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RESEARCH ARTICLE

Genome sequencing and analysis of a granulovirus isolated from the Asiatic rice leafroller, *Cnaphalocrocis medinalis*

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The complete genome of *Cnaphalocrocis medinalis* granulovirus (CnmeGV) from a serious migratory rice pest, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), was sequenced using the Roche 454 Genome Sequencer FLX system (GS FLX) with shotgun strategy and assembled by Roche GS De Novo assembler software. Its circular double-stranded genome is 111,246 bp in size with a high A+T content of 64.8% and codes for 118 putative open reading frames (ORFs). It contains 37 conserved baculovirus core ORFs, 13 unique ORFs, 26 ORFs that were found in all Lepidoptera baculoviruses and 42 common ORFs. The analysis of nucleotide sequence repeats revealed that the CnmeGV genome differs from the rest of sequenced GVs by a 23 kb and a 17kb gene block inversions, and does not contain any typical homologous region (*hr*) except for a region of non-*hr*-like sequence. *Chitinase* and *cathepsin* genes, which are reported to have major roles in the liquefaction of the hosts, were not found in the CnmeGV genome, which explains why CnmeGV infected insects do not show the phenotype of typical liquefaction. Phylogenetic analysis, based on the 37 core baculovirus genes, indicates that CnmeGV is closely related to *Adoxophyes orana* granulovirus. The genome analysis would contribute to the functional research of CnmeGV, and would benefit to the utilization of CnmeGV as pest control reagent for rice production.

KEYWORDS **baculovirus; granulovirus; *Cnaphalocrocis medinalis* granulovirus (CnmeGV); genome sequencing**

INTRODUCTION

Baculoviridae is a family of rod-shaped, enveloped, insect-specific viruses with a large, covalently closed, double-stranded DNA molecule ranging in size from 80 to 180 kilobases (Rohrmann, 2013). Based on the sequenced genomes and phylogenetic relationships within the family, *Baculoviridae* is subdivided into four genera: *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus* and *Deltabaculovirus* (Jehle et al., 2006). *Betabaculovirus,* which encompasses only lepidopteran-specific granuloviruses (GVs), is separated into three groups according to the pathological features of the insect hosts. Type I GV pathology is characterized by an infection constrained to the host's midgut and fat body and a relatively slow killing speed. Type II GVs are able to infect most of the host's tissues and exhibit a rapid killing. The third pathology, with a single representative, *Harrisina brillians* granulovirus, causes an infection confined to the midgut epithelium, resulting in the rapid death of the host (Federici, 1997). Because permissive cell lines are limited, GVs have been less well studied than lepidopteran-specific nucleopolyhedroviruses (NPVs), which be-

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long to *Alphabaculovirus* (Winstanley and Crook, 1993).

Cnaphalocrocis medinalis granulovirus (CnmeGV) was first isolated from Enping county, Guangdong province, China in 1979 (Pang et al., 1981). The CnmeGV strain is detectable in the population of *C. medinalis* larvae *in situ* even after 30 years since its first isolation (Zhang et al., 2014). As a highly specific and effective viral pathogen, CnmeGV is one of promising biological pesticides for controlling the rice pest. Studies on CnmeGV genome will be helpful for better understanding of its pathology and utilization. Here, we present the complete sequence and organization of the CnmeGV genome, and compare them to other baculoviruses using genomic and phylogenetic analyses.

MATERIALS AND METHODS

Viral DNA extraction

The strain of CnmeGV, designated as Enping strain, was isolated in 1979 and stored in our lab (Pang et al., 1981). The occlusion bodies (OBs) of CnmeGV were propagated in *C. medinalis* larvae and then were purified using differential centrifugation (O'Reilly et al., 1992). To extract viral DNA, the purified OBs were resuspended in 0.1 mol/L sodium carbonate solution [0.1 mol/L Na_2CO_3, 0.17 mol/L NaCl, 0.01 mol/L EDTA (pH 10.9)] and incubated at 37 overnight with 0.5 mg/mL proteinase K (Sigma-Aldrich®Shanghai,China) and 1% SDS. After extraction with phenol:chloroform: isoamyl alcohol (25:24:1, v/v), the viral DNA was precipitated with ethanol and then resuspended in TE buffer (10 mol/L Tris-HCl, pH 8; 1 mol/L EDTA).

Sequencing and bioinformatic analysis

The genome was sequenced with the Roche 454 GS FLX system by using shotgun strategy. The reads were assembled with Roche GS De Novo assembler software. Contigs assembly was performed with assistance of endonuclease restriction analyses. The genome sequence data was submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank).

Hypothetical ORFs were predicted by soft berry FGENESV program (http://www.softberry.com/berry.phtml) (Solovyev and Salamov, 1999) and defined by the standard ATG start, and a stop codon and potentially encode at least 50 amino acids. Gene annotation and comparisons were done with NCBI protein-protein BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Repeat structures were detected by BLAST alignment of two sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identity among homologous genes was calculated with MegAlign software using ClustalW with default parameters. Restriction sites were predicted by MapDraw software. Genome map framework was drawn with genomeVX (Conant and Wolfe, 2008).

Phylogenetic analysis

The phylogenetic analysis was performed based on amino acid sequences of 37 core genes form CnmeGV and the other 63 baculoviruses listed in the NCBI genome database (http://www.ncbi.nlm.nih.gov/nuccore/?term=baculovirus). All the sequences were linked by a stationary order and multiple alignments using ClusterW method with MEGA5 by using default settings. A phylogenetic tree was constructed by MEGA5 using Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992; Tamura et al., 2011). Phylogeny was tested by Bootstrap method with a value of 1000 (Sanderson and Wojciechowski, 2000).

RESULTS

Sequencing and general characteristics of the CnmeGV genome

To date, 74 baculovirus genomes have been fully sequenced (http://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10442) (Liu et al., 2014; Yin et al., 2015) and 18 of them belong to the *Betabaculovirus* genus. In the present study, the genome of CnmeGV was sequenced using the Roche 454 GS FLX system with shotgun strategy. Totally 692.5 times coverage of the genome was achieved by using the generated 77,041,934 nucleotides (nt) of raw data from 150,856 sequencing reads. The size of the assembled CnmeGV genome is 111,246 nt. It has a high A+T content of 64.8% which is similar to that of *Phthorimaea operculella granulovirus* (64.3%) (PhopGV, GenBank accession no. NC_004062) and *Adoxophyes orana granulovirus* (65.5%) (AdorGV, GenBank accession no.NC_005038) (Taha et al., 2000; Wormleaton et al., 2003). The coding sequences coverage 93% of the CnmeGV genome.

In total, 118 putative open reading frames (ORFs) were identified with at least 50 codons in length and minimal overlap. The *granulin* gene was defined as the first ORF and the adenine of its initiation codon was defined as the first nucleotide of the genome. As compared with other baculovirus genomes, the ORFs randomly distributed with 63 in the *granulin*-sense orientation and 55 in the opposite orientation. Among these ORFs, it contains the 37 conserved baculovirus core genes, 13 unique genes, 26 genes found in all sequenced lepidopteran baculoviruses, and 42 common genes that are present in a variety of baculoviruses (Figure 1).

Relationships with other baculoviruses

Phylogenetic analysis was performed based on 37 concatenated core genes of 64 representing baculovirus genomes (Figure 2). The obtained clad gram shows four groups of different genera, which were recognized in the current classification of the family (Jehle et al., 2006).

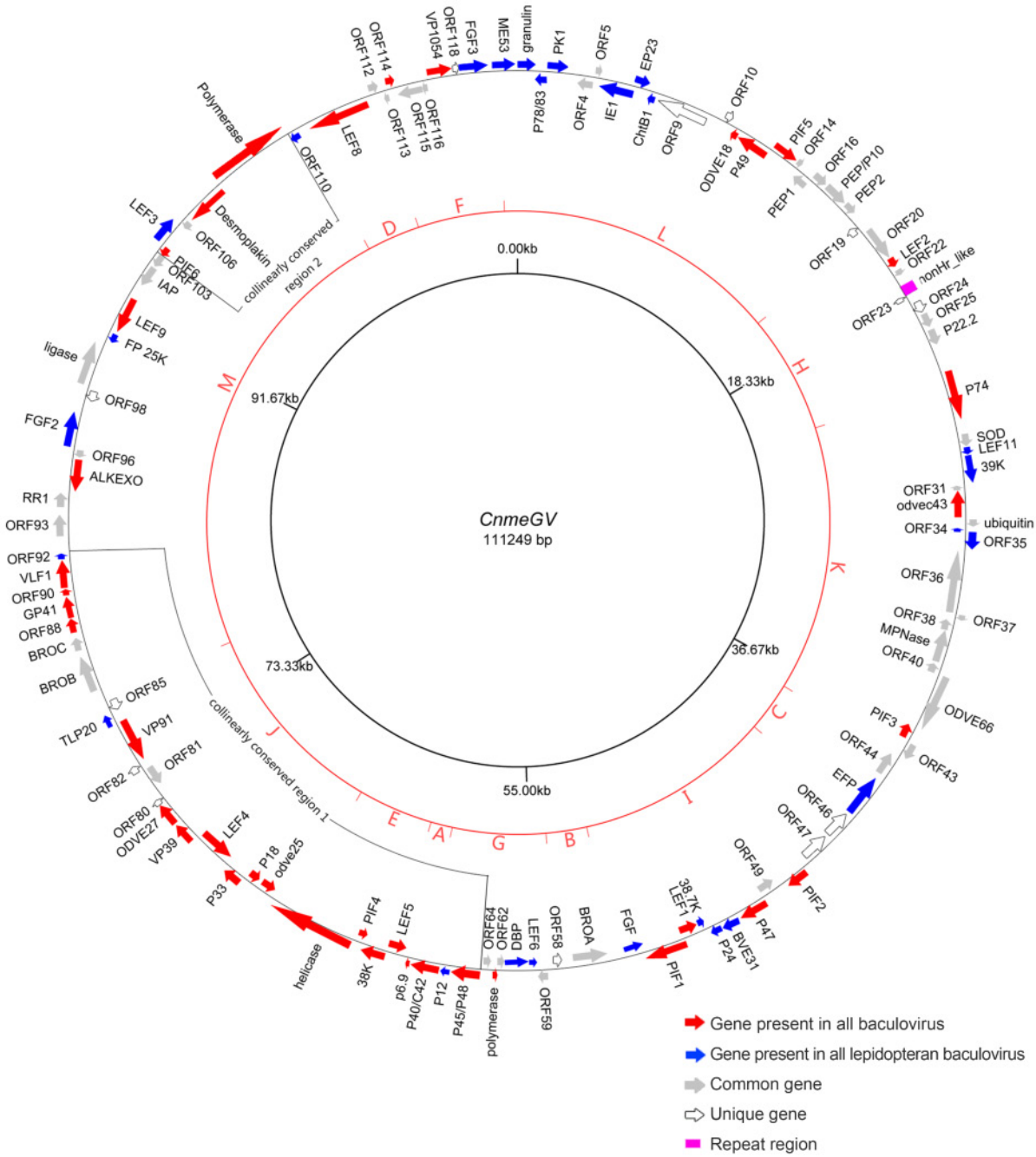

Figure 1. Genome map of CnmeGV. ORFs are indicated by arrows with a displayed name. Arrows also signify transcription directions. Red arrows represent core genes, blue represent genes present in all lepidopteron baculoviruses, gray represent baculoviral common genes and open arrows represent unique genes of CnmeGV. The pink square represent a repeat structure. The inner circle indicates genome scale position by 20 kb. *Hind* III restriction map is shown in the middle dark red circle. A region collinearly conserved in alpha- and betabaculoviruses is also shown.

CnmeGV is grouped in *Betabaculovirus* genus as expected. It forms a big clade with other seven GVs including AdorGV, *Clostera anastomosis* granulovirus (CaLGV), *Choristoneura occidentalis* granulovirus (ChocGV), *Clostera anachoreta* granulovirus (ClanGV), *Cydia pomonella* granulovirus (CpGV), *Cryptophlebia leucotreta* granulovirus (CrleGV) and *Pieris rapae* granulovirus (PrGV).

By using Gene Parity Plot (Hu et al., 1998), the gene colinearity of CnmeGV was compared to other se-

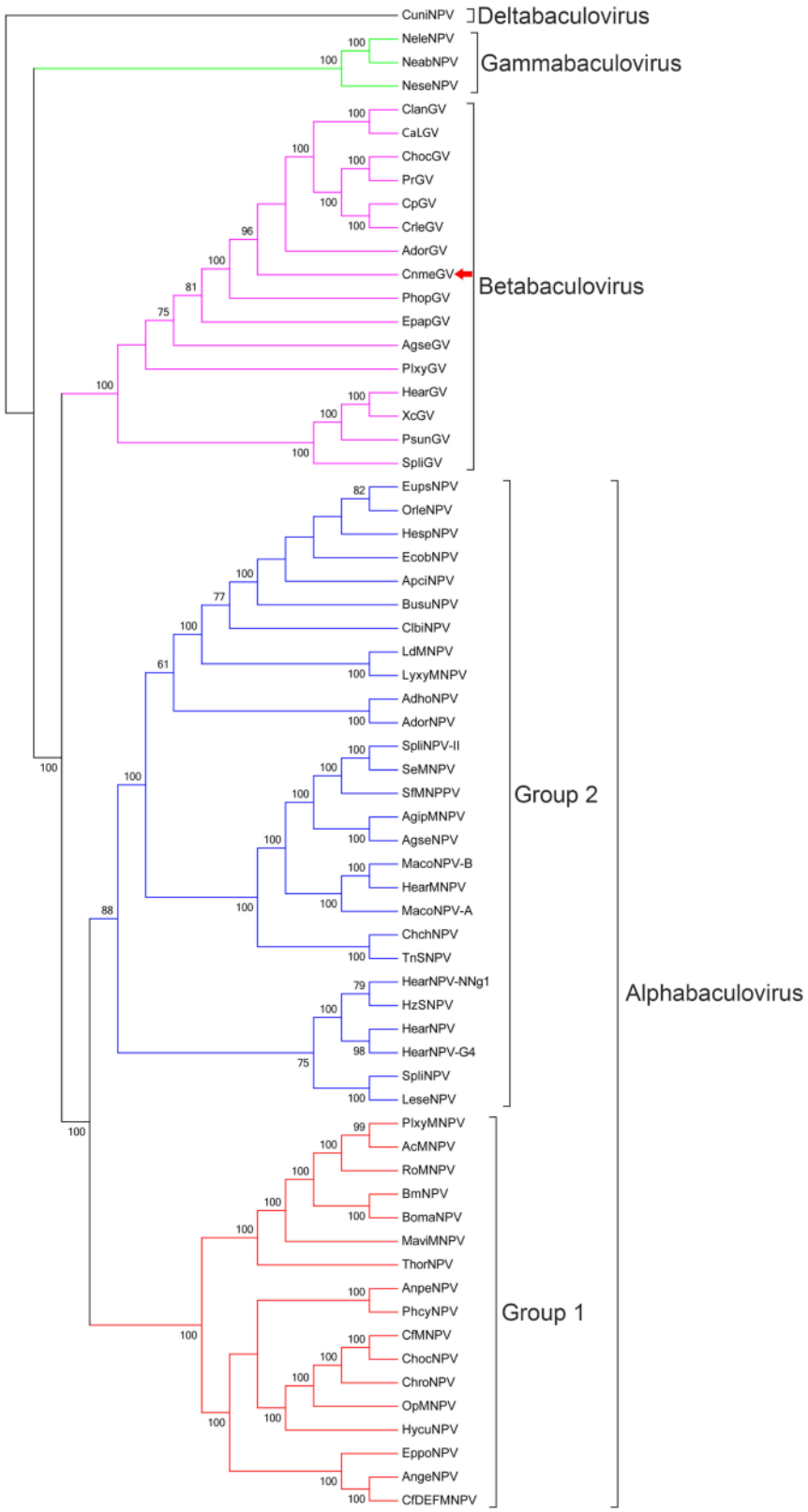

Figure 2. Phylogenetic tree using 37 core proteins of 64 sequenced baculoviruses based on Maximum Likelihood method. It tested by Bootstrap method with a value of 1000. The bootstrap values greater than 50% are showed in front of every nodes. Arrow points to CnmeGV.

quenced GVs. The result showed that CnmeGV differs from the rest of the GVs by a 23 kb gene block inversion from *Cnme25* to *Cnme48* and a 17kb gene block inversion from *Cnme92* to *Cnme108*. Gene-parity plots of CnmeGV with four closely related GVs including AdorGV, ClanGV, CpGV and PrGV based on ORF order are shown in Figure 3.

The nucleotide identities between the ORFs of CnmeGV and other representative sequenced GVs are shown in Supplementary Table S1. There are 72–95 homologous ORFs between CnmeGV and the 15 typical sequenced GVs, and the maximum and minimum homologies are with PrGV (95) and *Plutella xylostella* granulovirus (PlxyGV) (72), respectively. The genes present or missing in CnmeGV genome are summarized in Table 1.

CnmeGV genes grouped according to function

Repeat structure. Homologous repeated sequences (hrs) were supposed to be characteristic for many baculovirus genomes. *Hrs* seem absent from the CnmeGV genome, but a repeat sequence, which is probably able to form a hairpin structure, was detected in the CnmeGV genome from nt 18102 to 18575 with a high A/T content (75.5%) (Supplementary Table S1).

Transcription genes. Several genes required for baculovirus late gene transcription have been described (Lu and Miller, 1997). Like all other baculoviruses, CnmeGV codes for all four subunits of the viral encoded RNA polymerase, *lef-4* (Cnme76), *lef-8* (Cnme108), *lef-9* (Cnme100) and *p47* (Cnme50). Two other core genes, *lef-5* (Cnme69) and *vlf-1* (Cnme90), and three non-core genes, *lef-11* (Cnme29), *39k* (Cnme30) and *lef-6* (Cnme60), which may be also related to the transcription process, were found in the CnmeGV genome (Table 1). Of the baculoviral early transcription genes *ie-0*, *ie-1*, *ie-2* and *pe38*, only *ie-1* (Cnme6) is present in the CnmeGV genome and is poorly conserved (about 35.7% averaged amino acid identity to those of other GVs).

Replication genes. Six viral genes (*lef-1*, *lef-2*, *lef-3*, *dnapol*, *helicase* and *ie-1*) have been identified as essential genes for baculovirus DNA replication (Lu et al., 1997; Lu and Miller, 1997). Homologues of all these genes were found in the CnmeGV genome. It is noteworthy that CnmeGV has two *dnapol* genes (Cnme63 and Cnme107). Cnme63 encodes 62 amino acids and has only low identity to several NPVs whereas Cnme107 codes for 1086 amino acids and has homologues to all sequenced GVs with high identity.

Genes for enzymatic functions in nucleotide metabolism such as the large (*rr1*) and the small (*rr2*) subunits of ribonucleotide reductase (RNR) and deoxyuridyltriphosphate (dUTPase) have been reported in baculovirus gen-

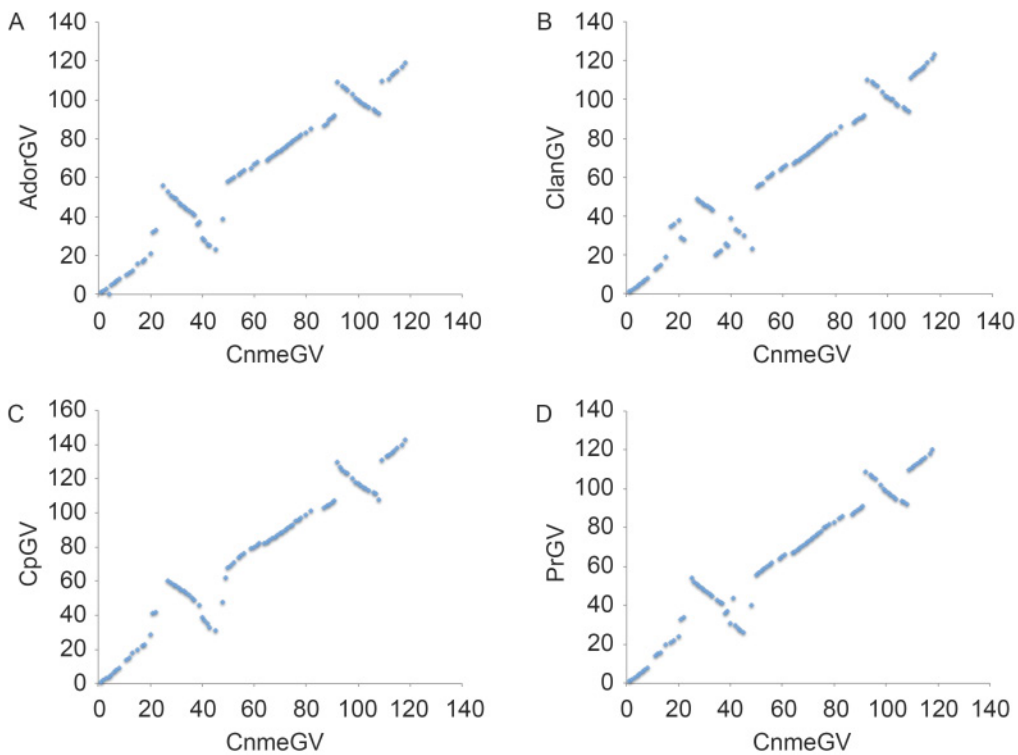

Figure 3. Gene-parity plots of CnmeGV with AdorGV (A), ClanGV (B), CpGV (C) and PrGV (D) based on ORF order.

Table 1. CnmeGV genes grouped according to function.

Gene function	Names of genes presented in CnmeGV(ORF numbers of CnmeGV)	Genes missing in CnmeGV
Transcription (9)	*lef-11*(*29*), *39k*(*30*), ***p47*(*50*)**, *lef-6*(*60*), ***lef-5*(*69*)**, ***lef-4*(*76*)**, ***vlf-1*(*90*)**, ***lef-9*(*100*)**, ***lef-8*(*109*)**	*pe-38*
Replication (12)	*ie-1*(*6*), ***lef-2*(*21*)**, ***lef-1*(*54*)**, *dbp*(*61*), ***dnapol*(*63, 107*)**, ***helicase*(*72*)**, *rr1*(*93*), *dnaligase*(98), *lef-3*(*104*), *me53*(*118*)	*rr2*
Structure (30)	*granulin*(*1*), *p78/83*(*2*), *pk-1*(*3*), ***odv-e18*(*11*)**, *pep-1*(*15*), *pep/p10*(*17*), *pep-2*(*18*), ***odv-ec43*(*32*)**, *odv-e66*(*41*), *efp*(*45*), *BV-e31*(*51*), *p24*(*52*), ***p6.9*(*68*)**, ***odv-e25*(*73*)**, ***p18*(*74*)**, ***p33*(*75*)**, ***vp39*(*77*)**, ***odv-e27*(*78*)**, *tlp20*(*83*), ***gp41*(*88*)**, *fp25k*(*99*), ***vp1054*(*115*)**	--
Per os infectivity	***pif-5*(*13*)**, ***p74*(*27*)**, ***pif-3*(*42*)**, ***pif-2*(*48*)**, ***pif-1*(*55*)**, ***pif-4*(*71*)**, ***vp91* (*82*)**, ***pif-6*(*103*)**	--
Auxiliary (11)	*sod*(*28*), *ubiquitin*(*33*), *mp-nase*(*39*), *fgf-1*(*56*), *bro-A*(*57*), *bro-B*(*85*), *bro-C*(*86*), ***alk-exo*(*94*)**, *fgf-2*(*96*), *iap-5*(*101*), *fgf-3* (*117*)	*chitinase*, *cathepsin*, *ctl*, *p35*, *lef-10*, *egt*, *gp37*, *enhancin*
Unknown (45)	*4*, *5*, *7*, *8*, ***p49* (*12*)**, *14*, *16*, *20*, *22*, *25*, *p22.2* (*26*), *31*, *34*, *35*, *36*, *37*, *38*, *40*, *43*, *44*, *49*, *38.7k* (*53*), *59*, *62*, *64*, ***p45/48*(*65*)**, *p12*(*66*), ***p40/c42*(*67*)**, *38k*(*70*), *80*, ***87***, ***89***, *91*, *92*, *95*, *102*, *105,* ***desmoplakin* (*106*)**, *108*, *110*, *111*, *112*, *113*, *114*	--
CnmeGV unique (13)	*9*, *10*, *19*, *23*, *24*, *46*, *47*, *58, 79*, *81*, *84*, *97*, *116*	--

Note: The ORFs that are presented in all sequenced baculoviruses are printed in bold.

omes (Taha et al., 2000; Luque et al., 2001; Ferrelli et al., 2012; Zhang et al., 2014). These enzymes can catalyze the reduction of host cell ribonucleotide diphosphates to yield deoxyribonucleotides (Lange and Jehle, 2003). Of these genes, CnmeGV contains only the *rr1* (Cnme93) gene, which has been also found in *Agrotis segetum* granulovirus (AgseGV), CpGV, *Epinotia aporema* granulovirus (EpapGV) and PhopGV (Taha et al., 2000; Luque et al., 2001; Ferrelli et al., 2012; Zhang et al., 2014).

CnmeGV also encodes a DNA ligase (*dnaligase*, Cnme98) which is present in all sequenced GVs. *dnaligase* seems to be linked to the appearance of a second helicase, *helicase-2* (Herniou et al., 2003), which is not found in CnmeGV. Other genes related to DNA replication, including DNA binding protein (*dbp*, Cnme61) and *me-53* (Cnme118), have also been identified in the CnmeGV genome.

Structural genes. In the CnmeGV genome, 19 core structural genes were identified, such as the core protein, *p6.9* (Cnme68); the tegument protein, *gp41* (Cnme88); the capsid-associated proteins, *38k* (Cnme70), *vp39* (Cnme77) and *vp1054* (Cnme115); the occlusion-derived virus (ODV) envelope proteins, *odv-e18* (Cnme11), *odv-e25* (Cnme73) and *odv-e27* (Cnme78); the *per os* infectivity factors *p74* (Cnme27), *pif-1* (Cnme55), *pif-2* (Cnme48), *pif-3* (Cnme42), *pif-4* (Cnme71), *pif-5* (Cnme13), *pif-6* (Cnme103) and *vp91* (Cnme82). Other core genes, including *p18* (Cnme74) which are related to the viral nucleocapsid and *p33* (Cnme75) which encodes a type of a sulfhydryl oxidase (Wu and Passarelli, 2010), were also identified.

Some non-core structural genes were found in CnmeGV genome, including *granulin* (Cnme1), *p78/83* (Cnme2), *pk-1* (Cnme3), *pep-1* (Cnme15), *pep/p10* (Cnme17), *pep-2* (Cnme18), *odv-e66* (Cnme41), *efp* (Cnme45), *BV-e31* (Cnme51), *p24* (Cnme52), *tlp20* (Cnme83) and *fp25k* (Cnme99), but CnmeGV lacks a homologue of odv-e56.

Auxiliary genes. Auxiliary genes are not essential for viral replication, but they can provide a selective advantage (O'Reilly, 1997). The CnmeGV genome contains neither a *chitinase* nor *cathepsin* gene (Table 1). That may explain why CnmeGV infected insects do not show the phenotype of typical liquefaction. Among the auxiliary genes, *ubiquitin* is the most conserved one and it has been found in all sequenced GVs. Cnme33 is an *ubiquitin* homologue, showing 80% mean amino acid identity to those of the sequenced GVs (Supplementary Table S1). All of the sequenced GV genomes encode three fibroblast growth factors (*fgf*s), and CnmeGV is not an exception, with Cnme56, 98 and 117 at the corresponding locations as seen in other GVs. CnmeGV also contains a *sod* (Cnme28) and a *iap* gene (*iap-5*, Cnme101). Three *baculovirus repeated orf* (*bro*) genes have also been found in CnmeGV including *bro-a*(Cnme57), *bro-b*(Cnme85) and *bro-c*(Cnme86). The absence or duplica-

tion of these genes is common in baculovirus (Zhou et al., 2012). BRO has been demonstrated to possess DNA binding activity, especially to single stranded DNA (Zemskov et al., 2000).

DISCUSSION

Rice is a very important crop all over the world. The Asiatic rice leafroller, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae), is the main migratory pest of rice with 1–11 generations per year, depending on its geographical distribution in rice-planting regions (Chai et al., 2011). The vast use of chemical insecticides for controlling *C. medinalis* results in increased insect resistance and environment pollution. After first isolated in *C. medinalis* larvae in 1979, CnmeGV still existed stably in the C. medinalis population *in situ* for more than 30 years (Zhang et al., 2014), which suggests that CnmeGV is a potential specific and effective pathogen to control the target pest in field. The genomic analyses of CnmeGV will be of great helpful for developing it as a biological pesticide for the control of *C. medinalis*.

Strong colinearity is observed in GV genomes sequenced to date (Luque et al., 2001; Lange and Jehle, 2003; Wormleaton et al., 2003; Hilton and Winstanley, 2008). However, CnmeGV differs from the rest of sequenced GVs by a 23 kb and a 17 kb gene block inversions. The first inversion begins with Cnme25 and ends with Cnme45. Except 3 core genes (*p74*, *odv-ec43*, *pif-3*), most of genes in this region are baculovirus common genes. The secondary inversion contain genes from Cnme92 to Cnme108. Four of the baculovirus core genes can be found in this region: *alk-exo, lef-9, pif-6 and desmoplakin*. Baculovirus genes with different functions are usually scattered throughout the genomes and ORFs are present on both strands of the DNA, so does the genes within the two fragments. Although the gene block inversion is uncommon in GVs, it is a common feature in NPVs, even in a same phylogenetic clade. No research result shows the function or effect of the inversion.

Homologous repeated sequences (*hrs*) are found in most baculovirus genomes with varying length (Ferrelli et al., 2012). *Hrs* were suggested to be origins of viral DNA replication (Kool et al., 1993; Hilton and Winstanley, 2008) and enhancers for transcription (Guarino et al., 1986; Choi and Guarino, 1995; Rodems and Friesen, 1995). However, the CnmeGV genome does not contain typical baculovirus *hrs* but probably contain a hairpin structure. A *non-hr* origin that contains palindromic and repetitive sequences in a complex organization was also demonstrated to initiate replication (Kool et al., 1993; Wu and Carstens, 1996). Whether this is a functional *non-hr* origin for CnmeGV needs further analysis.

Auxiliary genes are also compared and analyzed in this study. *Chitinase* and *cathepsin* genes are related to the breakdown of insect tissues at the end of infection. Deleting either the *chitinase* or *cathepsin* gene of AcMNPV results in the failure of host liquefaction (Slack et al., 1995; Hawtin et al., 1997). The absent of *chitinase* and *cathepsin* genes in CnmeGV genome explains why infection of CnmeGV cannot result in liquefaction of the host insects in a typical manner (Zhang et al., 2014).

In summary, the complete genome of CnmeGV was sequenced and analyzed in the present study. These results suggested that CnmeGV is a distinct species in *Betabaculovirus* and has closer relationship to AdorGV than to the other baculoviruses. CnmeGV genome differs from the rest of sequenced GVs by a 23 kb and a 17 kb gene block inversions. It does not contain typical baculovirus *hr*, but probably contains a new repeat structure. More interestingly, *chitinase* and *cathepsin* genes, which are reported to have major roles in the liquefaction of the hosts, were not found in the CnmeGV genome. This study, along with future studies about the characterization of CnmeGV infection, will be useful for the better understanding of the pathology caused by this virus and its potential utilization as a bioinsecticide.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

AUTHOR CONTRIBUTIONS

KY and FD designed the experiments. SZ, ZZ, SS and QC carried out the experiments. SZ, ZZ, FD, and KY analyzed the data. SZ, FD and KY wrote the paper. All authors have read and approved the final manuscript.

Supplementary Table S1 is available on the website of *Virologica Sinica*: www.virosin.org; link.springer.com/journal/12250.

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