Molecular Identification of a Novel Iflavirus in Brown-Spotted Pitvipers (Protobothrops Mucrosquamatus)

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Abstract

Background: Flaviviridae 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 iavirus (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 iavirus like virus (VVAIV) (58.6% in nt) and Lygus lineolaris virus (LyIV-1) (46.6% in nt) in nucleotides encoding polyproteins. It contained a single large ORF (304–9291 nt) encoding 2996 amino acids. The deduced amino acid sequences were compared with those of iavirus. 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 iavirus 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

Iavirus is a member of the family Flaviviridae, order Picornavirales, which has a positive-stranded RNA genome between 9 and 11 kilobases in length [1]. The iavirus genome organization is monopartite and monocistronic, encoding capsid proteins at the 5´ end and replicase proteins at the 3´ end [2]. All classified iavirus 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 (Chordopoxivirus 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], revovirus (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 picomavirus (Zamenis lineata) [26].

The present study first investigated the brown-spotted pitviper (Protobothrops mucrosquamatus) iavirus (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 iifa-like virus (VVAIV), which were isolated from insects and closely clustered with iavirus. This sequence analysis may contribute to understanding the evolution of iavirus 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} ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0, > 10 µg) 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 Biothcnology 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

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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.i3-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 iflaviruses 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 VAIVLV144 (35.71%), Aedes ifla-like virus (36.42%), Culex picomia-like virus 1 (36.75%), Fitzroy Crossing iflaviruses 1 (37.83%), Darwin bee virus 2 (36.39%) and Sanxia water strider virus 8 (37.06%), and was lower than that of LyIV-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 iflaviruses showed the highest sequence identity with the HUPV 36 (99.2% in nt and 99.6% in aa), LyIV-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 saccbrod 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 picomavirus. Three conserved helicase motifs (A, B and C) are present in the picomavirus and iflaviruses [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 KxxxxxXxxATN, in contrast to the consensus motif, KGxxxxSxxxxxSTN. Proteases were identified from aa 2174–2379 and showed 22.29% amino acid identity with 3C protease from coxsackievirus. The putative residues H2290, E2340 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, LyIV-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, LyIV-1 and VVIV, 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 iavirus strain (YB-PMP20) from brown-spotted pitvipers. Phylogenetic analysis indicated that YB-PMP20 belongs to the iavirus 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 iavirus 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

| Name | Sequence (5'→3') | Position |
|------|------------------|----------|
| 1F   | TTCTTACCCAAAAGGTAGGA | 1–20     |
| 1R   | CAACCAGCTAAATCTCACA | 990–1009 |
| 2F   | CTTTTCATTGAGGATCCAGG | 900–919  |
| 2R   | CTTACTATTGCTAACCTCACA | 1970–1989 |
| 3F   | TGTATTTGTCAAGAGCAAGCAG | 1800–1819 |
| 3R   | ATTTTTTGTCGAAATAAGTT | 2870–2889 |
| 4F   | CAGCACAAATACTAAGATTA | 2746–2765 |
| 4R   | ACTTCACCACAGTCTGTCAA | 3742–3761 |
| 5F   | TCATTAGGATCTGACGATTCA | 3471–3490 |
| 5R   | ATTCCTAACGAACAAAGAAG | 4268–4287 |
| 6F   | TTGGCAAAATACTTAAGCAG | 4034–4053 |
| 6R   | TTAACTAAGACGTCTCGTCT | 5014–5033 |
| 7F   | CTGAGCTCTACCAGCTGAAG | 4856–4875 |
| 7R   | AACAAGCCTTAATTACAAAC | 5842–5861 |
| 8F   | TGTAGCAAAATCTCCTGTGG | 5677–5696 |
| 8R   | ATATCGCTGTAGTTTTCAGC | 6514–6533 |
| 9F   | GCCTTGTAAACACTGTTGATT | 6387–6406 |
| 9R   | GCTCTAATTCTAGCTCCAGT | 7405–7424 |
| 10F  | TAGTCATATGATGAACTGA | 7242–7261 |
| 10R  | AGCGAAGGAGGAAATGAAA | 8205–8224 |
| 11F  | GGAGGCAAGGAATTTTAGCA | 8054–8073 |
| 11R  | CATATGATAGCAATAGGCTA | 9081–9100 |
| 12F  | TCTCCAAAACATGTCATTTT | 8922–8941 |
| 12R  | TTTATATGTTTTGTATTTT | 9789–9808 |

Table 2

|                      | YB-PMP20 | VVIV | AEIV | CUPV1 | DABV | FCIV1 | HUPV36 | LyIV1 | REBV | S |
|----------------------|----------|------|------|-------|------|-------|--------|-------|------|---|
|                      | nt%      |      |      |       |      |       |        |       |      |   |
| Polyprotein          | 100      | 100  | 58.6 | 48.6  | 43.7 | 24.6  | 44.1   | 42.4  | 29.1 | 37.1 | 24.5 | 37.1 | 22.7 |
|                      | 99.2     | 99.6 | 46.6 | 20.9  | 36.8 | 22.3  | 99.2   | 98.8  | 47.5 | 29.7 | 30.5 | 27.9 | 37.1 |
| VP1                  | 100      | 100  | 62.6 | 56.4  | 42.7 | 27.2  | 42.4   | 29.1  | 31.2 | 32.9 | 28.4 | 32.9 | 28.4 |
|                      | 99.2     | 98.8 | 47.5 | 29.7  | 30.5 | 27.9  | 99.2   | 98.8  | 47.5 | 29.7 | 30.5 | 27.9 | 37.1 |
| VP2                  | 100      | 100  | 64.3 | 65.2  | 40.1 | 25.7  | 41.4   | 25.7  | 35.4 | 30  | 31.6 | 17.4 | 99.5 |
|                      | 99.5     | 99.6 | 49.3 | 21.2  | 34.3 | 21.9  | 99.5   | 99.6  | 49.3 | 21.2 | 34.3 | 21.9 | 37.1 |
| 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 |
|                      | 99.3     | 100  | 46.1 | 29.2  | 36.4 | 26.8  | 99.3   | 100   | 46.1 | 29.2 | 36.4 | 26.8 | 37.1 |
| 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 |
|                      | 99.1     | 100  | 53.9 | 42.1  | 49.8 | 38.8  | 99.1   | 100   | 53.9 | 42.1 | 49.8 | 38.8 | 37.1 |
| Protease             | 100      | 100  | 58.6 | 50.5  | 47.4 | 25.6  | 46.9   | 25.6  | 40.6 | 22.8 | 38.1 | 22.9 | 99.5 |
|                      | 99.5     | 100  | 50.5 | 27    | 37.6 | 22.9  | 99.5   | 100   | 50.5 | 27    | 37.6 | 22.9 | 37.1 |
| 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 |
|                      | 99.5     | 99.8 | 45.3 | 33.2  | 37.6 | 34.3  | 99.5   | 99.8  | 45.3 | 33.2 | 37.6 | 34.3 | 37.1 |

Figures
Figure 1

Map of Laojun Mountain Nature Reserve.
Figure 2

Please see the Manuscript file for the complete figure caption.

Figure 3

Alignment of the conserved amino acid motifs of the Helicase (A), Protease (B) and RdRp (C).