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Isolation and identification of a new *Bombyx mori* nucleopolyhedrovirus strain isolated from Yunnan, China

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ABSTRACT: *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a silkworm pathogen that causes serious economic losses in sericulture. In this study, a new BmNPV strain, designated as BmNPV-YN1, from Yunnan (China), was isolated and identified through molecular identification. Sequence analysis results revealed that *polyhedrin* (*polh*) presented the highest identity among various BmNPVs; this gene indicated that BmNPV-YN1 is a BmNPV strain. The baculovirus repeated-ORF (*bro*) genes of this isolate were different from those of six BmNPV strains (BmNPV-T3, BmNPV-ZJ, BmNPV-GX, BmNPV-India, BmNPV-Cubic, and BmNPV-Brazilian). The BmNPV-YN1 *bro* family includes *bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e*, which are similar to those of BmNPV-T3. Sequence alignment results further showed the differences in the composition and sequences of the *bro* family members among various strains. The *bro-d* sequences were also phylogenetically analysed. Results demonstrate that BmNPV-YN1, BmNPV-Cubic, and BmNPV-India are closely related. Despite this close relationship, BmNPV-YN1 belongs to a distinct cluster. The BmNPV DNA was subjected to restriction endonuclease analysis and the obtained results provided additional evidence supporting the identity of the novel BmNPV-YN1 strain. Indeed, BmNPV-YN1 isolated from Yunnan is a new BmNPV strain. The proposed method can be used to rapidly and accurately detect and identify BmNPVs strains.

KEYWORDS: *Bombyx mori* nucleopolyhedrovirus, strain identification, *polh*, *bro*

INTRODUCTION

The *Baculoviridae* is a diverse family of insect viruses that contain double-strand circular DNA genomes ranging from 80–180 kb in length. This viral family can be classed into four genera, *Alphabaculovirus*, *Betabaculovirus*, *Gammabaculovirus*, and *Deltabaculovirus*, based on linkage evolutionary analysis of 31 conserved genes[1]. The importance of the study of the *Baculoviridae* resides in the fact that they can be used as biological control agent against insect pest and protein expression systems[2]. *Bombyx mori* nucleopolyhedrovirus (BmNPV) is a relatively narrow-host-range baculovirus, which is one of the most serious pathogens that cause annual economic losses in sericulture, and belongs to the genus *Alphabaculovirus*[3]. To date, there are six BmNPV strains which have been completely sequenced. The sizes of these six viral genomes differ; and among them, the first sequenced strain BmNPV-T3 genome was 128 413 bp long and contained 136 open-reading frames (ORFs)[4].

Numerous polyhedra are produced during an epizootic outbreak when an insect host is infected. Polyhedra are composed of polypeptide known as polyhedrin (polh), which is encoded by the *polyhedrin* (*polh*) gene. The polyhedra play an important role in protection of occluded viruses. The *polh* gene is highly conserved among baculoviruses; as such, more than 80% sequence identity has been detected among lepidopteran baculovirus Polhs[5]. The BmNPV polyhedrin gene is non-essential late gene for BmNPV replication and the ORF contains 738 nucleotides[6]. The *polh* gene has not only been used in phylogenetic studies but also in the molecular identification of baculoviruses specific to lepidoptera[7–10].

Baculovirus-repeated ORFs (*bro*) are widespread among invertebrate viruses. They comprise a class of complex gene family present in baculovirus and its copy number varies from 1–16 in different baculovirus genomes. The duplication and loss of *bro* genes among genotypic variants of baculovirus commonly occur[11,12]. As with these baculoviruses, BmNPV exhibits a divergence in *bro* genes, which is also one of the major differences among ORFs of

BmNPV strains[13–15]. The function of these genes during baculovirus infection remains unclear, although the properties of some individual bro proteins have been characterized[16–19].

In the current study, a new BmNPV strain named YN1 was identified through molecular identification. The highly conserved *polh* was used to identify BmNPV. The conserved *bro* family genes were also applied to identify the BmNPV strain. The results of restriction endonuclease analysis confirmed that YN1 is a new strain of BmNPV.

MATERIALS AND METHODS

Collection and isolation of the virus strain

Infected silkworm samples were collected from one sericultural farmer in Dali, Yunnan (China) on the basis of the classic symptom of BmNPV infection. The isolated sample was named BmNPV-YN1. Dead silkworm larvae were pounded using a mortar and pestle. The pounded samples were then sieved through three layers of gauzes with distilled water. The BmNPV polyhedra were isolated through differential centrifugation.

The viral polyhedra were layered onto four 50–60% (w/v) discontinuous sucrose gradients and were centrifuged at 2200*g* for 30 min. Then the polyhedra were resuspended in TE buffer and stored at 4 °C. The BmNPV strain was stored in our laboratory and used in accordance with the same procedure described above. The purity of the isolate was confirmed through the restriction endonuclease analysis.

Cloning and sequencing of *polh* and *bro* genes

Genomic DNA was extracted in accordance with the previously described procedure[10]. The fragments of *polh* and *bro* family members (*bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e*) for BmNPV-YN1 were cloned and sequenced. Primers were designed using the BmNPV-T3 sequence (GenBank accession No. NC_001962.1) available in the NCBI database. The PCR primers were designed by PRIMER PREMIER 5.0 and listed in Table 1.

Table 1 PCR primer pairs.

Target genes	Primer sequences
polh	F: ATGCCGAATTATTCATACACCC
	R: TTAATACGCCGGACCAGTG
bro-a	F: ATGGCTCAAGTTAAAATTGG
	R: TTACAAGTTAAAATTGTTATTC
bro-b	F: ATGGCTCAAGTTAAAATCGGGC
	R: TTAGTTTTGCGAGCAGTGGGGC
bro-c	F: ATGGCTCAAGTTAAAATTGG
	R: TGCTTAAACGCTTGACGACATA
bro-d	F: TATTGCGCCGCAGGAAGCCAT
	R: CGAATCGTCACGCGTCGTTGTA
bro-e	F: ATTAGTTTTGCGAGCAGTG
	R: ATGGCTCAAGTTAAAATTGG

Sequence alignment and phylogenetic analysis

The nucleotide sequences of the BmNPV-YN1 *polh* gene were aligned with those of the six other known BmNPVs by using CLUSTALX 2.0. The *polh* nucleotide of BmNPV-YN1 and the corresponding amino acid sequences were compared with those of other BmNPVs and lepidopteran NPVs through BLAST analysis. The *bro* sequences of BmNPV-YN1 were aligned with those of other BmNPV strains pairwise sequence alignment via BLAST. Phylogenetic trees were constructed on the basis of the *bro-d* nucleotide sequence from different BmNPV strains by using MEGA 5.0. The reliability of clusters in the tree was evaluated on the basis of 1000 bootstrap replications. The genome sequences of the BmNPV strains, namely, T3, ZJ, GX, Cubic, and India have been completely sequenced. These genome sequences are available in the NCBI database.

Analysis of restriction endonuclease digestion

The viral DNA was digested using three restriction enzymes (*Eco*RI, *Bam*HI, *Hind*III) in accordance with the manufacturer's instructions. The viral DNA (1 μg) was mixed with 10 U restriction enzyme and incubated for 5 h at 37 °C. Reactions were terminated by mixing the reaction mixture with one-sixth volume of 6× loading buffer solution. The samples were analysed through electrophoresis on 0.8% agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

Itshape larvae bioassays

Polyhedra of the BmNPV-YN1 and BmNPV-T3 were purified and suspended in distilled water. The newly moulted silkworm larvae of the 5th-instars were used for investigation of per oral infection efficiency. The polyhedral bodies (PIBs) were quantified by visual counting in a haemacytometer. They were diluted in distilled water to produce concentration 5×10^8, 4×10^8, 1×10^8, or 1×10^6 PIBs/ml and 10 μl of each dilution were fed orally to the larvae. Twenty larvae per group were used and each group was repeated in triplicate. Infected larvae were reared individually in culture dish and monitored daily until all larvae had either pupated or died.

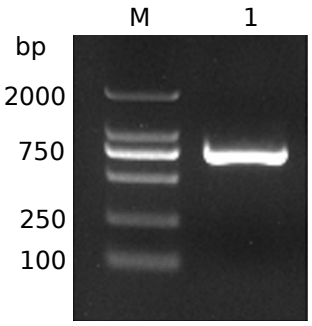

Fig. 1 PCR product of *polh* gene for the verification of BmNPV-YN1. M: DL2000 molecular marker; lane 1: DNA of BmNPV-YN1.

Dead larvae were counted and dose-mortality date was determined by Probit analysis using SPSS.

RESULTS

Identification, cloning, and analysis of the *polh* gene

The *polh* gene containing 738 bp was amplified successfully through PCR by using the BmNPV-YN1 genomic DNA as a template (Fig. 1). The *polh* gene of BmNPV-YN1 was cloned and sequenced. The sequences were registered in GenBank with the accession number of KR139829. The nucleotide sequence was further aligned with five other *polh* gene sequences of BmNPVs by using CLUSTALX 2.0. The result showed that the *polh* gene of BmNPV-YN1 exhibited 99.96% sequence identity to the six-*polh* genes of the other BmNPV strains (Fig. 2). The results of BLAST analysis also showed that the nucleotide and amino acid sequences of the *polh* gene of BmNPV-YN1 revealed high identity (99% for *polh* gene sequences and 100% for polh protein sequences) to those of other BmNPVs and low identities (between 74% and 90% for *polh* gene sequences and between 81% and 94% for polh protein sequences) to other lepidopteran-specific baculoviruses (Table 2). The high degree of sequence identity to the *polh* sequences of other BmNPVs suggested that these sequences are orthologs of the BmNPV *polh* gene in this study. The results of PCR verification and sequence alignment analysis confirmed that BmNPV-YN1 is a BmNPV strain.

Cloning, sequencing, sequence alignment of *bro* and phylogenetic analysis

Kang et al[16] found that *bro-a*, *bro-c*, and *bro-d* of BmNPVs are essential for the survival of BmNPVs. The *bro-d* gene is partially necessary to induce viral infection. All members of the *bro* family have been cloned in BmNPV-YN1, which is similar to BmNPV-T3 with five bro genes: *bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e*. The sequences of these genes were submitted to GenBank with the following accession numbers: KR139830, KR139831, KR139832, KR139833, and KR139834 for *bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e*, respectively. Using BmNPV-YN1 aligned with the six other BmNPV strain, we observed that *bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e* genes of BmNPV-YN1 share low identity to their corresponding *bro* genes in the six other BmNPV strains, whose homologies varied from 86% to 96% (Table 3). The results also indicated that the composition of the *bro* family

Table 2 Sequence identity of the *polh* gene or polh protein of BmNPV-YN1 to those of six other BmNPV strains and homologous genes from other lepidopteran-specific baculoviruses.

Viral strain	Gene Acc. No.*	SeqAl† (%)	
		NuS[a]	AmiA[b]
BmNPV-T3	NC-001962.1	99	100
BmNPV-ZJ	JQ991008.1	99	100
BmNPV-GX	JQ991011.1	99	100
BmNPV-Cubic	JQ991009.1	99	100
BmNPV-India	JQ991010.1	99	100
BmNPV-Brazilian	KJ186100.1	99	100
Philosamia cynthia ricini‡	JX404026.1	83	94
Maruca vitrata‡	EF125867.1	90	93
Orgyia pseudotsugata‡	U75930.2	82	91
Hyphantria cunea‡	AP009046.1	82	91
Autographa californica‡	L22858.1	79	86
Helicoverpa armigera‡	AF303045.2	74	83
Spodoptera litura‡	NC-003102.1	74	81

* Gene accession number.
† Sequence alignment with BmNPV-YN1.
‡ NPV.
[a] Nucleotide sequence identity.
[b] Amino acid sequence identity.

Table 3 Identity of the *bro* family members of BmNPV-YN1 to those of the six other BmNPV strains.

Strain	Nucleotide sequence identity (%)				
	bro-a	*bro-b*	*bro-c*	*bro-d*	*bro-e*
T3	95	95	86	94	91
ZJ	94	–	87	93	–
GX	96	93	90	94	–
Cubic	94	–	86	94	91
India	91	–	88	94	–
Brazilian	91	93	88	94	–

"–" indicates lack of gene.

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BmNPV-India ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
BmNPV-Cubic ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
BmNPV-GX    ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
BmNPV-T3    ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
BmNPV-ZJ    ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
BmNPV-YN1   ATGCCGAATTATTCATACACCCCCACCATCGGGCGTACTTACGTGTACGACAATAAATATTACAAAAACTTGGGCTGTCTTATCAAAAACGCCAAGCGCAAGAAGC   106
Consensus   atgccgaattattcatacaccccaccatcgggcgtacttacgtgtacgacaataaatattacaaaaacttgggctgtcttatcaaaaacgccaagcgcaagaagc

BmNPV-India ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
BmNPV-Cubic ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
BmNPV-GX    ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
BmNPV-T3    ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
BmNPV-ZJ    ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
BmNPV-YN1   ACCTAGTCGAACATGAACAAGAGGAGAAGCAATGGGATCTTCTAGACAACTACATGGTTGCCGAAGATCCCTTTTTAGGACCGGGCAAAAACCAAAAACTTACCCT   212
Consensus   acctagtcgaacatgaacaagaggagaagcaatgggatcttctagacaactacatggttgccgaagatccctttttaggaccgggcaaaaaccaaaaacttaccct

BmNPV-India TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
BmNPV-Cubic TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
BmNPV-GX    TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
BmNPV-T3    TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
BmNPV-ZJ    TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
BmNPV-YN1   TTTTAAAGAAATTCGCAGTGTGAAACCCGATACCATGAAGTTAATCGTCAACTGGAGCGGCAAAGAGTTTTTGCGTGAAACTTGGACCCGTTTTGTTGAGGACAGC   318
Consensus   ttttaaagaaattcgcagtgtgaaacccgataccatgaagttaatcgtcaactggagcggcaaagagtttttgcgtgaaacttggacccgttttgttgaggacagc

BmNPV-India TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
BmNPV-Cubic TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
BmNPV-GX    TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
BmNPV-T3    TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
BmNPV-ZJ    TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
BmNPV-YN1   TTCCCCATTGTAAACGACCAAGAGGTGATGGACGTGTACCTCGTCGCCAACCTCAAACCCACACGCCCCAACAGGTGCTACAAGTTCCTCGCTCAACACGCTCTTA   424
Consensus   ttccccattgtaaacgaccaagaggtgatggacgtgtacctcgtcgccaacctcaaacccacacgccccaacaggtgctacaagttcctcgctcaacacgctctta

BmNPV-India GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGCGGCGG   530
BmNPV-Cubic GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGTGGCGG   530
BmNPV-GX    GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGCGGCGG   530
BmNPV-T3    GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGCGGCGG   530
BmNPV-ZJ    GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGCGGCGG   530
BmNPV-YN1   GGTGGGAAGAAGACTACGTGCCCCACGAAGTAATCAGAATTGTGGAGCCATCCTACGTGGGCATGAACAACGAATACAGAATTAGTCTGGCTAAAAAGGGCGGCGG   530
Consensus   ggtgggaagaagactacgtgccccacgaagtaatcagaattgtggagccatcctacgtgggcatgaacaacgaatacagaattagtctggctaaaaaggg ggcgg

BmNPV-India CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
BmNPV-Cubic CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
BmNPV-GX    CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
BmNPV-T3    CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
BmNPV-ZJ    CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
BmNPV-YN1   CTGCCCAATCATGAACATCCACAGCGAGTACACCAACTCGTTCGAGTCGTTTGTGAACCGCGTCATATGGGAGAACTTCTACAAACCCATCGTTTACATCGGCACA   636
Consensus   ctgcccaatcatgaacatccacagcgagtacaccaactcgttcgagtcgtttgtgaaccgcgtcatatgggagaacttctacaaacccatcgtttacatcggcaca

BmNPV-India GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCGCCTCTGTTCACTGGTCCGGCGTATTA          737
BmNPV-Cubic GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCGCCTCTGTTCACTGGTCCGGCGTATTA          737
BmNPV-GX    GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCGCCTCTGTTCACTGGTCCGGCGTATTA          737
BmNPV-T3    GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCGCCTCTGTTCACTGGTCCGGCGTATTA          737
BmNPV-ZJ    GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCGCCTCTGTTCACTGGTCCGGCGTATTA          737
BmNPV-YN1   GACTCTGCCGAAGAAGAGGAAATCCTAATTGAGGTTTCTCTCGTTTTCAAAATAAAGGAGTTTGCACCAGACGCACCTCTGTTCACTGGTCCGGCGTATTA          737
Consensus   gactctgccgaagaagaggaaatcctaattgaggtttctctcgttttcaaaataaaggagtttgcaccagacgc cctctgttcactggtccggcgtatta
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Fig. 2 Multiple sequence alignment of the conservative *polh* gene in different BmNPV strains. Alignment was conducted using CLUSTALX.

members is diverse in various BmNPV strains. For example, *bro-d* and *bro*-e genes are absent in the BmNPV strains isolated in India and Thailand; in contrast, *bro-a*, *bro-b*, *bro-c*, *bro-d*, and *bro-e* genes are present in BmNPV-T3 and BmNPV-YN1.

The phylogenetic tree of the seven BmNPV strains was constructed on the basis of the *bro-d* gene by using MEGA 5.0 with the neighbour-joining method (Fig. 3). Based on the phylogenetic tree, two major groups were found in BmNPV strains. One group was composed of three strains, namely, T3, GX, ZJ, and Brazilian. The BmNPV-YN1 strain belonged to another group, which was further clustered into two clades. BmNPV-YN1 shares closer evolutionary relationships with the strains Cubic and India.

Restriction enzyme analysis

The viral DNA of BmNPV-YN1 and BmNPV-T3 were analysed by comparing the fragmentation profiles that obtained by digesting the viral DNA with *Eco*RI, *Bam*HI, and *Hind*III. In this procedure, *Hind*III-digested λ-DNA (Takara) was used as a molecular size marker. The image in Fig. 4 showed the distinct difference between the BmNPV-YN1 strain and the typical strain BmNPV-T3 in some digested fragments.

Biological activity in *B. mori* larvae

The study on comparative infections between BmNPV-YN1 and BmNPV-T3 showed that the LC_{50} values of BmNPV-YN1 and BmNPV-T3 were 1.34 ×

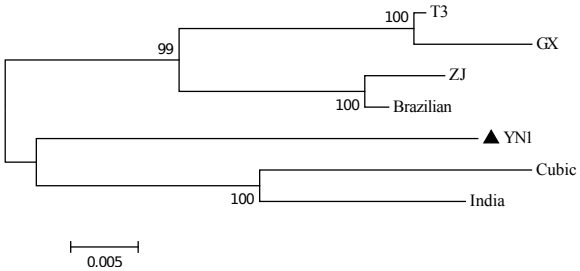

Fig. 3 Phylogenetic tree of various BmNPV strains constructed on the basis of the nucleotide sequences of *bro-d*. This tree was constructed in MEGA 5.0 by using the neighbour-joining method with 1000 bootstrap replicates.

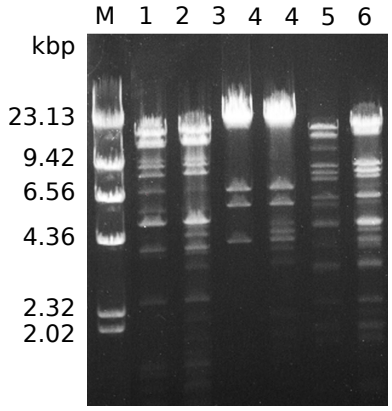

Fig. 4 Restriction endonuclease digestion profiles of the genomic DNA of BmNPV-YN1 and the typical BmNPV-T3. The viral DNA of BmNPV-YN1 (lanes 1, 3, and 5) and the typical BmNPV-T3 (lanes 2, 4, and 6) were digested with the restriction endonucleases *Eco*RI (lanes 1 and 2), *Bam*HI (lanes 3 and 4), and *Hind*III (lanes 5 and 6).

10^8 PIBs/ml and 1.44×10^8 PIBs/ml, respectively (Table 4). This result suggests that pathogenicity of BmNPV-YN1 is similar to that of BmNPV-T3.

DISCUSSION

Humans have reared *B. mori* for silk production. However, BmNPV infection has commonly occurred in silk-producing areas. Diverse baculovirus genomes can undergo deletion, point mutation, and recombination[20]. Likewise, the BmNPV genome undergoes similar processes. The identification of epidemic BmNPV strains is an important step to monitor and control the diseases. Five new strains of BmNPV (BmNPV-ZJ, BmNPV-GX, BmNPV-Cubic, BmNPV-Brazilian, and BmNPV-India) have been sequenced and identified since BmNPV-T3 was sequenced in 1999[4]. Although various genes, such as gene for DNA polymerase, *polh*, *ie-1*, *egt*, and *lef-8*, were used to classify viruses, baculovirus strains have rarely been identified[21–24]. Novel baculovirus strains are identified through genome sequencing or restriction endonuclease analysis, which are considered as major identification methods[25,26]. However, genome sequencing is costly and restriction endonuclease analysis cannot easily reveal small genome variation. Thus a simple and convenient identification method should be established. In the present study, a new strain of BmNPV was identified through molecular identification. Our preliminary study suggested that the highly conserved *polh* gene could be used to identify BmNPV; the conserved *bro* family genes can also be applied to identify BmNPV strains.

Table 4 LC_{50} of BmNPV-YN1 and BmNPV-T3 in *B. mori* larvae.*

virus	LC_{50} (PIBs/ml)	95% confidence limits	
		Lower	Upper
BmNPV-T3	1.44×10^8	1.05×10^8	1.93×10^8
BmNPV-YN1	1.34×10^8	9.72×10^7	1.80×10^8

* Fifth instar *B. mori* larvae were used.

The *polh* gene is highly conserved in lepidopteran-specific baculoviruses. This gene is used not only in phylogenetic studies but also in the molecular identification of lepidopteran-specific baculoviruses. The results of multiple sequence alignment analysis revealed that *polh* is highly conserved during BmNPV evolution; the *polh* gene of BmNPV-YN1 showed 99.96% identity to the six-*polh* genes of the other BmNPV strains. Nucleotide sequences were strongly conserved (99% identity) resulting in high amino acid sequence identity (100% identity). This study provided strong evidence supporting BmNPV-YN1 as a BmNPV strain. Indeed, *polh* sequences can be used to identify virus samples related to BmNPVs, this result is consistent with that of Liang et al[10].

The genomic divergence among isolated BmNPVs is mostly observed in *bro* genes and homologous regions[27,28]. In our study, the diversity of *bro* family was investigated. The results of cloning and sequencing of these genes showed the difference in sequence alignment between BmNPV-YN1 and other BmNPV strains. These results indicate that BmNPV-YN1 might be a new BmNPV strain. The sequence analysis results also show a difference in the composition and sequences of *bro* family members among various BmNPV strains. The phylogenetic tree of the *bro-d* gene of BmNPVs revealed that BmNPV-YN1 is

located in a separate branch. This result suggested that BmNPV-YN1 is a distinct strain. The diverse features of *bro* genes are also observed in different baculovirus species, such as *Helicoverpa armigera* nucleopolyhedrovirus[29] and *A. californica* multiple nucleopolyhedrovirus[30]. Thus the diversity of the *bro* gene family indicates that this gene could be used to identify a new BmNPV strain or some lepidopteran NPVs.

Restriction enzyme profiles further validated that the BmNPV-YN1 isolated from Yunnan was a new BmNPV strain. This method is an efficient and relatively simple method used to identify baculoviruses and constitute an important tool to distinguish NPV isolates[31,32]. Thus new BmNPV strain can be identified on the basis of the *bro* gene family.

In conclusion, the proposed molecular identification method was used successfully to identify and distinguish several isolates from a BmNPV collection. This study provide relevant information regarding the characteristics of our isolates and help us understand the diversity of BmNPV isolates.

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