifuged $2,000 \times g$ for 15 min at 4°C to remove cell debris. Viral DNA was extracted with Wizard plus SV miniprep kit (Promega, Madison, WI) as instructed by the manufacturer.

Oligodeoxyribonucleotide Design for Virus Diagnosis. For diagnosis of the diseased *A. dichotoma* larvae, three pairs of primers were designed based on the *OrNV* genome (GenBank accession no. NC_011588). Primer AdV-F1 is 5'-TCCGGAAATTACACGAGCCAC-3' corresponding from 58,961 to 58,981 bp of *OrNV* genome. Primer AdV-R1 is 5'-ATGCCGTACGAGAGTATAGGTCG-3', corresponding from 59,604 to 59,582 bp. Amplification using primer pair AdV-F1 and -R1 yields 644 bp fragment of lef-8 gene (OrNV_gp064). Primer AdV-F2 is 5'-TCGAATCCGTTTCCGATACTTACAG-3', corresponding from 23,249 to 23,273 bp, whereas primer AdV-R2 is 5'-TGAGTAGCGCTATAGACTGCTC-3', corresponding from 23,853 to 23,832 bp. Amplification between primer AdV-F2 and -R2 produces the 605 bp fragment of GrBNV_gp76-like protein (OrNV_gp025). Primer AdV-F3 is 5'-GGGTGTGACGAGAAAACAACGC-3' and corresponds from 48,009 to 48,030 bp. Primer AdV-R3

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Table 1. Three pairs of primers, AdV-F1, -R1, -F2, -R2, -F3, and -R3, were designed based on the *OrNV* genome for diagnosis of the diseased *A. dichotoma* larvae

Primers	GenBank accession no.	Primer sequence (5′→3′)	Location	Product (bp)
AdV-F1	KM233708	TCCGGAAATTACACGAGCCAC	lef-8 (YP_002321375)	644
AdV-R1	KM233708	ATGCCGTACGAGAGTATAGGTCG	lef-8 (YP_002321375)	
AdV-F2	KM233709	TCGAATCCGTTTCCGATACTTACAG	GrBNV_gp76-like protein (YP_002321336)	605
AdV-R2	KM233709	TGAGTAGCGCTATAGACTGCTC	GrBNV_gp76-like protein (YP_002321336)	
AdV-F3	KM233710	GGGTGTGACGAGAAAACAACGC	Ribonucleotide reductase (YP_002321362)	644
AdV-R3	KM233710	GCAGGCGTGTAATAAATGGCGG	Ribonucleotide reductase (YP_002321362)	

is 5'-GCAGGCGTGTAATAAATGGCGG-3', corresponding from 48,652 to 48,631 bp. Amplification between AdV-F3 and –R3 yields 644 bp fragment of ribonucleotide reductase gene (OrNV_gp051). Primers were custom-ordered and synthesized by MacroGen (Seoul, Korea). Sequence and the location of primers are listed in Table 1.

Direct Polymerase Chain Reaction. For fast diagnosis, the hemolymph extracted from diseased larva was diluted with distilled water. One twentieth dilution was found to be optimum ratio for subsequent polymerase chain reaction (PCR) diagnosis under following condition: a denaturation at 95°C for 3 min, 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. Three pairs of AdV primers were used for PCR diagnosis under the same condition. For amplification, AccuPower PCR premix (BioNeer, Seoul, Korea) was used as instructed by the manufacturer.

Results

Disease Symptom. The diseased larvae appear pale and milky, and by the terminal phase of disease, the larvae are fairly translucent. Their abdomens swell up due to the increase of hemolymph and the bodies become very soft and juicy. Occasionally, a larva with prolapsed rectum can be observed but due to the larva's rolled-up position, the rectum burst with the jaw soon (Fig. 1). These are clearly compared with the disease symptoms of *A. dichotoma* larvae caused by bacteria, *Bacillus thuringiensis* and *Serratia marcescens*, or fungi, *Metarhizium anisopliae* and *Beauveria bassiana*, which are known to cause disease in *A. dichotoma*.

Virus Virulence After Peroral Infection. Healthy third-instar larvae were inoculated by dropping 30 μl of hemolymph from diseased larvae on the mouthparts of the larvae, respectively. The inoculated larva was independently grown in plastic container filled with moist fermented sawdust from oak tree. The water and sawdust were sterilized before use to avoid other infector. The larvae began to develop the symptom after 3 wk and after 6 wk, about 62% of inoculated larvae died as shown in Fig. 2. Hemolymph was taken from all dead larvae, and the cadavers were diagnosed as *OrNV* infection by PCR with primers AdV-F1/R1, -F2/R2, and -F3/R3.

Virus Diagnosis Using PCR. Diseased *A. dichotoma* larvae showing the viral symptoms were collected on several locations nationwide including Youngdong County. Hemolymphs extracted from the examined larvae were used for amplification with AdV primers producing the expected size of DNA bands. To find out that the AdV primers are OrNV specific, other *A. dichotoma* pathogens, *B. thuringiensis*, *S. marcescens*, *M. anisopliae*, and *B. bassiana* were also tested for amplification with AdV primers along with these pathogen-specific primers (Yamada et al. 1999, Choi et al. 2004, Shin et al. 2011). The results are shown in Fig. 3. The collected samples were not amplified by other pathogen-specific primers.

The viral DNA fragment amplified by AdV-F1/R1, -F2/R2, and -F3/R3 was isolated from agarose gel, and DNA sequences were determined with an Applied Biosystems 3730xl DNA Analyzer (MacroGen, Seoul, Korea). Blast search with *OrNV* genome (NC_011588) revealed that the three sequences have 98%, 98%, and 99% identical to corresponding *OrNV* genes, respectively.

Fig. 1. Comparison of a diseased larva with viral infection (left) and a healthy larva (right). Diseased larva appears beige and milky, and its rectum is prolapsed. The abdomen of the diseased larva swells up, and the body is very soft.

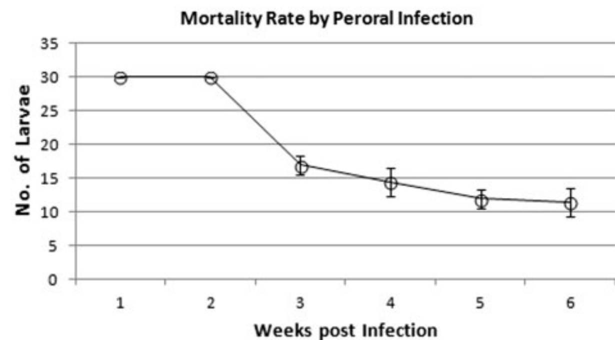

Fig. 2. Mortality rate after peroral infection. Thirty healthy larvae were infected with 30 μl of hemolymph from diseased cadaver, orally and after 6 wk, about 62% of larvae died with virus symptom.

The sequence data were submitted to GenBank (accession no. KM255708, KM255709, KM255710).

Discussion

Since it was first reported in 2012, the causation of mass loss of *A. dichotoma* larvae in Korea has been suspected as viral disease, and finally, it was turned out that that *OrNV* or at least *OrNV*-like virus may be the major pathogen for the viral disease. The PCR-based diagnose method makes it possible to detect the virus even in early stage of disease before symptom appears. Because in the farms rearing *A. dichotoma* larvae, dozens or hundreds of larvae grow together in a big plastic container filled with moist sawdust, a few viral-diseased larvae can easily infect the other larvae. Moreover, a cannibal behavior is often observed that a healthy larva ingests a diseased cadaver along with the sawdust. Many farmers trade their larvae for crossbreeding, and this also accelerates the fast spread of the disease. Also, there is growing concerns that this viral disease may be transmitted to the wild *A. dichotoma* because in some larvae farm located near mountain, the farmers lure the wild *A. dichotoma* for crossbreeding. Therefore, early

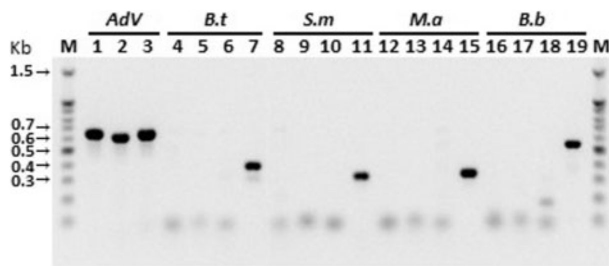

Fig. 3. The amplification of *OrNV* with AdV primers is species specific. Lanes 1, 2, and 3 are amplification of virus-infected larvae with AdV-F1/R1, -F2/R2, and -F3/R3, respectively, while a bacterial pathogen, *B. thuringiensis*, causing disease in *A. dichotoma*, was not amplified by AdV primers (lanes 4–6). Positive control in lane 7 for *B. thuringiensis* amplification with primer Bt-1 and Bt-2 yielded the expected size of DNA band. Another bacterial pathogen, *S. marcescens*, was also not amplified by AdV primers (lanes 8–10), while for the positive control in lane 11, *S. marcescens* was amplified with luxS-F and -R, correctly. Furthermore, two fungal pathogens, *M. anisopliae* and *B. bassiana*, were not amplified by AdV primers (lanes 12–14 and lanes 16–18). The fungal pathogens were amplified by their own primers Nc-F/R and Bb-P1/P3 as positive controls (lanes 15 and 19).

detection and removal of the diseased larvae from the breeding cage are extremely important at this stage.

The identification of this virus is not clear yet, and full-genome sequencing of the virus is planned for comparison with *OrNV* genome. If it is turned out the origin of this virus is *OrNV*, a further study should be carried out immediately how this viral epidemic has landed on Korea and how to block the epidemic route.

Acknowledgments

This study was supported by a grant (P009608) from the Agenda program, Rural Development Administration, Korea.

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Received 30 November 2014; accepted 3 January 2015.