Molecular Identification of a Novel Iflavirus in Brown-Spotted Pitvipers (*Protophrops mucrosquamatus*)

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Abstract

Background: *Iflaviridae* is a family of small non-enveloped viruses with monopartite, positive-stranded RNA genomes, which are identified in arthropod hosts, primarily infecting insect species. Herein, we firstly identify the sequence of an iflavirus (YB-PMP20) found in brown-spotted pitvipers in China.

Results: The sequence of YB-PMP20 showed high identity to the sequences of Hubei picorna-like virus (HUPV) (99.2% in nt), *Vespa velutina*-associated iflavirus like virus (VVAIV) (58.6% in nt) and *Lygus lineolaris* virus (LyIV-1) (46.6% in nt) in nucleotides encoding polypeptides. It contained a single large ORF (304–9291 nt) encoding 2996 amino acids. The deduced amino acid sequences were compared with those of iflavirus. Helicase, protease and the RdRp domain were found to be located at the 3′ end, and structural genes (VP1, VP2 and VP3) were found to be located at the 5′ end. Phylogenetic analysis indicated that YB-PMP20 belongs to the iflavirus cluster, and is similar to HUPV, LyIV-1 and VVAIV.

Conclusion: The present study described the genetic characterization of a PmIFV strain in brown-spotted pitvipers. Our genomic data extend knowledge of the diversity of viruses in snakes.

1. Introduction

Iflavirus is a member of the family *Iflaviridae*, order *Picornavirales*, which has a positive-stranded RNA genome between 9 and 11 kilobases in length [1]. The iflavirus genome organization is monopartite and monocistronic, encoding capsid proteins at the 5′ end and replicase proteins at the 3′ end [2]. All classified iflavirus species infect arthropod hosts, mostly insects, including the honey bee [3], planthopper [4], soybean thrip [5], Varroa destructor [6], aedes mosquito [7, 8], sogatella furcifera [9] and leafhopper [10].

Many viruses have been described in diverse snake species, for example, ranavirus (*Chondropython viridis*) [11], erythrocytic necrosis virus (*Thamnophis sauritus*) [12], herpesvirus (*Boa constrictor*) [13], adenovirus (*Python regius*) (*Pantherophis guttatus*) [14, 15], parvovirus (*Pantherophis guttatus*) [16], circovirus (*Aspidites melanocephalus*) [17], retrovirus (*Vipera russelli*) [18], reovirus (*Python regius*) [19], paramyxovirus (*Bothrops alternatus*) [20–22], calicivirus (*Crotalus unicolor*) [23], Japanese encephalitis virus (*Zaocys dhumnades*) [24], Western equine encephalitis virus (*Thamnophis*) [25] and picornavirus (*Zamenis lineata*) [26].

The present study first investigated the brown-spotted pitviper (*Protobothrops mucrosquamatus*) iflavirus (PmIFV), denoted YB-PMP20, which was identified from the feces of *P. mucrosquamatus* (*Crotalinae*, *Viperidae*) through high-throughput sequencing. Sequence and phylogenetic analyses indicated that YB-PMP20 showed high sequence identity with Hubei picorna-like virus 36 (HUPV 36) and *Vespa velutina* associated ifla-like virus (VVAIV), which were isolated from insects and closely clustered with iflavirus. This sequence analysis may contribute to understanding the evolution of iflavirus in snakes.

2. Materials And Methods

2.1 Sample collection

From July to September 2020, we collected seven anal swabs from brown-spotted pitvipers with lassos from quebrada and bamboo forests located in the Laojun mountains, 110 kilometers from Yibin city in Sichuan province (Fig. 1). To prevent sample contamination, we placed the animals individually in sterilized tubs and cleaned their skins with 75% alcohol. All samples were collected opportunistically with sterilized swabs in areas where pitvipers were captured. The swabs were placed in RNase-free tubes and immediately transported on dry ice to Shanghai Biozeron Biothchnology Co., Ltd. (Shanghai, China.) the same day. The pitvipers were then released back into the wild.

2.2 RNA extraction

Total RNA was extracted from tissue with TRIzol® Reagent according to the manufacturer's instructions (Invitrogen), and genomic DNA was removed with DNase I (TaKara). Then RNA quality was determined with a 2100 Bioanalyzer (Agilent) and quantified with an ND-2000 spectrophotometer (NanoDrop Technologies). High-quality RNA samples ($OD_{260/280} = 1.8–2.2$, $OD_{260/230} \geq 2.0$, $RIN \geq 6.5$, $28S:18S \geq 1.0$, $> 10 \mu g$) were used for sequencing library construction.

2.3 Library preparation and Illumina HiSeq sequencing

Metatranscriptome libraries were prepared with a TruSeq™ Stranded Total RNA Sample Preparation Kit from Illumina (San Diego, CA), using 5 μg of total RNA. Subsequently, rRNA removal was performed with Ribo-Zero™ rRNA Removal Kits from Illumina (San Diego, CA), and fragmentation was performed with fragmentation buffer. cDNA synthesis, end repair, A-base addition and ligation of the Illumina-indexed adaptors were performed according to Illumina's protocol. Libraries were then size selected for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose and then amplified with PCR using Phusion DNA polymerase (NEB) for 15 PCR cycles. Metatranscriptomic sequencing was performed by Shanghai Biozeron Biothchnology Co., Ltd. (Shanghai, China) on an Illumina Novaseq 6000 instrument. All samples were sequenced on the Illumina HiSeq 2500 instrument. Libraries were prepared with a fragment length of approximately 450 bp. Paired-end reads were generated with 150 bp in the forward and reverse directions.

2.4 Read quality control and mapping

The raw paired end reads were trimmed and subjected to quality control in Trimmomatic with parameters (SLIDINGWINDOW:4:15 MINLEN:75) (version 0.36 <http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic>). Then, clean reads that aligned to the host genome were also removed. This set of high-quality reads was then used for further analysis. A total of 10.0 gigabases (Gb) of paired-end reads was obtained for the sample.

2.5 Metatranscriptome assembly

The clean reads were aligned to the SILVA SSU (16S/18S) and SILVA LSU (23S/28S) databases to remove rRNA related reads in SortMeRNA (<http://bioinfo.lifl.fr/RNA/sortmerna/>) software. Then, clean data from all samples were used for assembly with megahit (<http://www.l3-bioinfo.com/products/megahit.html>).

All genes were predicted by METAPRODIGAL (<http://compbio.ornl.gov/prodigal/>). Then, a non-redundant gene catalog was constructed with 95% identity and 90% coverage by CD-HIT (<http://www.bioinformatics.org/cd-hit/>).

2.6 Identification of the genome

Reverse transcription of 1 µg RNA from feces was conducted with random primers according to the manufacturer's protocol (TAKARA). Twelve pairs of primers were developed for PCR amplification, as previously described [27].

2.7 Sequence alignment and phylogenetic analysis

Sequence data were assembled and analyzed in Clustal X software and DNASTAR. To determine the relationship between the iflavirus representative isolates and YB-PMP20 strain, phylogenetic trees based on the whole gene sequence were constructed in molecular evolutionary genetics analysis (MEGA) software (version 6.0) with the maximum-likelihood method. Bootstrap values were estimated for 1,000 replicates. The sequences obtained in this study were assembled and submitted to GenBank under the accession number MZ005704.

3. Results

The genome of YB-PMP20 was 9808 nucleotides (nt) in length, with a nucleotide composition of 2827, 2329, 2680 and 1972, A, G, T and C nucleotides, respectively. The G + C content of the YB-PMP20 genome was 43.85% higher than that of other iflaviruses, including VVAILV144 (35.71%), Aedes Iffa-like virus (36.42%), Culex picorna-like virus 1 (36.75%), Fitzroy Crossing iflavirus 1 (37.83%), Darwin bee virus 2 (36.39%) and Sanxia water strider virus 8 (37.06%), and was lower than that of Lylv-1 (46.15%). The genome has a 5'-untranslated region (UTR) followed by a single open reading frame spanning 8988 nt from position 304 to 9291, and a 3'-UTR region. A consensus invertebrate initiation sequence (ANNAUGG; N = any nucleotide) is located in nucleotide position 301–307 nt, and a translation initiation codon (AUG) is present at nucleotide position 304–306 nt. The polyprotein of 2995 amino acids has a calculated molecular mass of 335.3 kDa, an isoelectric point of 6.709 and a charge of -8.611 at pH 7.

Alignment of the polyprotein sequences of YB-PMP20 and iflavirus showed the highest sequence identity with the HUPV 36 (99.2% in nt and 99.6% in aa), Lylv-1 (46.6% in nt and 20.9% in aa), VVILV (58.6% in nt and 48.6% in aa) and AEIV (43.7% in nt and 24.6% in aa) sequences.

The peptide domains of capsid proteins, helicase, peptidase and RNA directed RNA polymerase (RdRp) of YB-PMP20 were identified through similarity searches with the Simple Modular Architecture Research Tool (SMART: <http://smart.embl-heidelberg.de>), Conserved Domain Database (CDD: <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and SWISS-MODELbioinformatics server [28, 29]. YB-PMP20 polyprotein amino acid regions 130–382, 429–680 and 747–992 were identified as capsid proteins (VP) 2, 3 and 1, which shared lower protein sequence identities (30%) with the sacbrood virus. However, the deduced VP4 was not found in the consensus sequences. RNA helicase domains were identified in the polyprotein from 1437–1583 aa, and showed 25.93% amino acid identity with 2C helicase from enterovirus 71 (EV71) and 2C ATPase of picornavirus. Three conserved helicase motifs (A, B and C) are present in the picornavirus and iflavirus [30]. The highly conserved amino acids within motif A (GxxGxxGKS) and motif B (QxxxxxDD) were identified in the YB-PMP20 sequence, between amino acids 1449–1456 and 1495–1503. In YB-PMP20, the amino acids within motif C were KKxxxxPxxxxxATN, in contrast to the consensus motif, KGxxxxSxxxxSTN. Proteases were identified from aa 2174–2379 and showed 22.29% amino acid identity with 3C protease from coxsackievirus. The putative residues H²²¹⁷, E²²⁹⁰ and C²³⁴⁰ may form the catalytic triad in the protease. RdRp domains were identified in aa 2416–2974, and showed 20.86% amino acid identity with RdRp of sapporovirus. Eight conserved RdRp amino acid motifs are found in RNA viruses [30]. The putative RdRp conserved domains are shown in Fig. 3C. Furthermore, we constructed phylogenetic trees based on the full sequences, by using the maximum likelihood method. The iflaviruses clustered in a large clade, and two subclades were present. The phylogenetic trees indicated that YB-PMP20 clustered in the same subclade with HUPV 36, Lylv-1 and VVILV.

4. Discussion

In present study, we first identified and characterized an iflavirus strain (YB-PMP20) from brown-spotted pitvipers without apparent clinical symptoms, which shows characteristics typical of the family *Iflaviridae*, including the capsid protein, helicase, protease and RdRp domains. Sequence analysis suggested that YB-PMP20 is similar to HUPV 36, Lylv-1 and VVAIV, identified in Diptera, Lygus lineolaris and Vespa velutina nigrithorax, respectively.

Metagenomic studies of invertebrate viruses have recently been undertaken. More than 220 invertebrate species, including 9 metazoan phyla, were identified at least 1,445 distinct virus genomes segments [31]. It indicated that invertebrates acted as viral vectors, could play a key role in transmit and reservoir of pathogens.

Snakes may be a common predator of monkeys [32], shell snails [33], kangaroos [33], fishes [33], leeches [33], earthworms [33], frogs [33], tadpoles [33], fish eggs [34], lizards [35, 36], field voles [36] and shrews [36]. There are no reports to support the idea that snakes prey on insects. We speculated that 1) snakes may occasionally prey on insects, thus, resulting in transmission of the virus from insects to snakes; 2) generally, amphibians including frogs feed on insects, thus, resulting in pathogen transmission from insects to amphibians. Then, snakes preying on amphibians might become a reservoir of insect and amphibian pathogens. To investigate the epidemiology of iflavirus in snakes, a large-scale survey is needed. Such an investigation would aid in understanding the prevalence of iflavirus and across the range of all snake species.

5. Conclusion

We first identified and characterized an iflavirus strain (YB-PMP20) from brown-spotted pitvipers. Phylogenetic analysis indicated that YB-PMP20 belongs to the iflavirus cluster, and is similar to HUPV, LyIV-1 and VVAIV. These findings contribute to our understanding of the prevalence of viruses in snake species.

Abbreviations

Hubei picorna-like virus (HUPV), *Vespa velutina*-associated iflavirus like virus (VVAIV), *Lygus lineolaris* virus (LyIV-1), gigabases (Gb), molecular evolutionary genetics analysis (MEGA), RNA directed RNA polymerase (RdRp), enterovirus 71 (EV71).

Declarations

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Yibin University, Yibin, China, according to the OIE standards for use of animals in research and education.

Consent for publication

Not applicable.

Availability of data and materials

The complete sequences obtained in this study have been submitted to the GenBank database (accession number: MZ005704).

Competing interests

The authors declare no competing interests.

Author Contributions: Conceptualization, HXL, GP; methodology, TZG., DY, CHZ; formal analysis, TZG, HXL, GP; investigation, DY, ZMM, TT, JFR, ZAT, CHZ, LY; resources, DY, ZMM, TT, JFR, ZAT and XX; writing original draft preparation, TZG; writing review and editing, TZG, GP; supervision, HXL, GP; project administration, HXL; funding acquisition, HXL, GP.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Tables

Table 1
Primers used for identifying and sequencing the YB-PMP20 strain

Name	Sequence (5'-3')	Position
1F	TTCTTACCCCAAAGGTAGGA	1–20
1R	CAACCAGCTTAAATTCTACA	990–1009
2F	CTTTTCATTGAGGATCCCAG	900–919
2R	CTTAACAATGTCAAATCAT	1970–1989
3F	TGATATTGCTAAGAAGCAGA	1800–1819
3R	ATTTTTTGGTCGAATAAGTT	2870–2889
4F	CAGCACCAATAACTAAGAT	2746–2765
4R	ACTTCACCCAGATCTGTCAA	3727–3746
5F	GCATACGATCTATGATCTTA	3471–3490
5R	ATTCCTAACGAACAAGAAAG	4268–4287
6F	TTGGCAAATACTTAAGCAG	4034–4053
6R	TTAACTAAGACGTCTCGTCT	5014–5033
7F	CTGAGCTCTACCAGCTGAAG	4856–4875
7R	AACAAGCTCATAATTACAAC	5842–5861
8F	TGTAGCAAATCTCCTGTGG	5677–5696
8R	ATATCGCTGTAGTTTTCAGC	6514–6533
9F	GCCTTGTAACACTACTGATT	6387–6406
9R	GCTCTAATTCTAGCTCCAGT	7405–7424
10F	TAGTGCATATGATGAAGTGA	7242–7261
10R	AGCGAAGGTGAGAAGTAAAA	8205–8224
11F	GGAGGCAAGGAATTTTAGCA	8054–8073
11R	CATATGATAGCAATAGGCTA	9081–9100
12F	TCTCCAAAACATGTCATTTT	8922–8941
12R	TTTATATTGTTTTGTATTT	9789–9808

Table 2
Comparison of amino acid and nucleotide sequence identity between YB-PMP20 and other representative Iflaviruses

	YB-PMP20		VVIV		AEIV		CUPV1		DABV		FCIV1		HUPV36		LyIV1		REBV		S
	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%	aa%	nt%
Polyprotein	100	100	58.6	48.6	43.7	24.6	44.1	24.5	37.1	22.7	36.8	22.3	99.2	99.6	46.6	20.9	36.8	22.9	3
VP1	100	100	62.6	56.4	42	27	42.4	26.2	29.1	31.2	32.9	28.4	99.2	98.8	47.5	29.7	30.5	27.9	30
VP2	100	100	64.3	65.2	40.1	25.7	41.4	25.7	35.4	20	31.6	17.4	99.5	99.6	49.3	21.2	34.3	21.9	30
VP3	100	100	64.1	64.3	43.1	28.5	43.3	28.1	36.3	26.8	38.6	29.6	99.3	100	46.1	29	36.4	26.8	30
Helicase	100	100	70.2	78.9	34.4	29.3	36.3	29.3	45.8	40.1	50.2	37.4	99.1	100	53.9	42.1	49.8	38.8	40
Protease	100	100	58.6	50	47.4	25.6	46.9	25.6	40.6	22.8	38.1	22.9	99.5	100	50.5	27	37.6	22.9	3
RdRp	100	100	58.6	56.5	47.4	40.1	46.9	40.5	40.6	36.1	38.1	32.9	99.5	99.8	45.3	33.2	37.6	34.3	3

Figures

Figure 1

Map of Laojun Mountain Nature Reserve.

Figure 2

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