

Molecular epidemiology of *Bombyx mori* nucleopolyhedrovirus in Yunnan sericulture region, China

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Received 7 Dec 2017

Accepted 31 Aug 2019

ABSTRACT: Sericultural production is seriously influenced by the blood pathogens of grasserie, *Bombyx mori* nucleopolyhedrovirus (BmNPV). To investigate the infectious condition and define an effective control method for BmNPV, 233 from 240 suspected samples infected with BmNPV were detected using PCR identification. One sample was selected from each county in the positive sample collection area, 56 isolates of which were used as experimental objects. The homology of the *bro-d* nucleotide sequence was at least 91% among the 56 BmNPV isolates. However, the conserved 7 amino acids (percentage of amino acid constant sites, PAACS) at the position 217–224 were absent for YNQJLL001 and YNZTQJ074 pandemic isolates. Phylogenetic tree analysis of *bro-d* gene separated the BmNPV isolates from Yunnan into 4 main clades: I, II, III, and IV. Clade I was the dominant clade containing 32 local epidemic isolates. Clades II, III, and IV contained 5, 16, and 3 pandemic isolates, respectively. Geographical distribution of clade I in Yunnan showed a concentrated pattern, while the remaining members of the other three clades depicted no direct contact in relation to their locations. This study may provide valuable molecular types on BmNPV isolate distribution in the silkworm-raising regions, also be helpful for evaluating BmNPV pathogenesis and epidemic situations in the Yunnan sericulture region.

KEYWORDS: *Bombyx mori* nucleopolyhedrovirus, *bro-d*, evolutionary analysis, BmNPV

INTRODUCTION

Bombyx mori nucleopolyhedrovirus (BmNPV) belongs to the baculovirus division (Baculoviridae), with inclusions in the true baculovirus subfamily (*Eubaculovirinae*) karyotype polygonal body virus (nucleopolyhedrovirus)¹. Silkworm is infected by BmNPV, causing blood grasserie, and its first symptom is a colour change of the epidermis from white to yellowish white, followed by the swelling of body segments, and finally to a state of purulence and death². The disease causes serious harm in sericulture-prevalent regions of the world. While this disease occurs in varying seasons among some sericultural regions of China, it particularly affects the Yunnan region during the summer. This accounts for 70–90% of the total loss in cocoon production³. The average incidence rate is about 5% in Yunnan sericulture regions. The disease incidence has risen in recent years; for example, some regions have experienced an incidence rate of more than 13%⁴.

BmNPV can generate two kinds of virus particles

in the developmental cycle. A sprouting circulation type virus (budded virions, BV) and an embedding virus (occluded virions, ODV) alternately infect the silkworm completely⁵. The silkworm lacks immunity required to combat this two-phase attack of alternating infection. Hence the study of BmNPV itself is focused on its prevention and cure. In recent years, the research of BmNPV genome and functional gene identification showed significant progress. However, few reports exist that focus on transgenerational BmNPV genetic traits from isolated geographical environments (e.g., morphology, pathogenicity, serological reaction, biological physicochemical properties, and so on) and on determining whether differences between BmNPV from various ecological and geographic regions exist, or if they are identical strains. A study performed by Qi found that the *vp39* gene homology of BmNPV isolates from China and Japan were above 98%⁶, and polyhedra production and gene structures showed differences among the three BmNPV isolates were from Korean farm⁷. The evolution

analysis found that Guangxi BmNPV isolates were divided into three groups based on the *polyhedrin* and *p10* genes, which suggested that the Guangxi sericulture regions had distributed with different pandemic strains⁸.

Some DNA viruses are diverse due to their high levels of adaptability and rapid evolution⁹. Baculoviruses pathogenic to lepidopteran larvae (silkworms) exhibit considerable genetic diversity. BmNPV run parallel with one another in genome evolution¹⁰ and host selection pressure results in either a BmNPV gene loss or increase. Some genes of BmNPV are deleted or increased, thus improving the ability of virus to infect the host and adapt to the environment and result in viral genome diversity. Genome evolution is mainly caused by gene duplication and horizontal gene transfer¹¹. Gene duplication creates BmNPV diversity between genome strains¹². The *bro* (baculovirus repeated open reading frames) gene family is typical of the insect baculovirus genome, and they are widely distributed in the insect baculovirus genome. However, gene fragment size and copy number for the type of virus is different. Different *bro* genes in the virus genome copy numbers were 1–16^{13–16}, and this is often used to distinguish between BmNPV strains¹⁷. The *bro* gene exists in insect baculovirus, which is not only found in the insect iridescent virus (Iridovirus), but with high homologous genes as well¹⁸. With NPV as the host of the silkworm genome, the *bro* gene copy number is also different (such as Zhou by Japanese seedlings BmNPV, BmNPV-T3 and BmNPV-Thailand, Chongqing plant BmNPV-CQ1, BmNPV-GD, French seedlings BmNPV-SC7)¹⁹. The comparative analysis of the different BmNPV strains found that the *bro-a*, *bro-b*, *bro-c*, *bro-d*, *bro-e* 5 gene copies were present in the BmNPV-T3 strain, where the *bro-a* gene was present in one region of the genome alone, and *bro-b* and *bro-c* were present in a region of the genome in series. *Bro-d* and *bro-e* are also present in the other region of the genome in series. *Bro* genes are highly conserved in many BmNPV genomes. The percentage of amino acid conserved sites (PAACS) of *bro* gene in the genome of different BmNPV strains was over 82%. The *bro-a*, *bro-c*, and *bro-d* genes make up 83%, 87%, and 83%, respectively²⁰. Deletion mutants of the *bro* gene constructed in the BmNPV genome in which the *bro-a*, *bro-b*, *bro-c*, and *bro-e* genes individually inactivated are successfully isolated. However, exhaustive efforts failed to isolate a *bro-d* deficient mutant. It may be possible to isolate a double-deletion textitbro-a and *bro-c* mutant. This shows that the *bro-d* gene plays an

irreplaceable role in the infection of silkworms by this virus²¹.

Blood grasserie, a common disease in Yunnan sericulture regions, presents a serious threat to cocoon production in that province. The Yunnan province located in southwest China with an average elevation of about 2000 m is abundant in biological, geographical, and ecological diversity. A diverse population of insects could be infected by BmNPV in mulberry orchards. There are various geographical conditions and climate in Yunnan sericulture region, such as Puer, tropical region; Honghe-Mengzi, subtropical region; Qujing, northern subtropical or temperate region; and Dali-Heqing, high-altitude and cold region. Some questions regarding whether these factors may have led to the genetic diversity of BmNPV strains, or that isolate would be capable of causing a major epidemic existence. Here, the collections of 240 samples were based on suspected symptoms of BmNPV infection. *Polyhedrin* gene, a conservative gene, was used for molecular identification. The *bro-d* gene was cloned and sequenced. Sequence alignment, phylogenetic analysis based on the ORF of *bro-d*, and the distribution of BmNPV isolates from Yunnan sericulture regions were determined. This has provided useful data for assessment of the manner of transmission, development of effective control, understanding of the evolution of BmNPV, and the breeding of resistant silkworms suited to Yunnan's various climates.

MATERIALS AND METHODS

Sample collection and treatment

According to the geographical distribution of Yunnan sericulture regions, we chose silkworm-raising intensive regions located at different latitudes as collection points. Two-hundred and forty suspected BmNPV disease samples of silkworm were collected and ground with sterilized water. The mixed slurries were then filtered through gauze, and the filtrations were separated by two cycles of differential centrifugation at 4000g for 10 min. White polyhedra layers at the bottom of the centrifuge tube were collected, and the polyhedra layers were resuspended in 1% (W/V) sodium dodecyl sulphate (SDS) and incubated for 30 min. The polyhedra were then purified by different concentration of sucrose solution (mass fraction 30%, 40%, 50%, 60%).

Pathogen detection of silkworm infected by BmNPV

The purified polyhedra were suspended in 0.32 M NaCl-0.2 M Na₂CO₃ alkaline solution (pH 10.8) at 37 °C for 30 min to dissolve the polyhedrin matrix. The adequate mixtures were incubated with 1 ml genome extraction fluid (10 mmol/l Tris-HCl, pH 8.0; 0.1 mol/l EDTA, pH 8.0; 0.5% SDS) and protease K (20 mg/ml). The solutions were then incubated at 55 °C for 2 h, and viral DNA was extracted once with an equal volume of Tris-saturated phenol buffer twice with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform. The aqueous phase containing viral genomic DNA was precipitated by absolute ethanol, washed twice with 70% ethanol, air dried, and resuspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). These solutions were then used for PCR detection. According to the ORF sequence of the *polyhedrin* gene, which GenBank logged in as a BmNPV-T3 genome (L33180.1), complete ORF was amplified using the following primers: *polh*-F, 5'-ATGCCGA ATTATTCATACACCC-3'; *polh*-R, 5'-TTAATACGCC GGACCACTG-3' in 20 µl PCR reactions with the different genome as a template²³. Reaction system: 10 × PCR buffer (Mg²⁺ plus) 5 µl, 2.5 mM dNTPs 4 µl, 250 U/µl r-Taq polymerase (Takara, Japan) 0.25 µl, 10 µmol/l forward and reverse primers 1 µl, genomic DNA template 1 µl, add distilled water to 20 µl was used. PCR conditions: 94 °C pre-denaturation for 3 min; 94 °C denaturation for 30 s; annealing 55 °C for 30 s; 72 °C for 1 min, 30 cycles; final extension 72 °C for 10 min. The PCR products were detected on 1% agarose gel.

Cloning and sequencing of *bro-d* gene

The *bro-d* ORF was amplified using the following primers²³: *bro-d*-F, 5'-TATTGCGCCGAGGAAGCC AT-3'; *bro-d*-R, 5'-CGAATCGTCACGCGTCGTTGT A-3', 50 µl PCR reactions with Ex-Taq polymerase (Takara, Japan). PCR products were purified using a Sangon Gel Extraction Kit (Sangon Biotech, China Shanghai) and ligated into the cloning vector pMD19-T, then sequenced by Sangon Biotech to confirm the ORF sequences of the *bro-d* genes of each isolate.

Sequence alignment and phylogenetic analysis

The *bro-d* ORF sequences of different BmNPV isolates from Yunnan silkworm-raising regions were used for sequence alignment and phylogenetic analysis. Alignments of *bro-d* sequences were carried

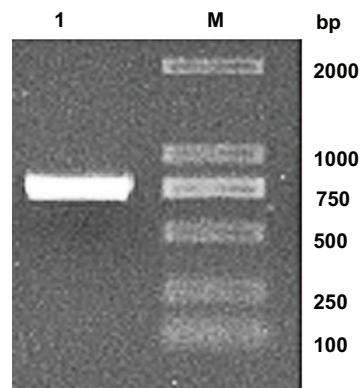


Fig. 1 *polh* PCR amplification of diseased silkworm genome. 1: diseased silkworm genome; M: DNA marker DL2000 marker.

out using BIOXM 2.6 software. The *bro-d* gene homology was analysed using DNAMAN 6.0 software. The *bro-d* nucleotide sequences of BmNPV-T3 (L33180.1), BmNPV-Thailand (JN862927.1), *B. mandarina* nucleopolyhedrovirus S1 (BomaNPV-S1, FJ882854.1), BomaNPV-S2 (JQ071499.1), and *Lymantria dispar* multiple nucleopolyhedrovirus isolate 2161 (LdMNPV-2161, KF695050.2) were downloaded from the NCBI database. BmNPV-T3 and BmNPV-Thailand were selected as representative strains for comparison. To understand the genetic relationship between BomaNPV and BmNPV, and *B. mori* domesticated from *B. mandarina*, the BomaNPV-S1 and the BomaNPV-S2 were selected as representative strains. Phylogenetic analysis of aligned nucleotide sequences generated from the isolates was carried out using MEGA 5.0 software package (MEGA Software, US) based on the Kimura 2-parameter model under the Neighbour Joining (NJ) method and adjustment of 1000 times the bootstrap²⁴. The homologous sequence of LdMNPV-2161 was used as outgroup.

RESULTS

Isolation and identification of BmNPV in different areas

Two-hundred and forty samples of diseased silkworm were collected from different silkworm-raising regions of the Yunnan province, and genomic DNA was extracted from each sample. PCR was performed using specific primers of the *polyhedrin* gene; the size of PCR products was the same as the *polyhedrin* gene by electrophoresis detection (approximately 738 bp, shown in Fig. 1). PCR results showed that 233 of the 240 samples were positive.

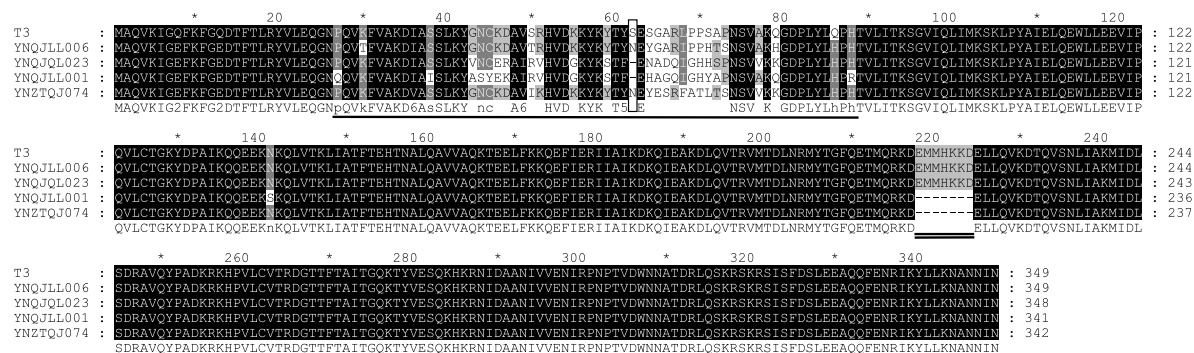


Fig. 2 Alignment of BRO-d amino acid sequence of BmNPV isolates. Identical and similar amino acid residues were indicated by black and grey highlights, respectively. The numbers indicated the position of the amino acid sequence. Underline indicated main different regions of amino acid sequences, rectangle indicated the position of N⁶² amino acid, double underline indicated the deletions of 7 amino acids located in 217–224 aa region.

Table 1 BmNPV isolates detected by PCR method.

Source	No. of samples	Positive No. of BmNPV
Kunming	6	6
Qujing	64	61
Puer	41	38
Zhaotong	23	23
Dali	31	30
Honghe	12	12
Chuxiong	51	50
Diqing	1	1
Baoshan	8	8
Lijiang	1	1
Wenshan	1	1
Lincang	1	1
Total	240	233
Positive rate	–	97%

Detection rate of about 97% (Table 1) furtherly confirmed that the widespread blood grasserie had occurred in Yunnan sericulture regions.

According to geographical distribution of Yunnan sericulture regions and outbreak situation of the blood pathogens of grasserie, 56 genomic samples which had been already identified by PCR were selected to experimental object. Among the 233 positive BmNPV genomes, 56 BmNPV diseased samples were from different counties with the condensed silkworm husbandry in Yunnan Province (Table 2).

Sequence analysis of *bro-d* gene

The sequences of *bro-d* genes were submitted to GenBank, and the accession numbers were presented in Table 2. Comparison of the *bro-d* coding

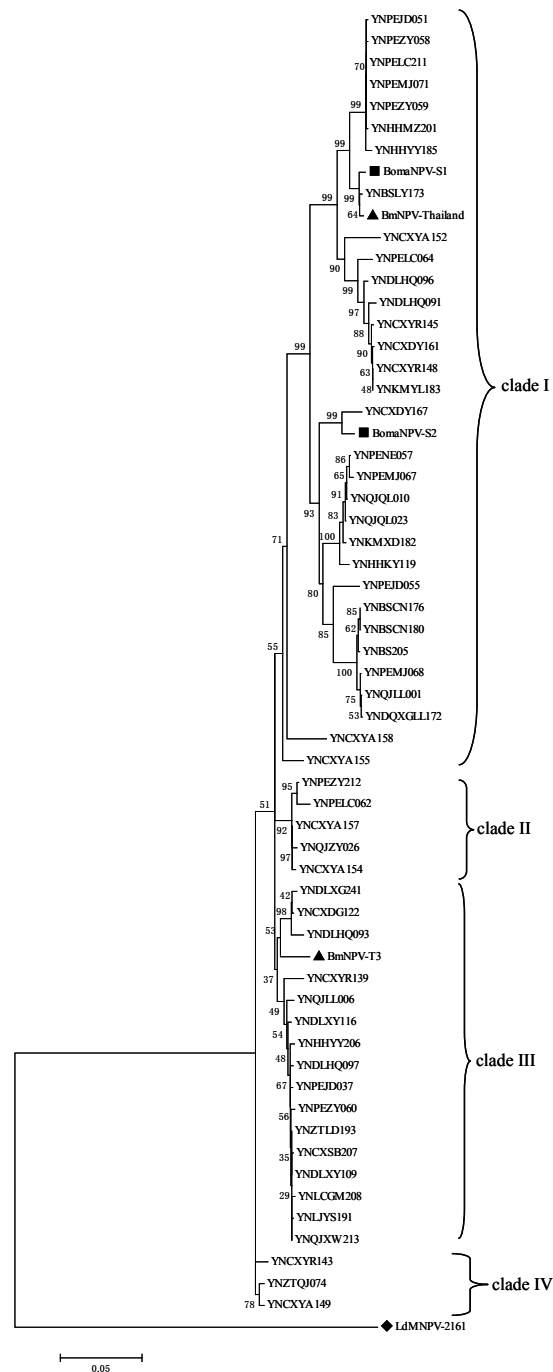
region sequence showed that the similarity of *bro-d* nucleotide sequence was above 91% among the different BmNPV isolates (Table 3). BmNPV-T3 *bro-d* ORF consisted of 1050 nt and encoding 349 amino acid residues. The full length of *bro-d* ORF of the BmNPV-Thailand strain was 1047 nt. The ORF analysis indicated that the full length of *bro-d* had mostly been 1050 nt and 1047 nt among the 56 different BmNPV isolates, whereas 1026 nt was found in YNQJLL001 and YNPEMJ067. Base insertions of ORF had not been detected. The C-terminal of BRO-d amino acid sequences were highly conserved, whereas the differences existed mainly in other regions²¹. Amino acid sequence alignment of BRO-d among the BmNPV-T3 strain and YNQJLL001, YNQJLL006, YNQJL023, YNZTQJ074 isolates, the differences in amino acid sequence located in 27–88 aa; deletions of 7 amino acids occurred in 217–224 aa between YNQJLL001 and YNZTQJ074 were found. N or Asparagine at the amino acid position 62 was absent in both YNQJLL001 and YNQJL023 isolates (Fig. 2).

Phylogenetic tree analysis

Genetic diversity of NPV was mainly caused by *bro* gene. The *bro-d* gene was a highly conserved gene in insects NPV genome and necessary for viral replication. Base on the coding regions of the *bro-d* gene, evolutionary relationship of BmNPV isolates was analysed by NJ method (Fig. 3). The phylogenetic tree revealed that BmNPV strains in the Yunnan silkworm-raising regions had significant genetic diversity. The BmNPV isolates were separated into four main clades: Clade I contained 32 local epidemic strains and was the branch of most members.

Table 2 Origins of BmNPV isolates from Yunnan used in this study.

Isolate	District	Collection date	GenBank No.
YNQJQL001	Qujing Qilin	2012.10	KX507164
YNQJLL006	Qujing Luliang	2012.10	KX507165
YNQJQL010	Qujing Qulin	2013.09	KX507166
YNQJQL023	Qujing Qilin	2013.10	KX507167
YNQJZY026	Qujing Zhanyi	2013.09	KX507168
YNPEJD037	Puer Jingdong	2012.10	KX507169
YNPEJD051	Puer Jingdong	2012.10	KX507170
YNPEJD055	Puer Jingdong	2013.08	KX507171
YNPENE057	Puer Ninger	2013.08	KX507172
YNPEZY058	Puer Zhenyuan	2013.08	KX507173
YNPEZY059	Puer Zhenyuan	2013.08	KX507174
YNPEZY060	Puer Zhenyuan	2013.08	KX507175
YNPELC062	Puer Lancang	2013.08	KX507176
YNPELC064	Puer Lancang	2013.08	KX507177
YNPEMJ067	Puer Mojiang	2013.08	KX507178
YNPEMJ068	Puer Mojiang	2013.08	KX524427
YNPEMJ071	Puer Mojiang	2013.08	KX524428
YNZTQJ074	Zhaotong Qiaojia	2012.10	KX524429
YNDLHQ091	Dali Heqing	2013.09	KX524430
YNDLHQ093	Dali Heqing	2013.09	KX524431
YNDLHQ096	Dali Heqing	2012.10	KX524432
YNDLHQ097	Dali Heqing	2013.09	KX524433
YNDLXY109	Dali Xiangyun	2013.09	KX524434
YNDLXY116	Dali Xiangyun	2013.09	KX524435
YNHHKY119	Honghe Kaiyuan	2013.09	KX524436
YNCXDGI22	Chuxiong Donggua	2013.09	KX524437
YNCXYR139	Chuxiong Yongren	2013.10	KX524438
YNCXYR143	Chuxiong Yongren	2013.10	KX524439
YNCXYR145	Chuxiong Yongren	2013.10	KX524440
YNCXYR148	Chuxiong Yongren	2013.10	KX524441
YNCXYA149	Chuxiong Yaoan	2013.10	KX524442
YNCXYA152	Chuxiong Yaoan	2013.10	KX524443
YNCXYA154	Chuxiong Yaoan	2013.10	KX524444
YNCXYA155	Chuxiong Yaoan	2013.10	KX524445
YNCXYA157	Chuxiong Yaoan	2013.10	KX524446
YNCXYA158	Chuxiong Yaoan	2013.10	KX524447
YNCXDY161	Chuxiong Dayao	2013.10	KX524448
YNCXDY167	Chuxiong Dayao	2013.10	KX524449
YNDQXGLL172	Diqing Xianggelila	2013.10	KX524450
YNBSLY173	Baoshan Longyang	2013.10	KX524451
YNBSN176	Baoshan Changning	2013.10	KX524452
YNBSN180	Baoshan Changning	2013.10	KX524453
YNKMXD182	Kunming Xundian	2012.10	KX524454
YNKMYL183	Kunming Yiliang	2013.07	KX524455
YNHHY185	Honghe Yuanyang	2013.07	KX524456
YNLJYS191	Lijiang Yongsheng	2014.07	KX524457
YNZTLD193	Zhaotong Ludian	2014.07	KX524458
YNHHMZ201	Honghe Mengzi	2014.09	KX524459
YNBS205	Honghe Mengzi	2012.01	KX524460
YNHHY206	Honghe Yuanyang	2014.09	KX524461
YNCXSB207	Chuxiong Shuangbai	2014.09	KX524462
YNLCGM208	Lincang Gengma	2014.09	KX524463
YNPELC211	Puer Lianchang	2014.05	KX524464
YNPEZY212	Puer Zhenyuan	2014.05	KX524465
YNQJXW213	Qujing Xianwei	2014.05	KX524466
YNDLXG241	Dali Xiaguan	2014.09	KX524467



Clade IV was a branch of the least number of members and contained 3 epidemic strains. Clades II and III contained 5 and 16 strains, respectively. BmNPV-T3 and BmNPV-Thailand distributed in clade III and clade I were selected as representative isolates for comparison. BomaNPV-S1 and BomaNPV-S2 which parasitized *B. mandarina* located in the same clade I branch had a close genetic relationship with BmNPV. Two viruses crossly transmitted between

Fig. 3 Phylogenetic tree based on the *bro-d* gene of BmNPV isolates. Black triangles represented BmNPV *bro-d*, as benchmark. Black square represented BomaNPV *bro-d*. Black rhombus represented LdMNPV *bro-d*, as outgroup. Numbers above branches indicated reliability from bootstrapping test using 1000 replicates. The bar represented a genetic distance of 0.05.

Table 3 Homologies of bro-d gene of BmNPV isolates from Yunnan province (%).

	006	010	026	037	051	059	060	062	074	091	143	148	149	152	154	157	172	176	183	191	193	201	205	206	207	208	212	Isolate
006	—																											YNQJLL006
010	94.7	—																										YNQJQL010
026	97.8	95.2	—																									YNQJZY026
037	99.1	94.8	98.1	—																								YNPEJD037
051	93.6	95.1	93.3	93.5	—																							YNPEJD051
059	93.7	95.2	93.4	93.6	99.9	—																						YNPEZY059
060	99.0	94.5	97.9	99.4	93.7	93.8	—																					YNPEZY060
074	97.0	94.3	98.6	97.2	92.5	92.6	97.0	—																				YNPELC062
091	93.2	93.2	92.4	93.5	96.3	96.4	93.7	91.9	92.6	—																		YNZTQJ074
143	97.2	94.6	97.1	97.3	93.8	93.9	97.3	96.3	98.8	92.8	—																	YNDLHQ091
148	93.6	93.4	92.6	93.9	96.1	96.2	93.7	92.1	93.4	99.2	93.4	—																YNCXYR143
149	97.2	94.7	97.3	97.5	94.0	94.1	97.3	96.5	99.4	92.9	98.7	93.3	—															YNCXYR148
152	93.0	93.1	92.8	93.3	95.2	95.3	93.0	92.7	92.9	96.0	92.2	96.2	92.9	—														YNCXYA149
154	97.9	95.2	99.5	98.2	93.4	93.5	98.0	98.7	97.4	92.5	97.2	92.6	97.4	92.9	—													YNCXYA152
157	98.1	95.4	99.7	98.4	93.6	93.7	98.2	98.9	97.6	92.6	97.4	92.8	97.6	93.0	99.8	—												YNCXYA154
172	94.2	96.4	93.2	94.1	93.5	93.6	93.7	92.3	93.5	93.7	92.5	93.9	93.5	93.1	93.4	93.6	—											YNCXYA157
176	94.3	96.2	93.4	94.2	93.5	93.6	93.9	92.5	93.7	93.5	92.9	93.9	93.5	93.1	93.4	93.6	99.4	—										YNDQXGLL172
183	93.6	93.4	92.6	93.9	96.1	96.2	93.7	92.1	93.4	99.2	99.8	93.3	96.2	92.6	93.4	93.0	96.3	93.9	—									YBSCN176
191	99.2	94.7	98.0	99.7	93.4	93.5	99.5	97.1	97.2	93.6	97.4	94.0	97.4	93.2	98.1	98.3	94.0	94.2	94.0	—								YNKMYL183
193	99.3	94.8	98.1	99.8	93.5	93.6	99.6	97.2	97.3	93.7	97.5	94.1	97.5	93.3	98.2	98.4	94.1	94.2	94.1	99.9	—							YNLJYS191
201	93.5	95.0	93.2	93.4	99.8	99.9	93.6	92.3	93.7	96.2	93.7	96.0	93.9	95.1	93.3	93.5	93.5	96.0	93.3	93.4	—							YNHMHZ201
205	94.5	96.3	93.5	94.4	93.6	93.7	94.0	92.6	93.7	93.8	93.0	94.0	93.6	93.2	93.5	93.7	99.6	99.8	94.0	94.3	94.4	93.5	—					YNHHS205
206	99.2	94.7	98.0	99.5	93.2	93.3	99.3	97.1	97.2	93.4	97.4	93.8	97.4	93.0	98.1	98.3	94.0	94.2	93.8	99.6	99.7	93.1	94.3	—				YNHHY206
207	99.2	94.7	98.2	99.7	93.4	93.5	99.5	97.1	97.2	93.6	97.4	94.0	97.4	93.2	98.1	98.3	94.0	94.2	94.0	99.8	99.9	93.3	94.3	99.6	—			YNCXSB207
208	99.1	94.7	97.9	99.6	93.3	93.4	99.4	97.0	97.1	93.5	97.3	93.9	97.3	93.1	98.0	98.2	93.9	94.1	93.9	99.7	99.8	93.2	94.2	99.5	99.7	—		YNLCGM208
212	97.7	95.0	99.3	98.0	93.2	93.3	97.8	99.1	97.2	92.3	97.0	92.5	97.2	92.7	99.4	99.6	93.0	93.2	92.5	97.9	98.0	93.1	93.3	97.9	97.9	97.8	—	YNPEZY212
213	99.2	94.7	98.0	99.7	93.4	93.5	99.5	97.1	97.3	93.6	97.4	94.0	97.4	93.2	98.1	98.3	94.1	94.2	94.0	99.8	99.9	93.4	94.3	99.6	99.8	99.7	97.9	YNQJXW213

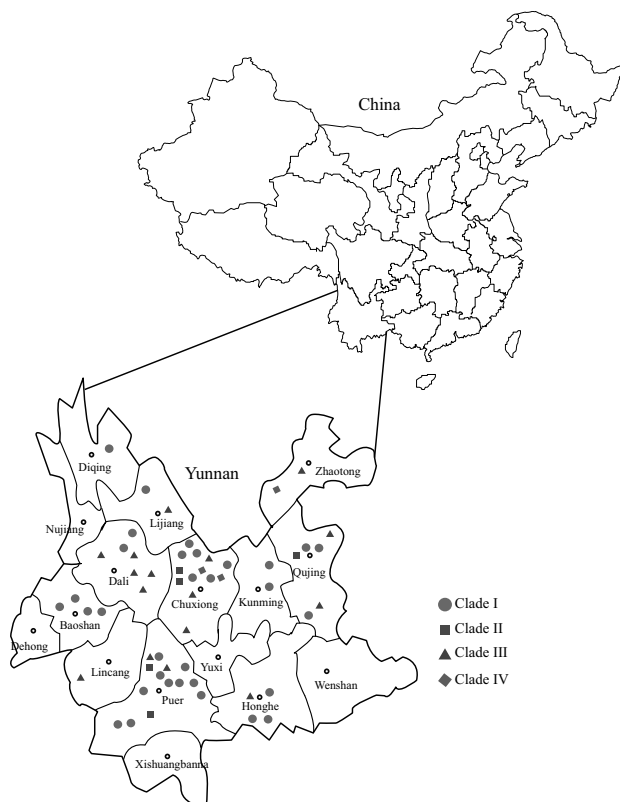


Fig. 4 Geographical distribution of the BmNPV isolates from Yunnan. The distribution of 56 BmNPV isolates in clade I (●), clade II (■), clade III (▲) and clade IV (◆).

B. mori and *B. mandarina*²⁵, leading to speculation that either the BomaNPV parasitized *B. mandarina* from a local mulberry field was an ancestor of the BmNPV source, or two virus types had originated from the same ancestor. BmNPV and BomaNPV were single-grain embedding virus, while LdMNPV was a multiple-grain embedding virus; there were distant relatives between the two kinds of viruses.

Geographical distribution of Yunnan BmNPV isolates

Geographical distribution of different BmNPV isolates in each inferior branch of clade I was relatively concentrated and adjacent, mainly distributed in Kunming, Qujing, Puer, Baoshan, Dali, Chuxiong, Honghe, and Diqing. Local transmission of this pathogen clearly occurred in the silkworm-raising regions in Yunnan. The isolates of other clades were irregularly scattered throughout Yunnan (Fig. 4), which suggested that long-distance transmission might have occurred. The isolates of clade II were mainly distributed in Puer, Chuxiong, and Qujing.

The regional climate was quite different among the three areas. The genetic relationship of BmNPV isolates within those locations had no direct contact; only clade IV contained YNCXYR143, YNZTQJ074, and YNCXYA149, and three members had distant relatives with other strains.

DISCUSSION

The silkworm industry is a traditional, pillar industry in China that makes important contributions to the development of its national economy and social culture. Sericulture is also a speciality industry in the Yunnan province. Located in southwest China, it has a variety of ecological environments, such as tropical regions in Puer, subtropical regions in Mengzi, semi-tropics or temperate zone regions in northern Qujing, and high elevation regions in Heqing. Sericulture regions are widely distributed here, as these suitable climatic conditions are conducive to silkworm breeding. This climate is also favourable for the breeding and spread of viral disease. The blood pathogens associated with grasserie outbreaks may cause serious economic losses in areas of Yunnan sericulture. The Yunnan area is particularly affected by the silkworm *B. mori* viral disease during the summer and fall seasons. This study focused on samples collected between 2012 and 2014. The blood pathogens associated with grasserie do the most damage in Puer and Honghe, which are humid, tropical parts of Yunnan, though some temperate zones also showed infestation. The viral disease mainly broke out in fifth-instar silkworm larvae. It was rarely seen in younger worms. The diseased silkworm samples were mainly collected from local households. Silk-grower may overlook diseased silkworms during silkworm husbandry, and the potency of various treatment technologies were not enough to cut off the BmNPV route of transmission in a timely manner. There was an explosion of the disease because the viral particles remained in the environment for a long time.

PCR technology is widely used to rapidly diagnose pathogenic microorganisms, such as in the parasitic silkworm, as it holds the characteristics of rapid trace and sensitive specificity. The design of specific primers is the key to use PCR for diagnosis. The *polyhedrin* gene was a candidate gene for BmNPV identification²⁶. Tang detected infected silkworms through BmNPV *polyhedrin* gene specific primers successfully²³. In this study, PCR was used for rapid detection of the collected 240 samples of sick silkworm, 233 were positive with BmNPV

infection. The detection rate is as high as 97%. If measures are not taken to improve the disinfection of silkworm diseases, the blood pathogens associated with grasserie can sustain an outbreak for a long time in areas of sericulture in Yunnan.

Bro-d is a highly important gene expressed during early stages of viral replication. Bideshi found there to be considerable homology between single copies of the *bro* gene (*ac-bro*) in the AcMNPV and BmNPV *bro-d*¹⁴. The *ac-bro* gene was found to directly and indirectly influence the number of viral particles in infected hosts, which suggests that *bro-d* is related to virulence in more than one BmNPV isolate. The variations in the coding region of *bro-d* gene may cause differences in virulence in different BmNPV isolates. An assessment of homology showed that the nucleotide similarity of *bro-d* exceeded 91% in 56 BmNPV isolates (Table 3) and a minimum of the *bro-d* homology was 92% between YNPELC062 and YNDLHQ091. The full length of *bro-d* ORF was 1050 bp from BmNPV-Guangxi (JQ991011.1) and BmNPV-Zhejiang (JQ991008.1) of China local BmNPV epidemic isolates, and the *bro-d* gene ORF of BmNPV-Thailand was 1047 bp. The full lengths of *bro-d* ORFs were mainly 1050 bp and 1047 bp among the 56 BmNPV isolates, whereas 1026 bp were in YNQJLL001 and YNPEMJ067. The ones of YNZTQJ074, and YNCXYA155 were 1029 bp. The genetic phenomenon of base deletion and insertion had not been found in the pandemic isolates. While compared the *bro-d* ORF sequence of BmNPV-T3 strain with YNQJLL001 and YNZTQJ074, there was the deletion of 7 amino acids occurred at 217–224 aa. The differences of BRO-d protein mainly focused on the N-terminal. BRO proteins have been identified as nucleocytoplasmic shuttle proteins in CRM-1-mediated nuclear export pathways because the leucine-rich region in their N-terminals plays the same role as CRM-1-dependent nucleus export signal²⁷. For the same gene, the absence and insertion of gene fragment could affect the virulence of different virus isolates²⁸; for example, the absence of these amino acids of BRO-d among the different BmNPV isolates. However, further work must be performed to determine whether the absence of these amino acids truly affects the virulence of the virus.

Yunnan province has a unique geographic position, the silkworm-raising areas distributed in dam, hills and other uplands. These silkworm-raising regions include both areas with a long history situated in optimal climate and new silkworm-raising regions situated in moist, hot areas. BmNPV iso-

lates showed a large amount of genetic diversity because of this broad geographic variety. The genetic differences in BmNPV mainly concerned the *bro* gene families²⁹. The genetic diversity of the virus was mainly caused by the *bro* gene³⁰. *Bro-d* was found to be a highly conserved gene from insect NPV. The 56 BmNPV isolates from Yunnan were separated into four main clades (I, II, III, and IV). This *bro-d* gene phylogenetic tree contained four clades that had 32, 5, 16, and 3 local epidemic isolates, respectively. The geographical location of 56 BmNPV isolates indicated that the co-relation between evolution of *bro-d* and geographical location was not obvious. The geographic distribution of clade I isolates in Yunnan showed a concentrated pattern. Local transmission of this pathogen took place in these silkworm-raising regions. The isolates from the other three clades were irregularly scattered throughout Yunnan, which suggested that long-distance transmission was taking place. This phenomenon may have been caused by the dissemination medium, mulberry field insects, human factors and others. Clade IV contained three BmNPV isolates, the samples of YNCXYR143, YNZTQJ074, and YNCXYA149 were collected in Menghu town, Yongren County; Dongchuan town, Yaoan County within the Chuxiong autonomous prefecture, and Baohetan township, Qiaojia County in Zhaotong prefecture-level city, respectively. The topography and climate of these three sericulture regions were very similar. Three pandemic isolates retained the original genetic information, and their more distant relatives contained other BmNPV isolates.

This work presents previously undiscovered molecular epidemiological data concerning BmNPV in China's Yunnan Province. The study mainly includes PCR detection of diseased silkworm samples from sericulture regions and analysis of the genetic relationships among BmNPV strains based on *bro-d* genes. These results may provide a better evaluation of epidemic regularity of BmNPV and facilitate the discovery of new method of curb the spread of the virus in areas of sericulture within Yunnan. So far, there are no effective drugs for preventing or treating BmNPV infection pathogens accumulated in the natural environment of the Yunnan sericulture area. Hence the process of silkworm feeding, reducing the spread of virus particles and strengthening environmental disinfection are helpful to decrease the incidence of BmNPV disease. In addition, adequately mastering the molecular mechanisms of BmNPV pathogenicity is the key to prevent BmNPV infection for sericulture.

Acknowledgements: This study was supported by the Foundation for the National Natural Science Foundation of China (Grant No. 31260583 and No. 31560675). We would like to thank members of the research group on pathology of silkworm and all the silkgrowers in the silkworm-raising regions.

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