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Research Paper

# Krykféie dicistrovirus: A novel dicistrovirus in velvety free-tailed bats from Brazil

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ABSTRACT

The *Dicistroviridae* family comprises positive single-stranded RNA viruses that are classified into *Picornavirales* order. These viruses are identified in arthropod hosts, including some having devastating economic consequences. Here, we described and characterized a novel nearly complete dicistrovirus genome identified in liver samples of velvety free-tailed bats (*Molossus molossus*) collected in June 2010 in Araçatuba city, São Paulo State, Brazil. This novel virus presents a genome of 9262 nucleotides in length and a typical dicistrovirus genome organization. Based on our phylogenetic analysis and ICTV criteria, we propose this virus as a novel species into the *Triatovirus* genus. Attempts of viral propagation in Vero E6 and C6/36 cell lines were unsuccessful. The novel dicistrovirus was detected only in one out of nine liver bat samples, representing for the first time an internal organ detection from a representative of this virus family.

## 1. Introduction

*Dicistroviridae* is a family of small non-enveloped viruses that belong to *Picornavirales* order. Members of this family possess linear, positive-sense RNA genome with ~8.5 to 10 kb in length (Valles et al., 2017). The *Discistroviridae* genomes exhibit a typical organization containing two open reading frames (ORFs) that encodes the non-structural (ORF1) and structural (ORF 2) proteins. The ORF1 includes domains 2C-like helicase, 3C-like protease, and 3D-like RNA-dependent RNA polymerase, and the ORF2 contains the capsid domain. Also, the dicistroviruses have two internal ribosome entry sites (IRES), one for translation of the replicase in ORF1 protein, and another in the intergenic region, for translation of capsid protein in ORF2 (Nakashima and Uchiumi, 2009).

The *Dicistroviridae* family is composed of three genera, including *Apavirus* genus with six species, the *Cripavirus* genus with four species and the *Triatovirus genus* with five species (Valles et al., 2017). These viruses have been detected predominantly in invertebrate hosts, such as aphids, leafhoppers, flies, bees, ants, silkworms and shrimps. Some of them are pathogenic to their hosts, leading to devastating economic consequences such as the *Israeli acute paralysis virus* (IAPV) infecting honeybees and *Taura syndrome virus* (TSV) in shrimps (Bonning and Miller, 2010). Recently, new dicistroviruses have been detected in feces samples of vertebrate hosts such as goose and bats (Greninger and Jerome, 2016; Reuter et al., 2014; Yinda et al., 2017). Here, we report the identification and characterization of a novel dicistrovirus identified in a tissue sample of a velvety free-tailed bat (*Molossus molossus*) captured in Brazil.

## 2. Material and methods

### 2.1. Bat samples, viral genome sequencing and assembly

In June 2010, we captured 42 bats in rural areas of Araçatuba city, São Paulo State, Brazil. Bats were captured using mist nets, euthanized, and necropsied and were identified on the basis of morphological criteria. These animals were classified into five different species; *Molossus rufus*, *Artibeus lituratus*, *Carollia perspicillata*, *Glossophaga soricina* and

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*Desmodus rotundus* (Norberg and Rayner, 1987). Kidney and liver samples were collected for virological analyses. Sample collection and handling procedures were approved by the Brazilian Committee on Animal Experimentation (protocol number 00858-2012) and Chico Mendes Institute for the Conservation of Biodiversity; protocol numbers 12.751-3/2009 and 27.346-1/2011. Samples were separated into eight pools clustered by species and sample type. The sample pools were homogenized using a 5 mm stainless steel bead on Tissuelyser II equipment (Qiagen, Hilden, Germany) during 10 min at 30 Hz.

The sample supernatant was filtered using a 0.22 µm-pore-size polyvinylidene difluoride filter (Merck Millipore, Burlington, USA). The viral RNA was extracted from 140 µl of sample supernatant using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), eluted in 60 µl of elution buffer and then performed the synthesis of cDNA using SuperScript II (Invitrogen, Carlsbad, USA). The samples were sequenced using the TruSeq RNA sample preparation kit in an Illumina HiSeq 2500 instrument (Illumina, USA) with a paired-end and 150-base-read protocol in *RAPID* module. Sequence reads were assembled *de novo* using the metaViC pipeline as previously described (de Souza et al., 2018a; de Souza et al., 2018b).

### 2.2. Genome characterization and phylogenetic analysis

Viral genomes were assessed for genome size and ORFs prediction using Geneious 9.1.2 (Biomatters, New Zealand). The ORFs were also confirmed using the BLASTX database. Also, protein domains were screened using Pfam (Finn et al., 2014). The nucleotide sequence determined in this study was deposited in GenBank under the accession number MH370347.

Maximum likelihood (ML) phylogenetic trees were inferred using a protein alignment of 316 amino acids of the RNA-dependent RNA polymerase region – RdRP (ORF1: 4952 to ORF1: 5377, numbered according to the virus identified in this study) and 673 amino acids of Capsid protein. The final alignment contained sequences of the viruses identified in the present study and sequences obtained from representative members of the *Picornavirales* order. The multiple sequence alignments (MSAs) were carried out using PROMALS3D (Pei et al., 2008) and the ML trees were inferred using IQ-TREE version 1.6.0 software using LG + I + G4 to RdRp region and LG + F + I + G4 substitution model with 1000 ultrafast bootstraps (Nguyen et al., 2015). The best-fit models were selected based on 168 reversible amino acids substitution models based on Bayesian Information Criterion (Kalyaanamoorthy et al., 2017). Statistical support for individual nodes of the phylogenetic tree was estimated using the bootstrap value. The phylogenetic tree was visualized using FigTree v.1.4.2.

### 2.3. RT-PCR to new dicistrovirus

To determine the authenticity and frequency of viral genomes of the new dicistrovirus, we designed primer sets to specifically amplify an 1294 bp long sequence located in ORF 1 gene, based on the virus identified in this study (forward primer: 5′-GCGTTTAGGCGGTGTCC CGC-3′ - Position 4578 to 4597; reverse primer: 5′ -CAATATGGGCGA CCACACGTCCC -3′ - Position 5849 to 5871). Then, the viral RNA of individual bats samples were extracted using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) and converted to cDNA using Superscript III (Invitrogen, Carlsbad, USA) with random hexamers (Invitrogen, Carlsbad, USA), following the manufacturer's instructions. Subsequently, PCR was performed using Platinum Taq DNA polymerase High Fidelity (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. The cycling conditions were: 98 °C for 30 s followed by 35 cycles at 98 °C for 15 s, 63 °C for 30 s and 68 °C for 2 min, followed by a final extension of 68 °C for 5 min. Amplicons were visualized by 2% agarose gel electrophoresis. All PCR products were verified by dideoxy sequencing using ABI 3730 genetic analyzer (Applied Biosystems, Foster City, USA).

### 2.4. Viral propagation in culture cell

The positive sample supernatant was filtered through 0.22 µm-pore-size polyvinylidene difluoride syringe filter (Merck Millipore, Burlington, USA) and 200 µl was inoculated into Vero E6 and C6/36 cells monolayer in T-25 cell culture flasks. Viral adsorption was carried out at 37 °C under gently rocking during 1 h, followed by addition of 6 ml of cell culture medium, Dulbecco's modified Eagle's medium (DMEM) for Vero E6 or Leibovitz's L-15 medium for C6/36 cell line, with 2% fetal bovine serum supplementation. Infected cells were incubated during seven days (37 °C and 5% $CO_2$ atmosphere for Vero E6 and 28 °C for C6/36), and then 200 µl of infected cell culture supernatant was collected and used for blind infection passage into new cell culture monolayer in a T-25 flask. Three blind viral infection passages were attempted in each cell line, viral RNA was extracted from each passage and RT-PCR for dicistrovirus performed as previously described.

## 3. Results and discussion

We have identified a nearly complete genome of a novel dicistrovirus, which has been tentatively designated as Krykféie dicistrovirus (KRYV), which is a tribute to the indigenous tribe of the Kaingang that inhabited the region of Araçatuba. Krykféie means bat in Kaingang language. The KRYV genome was obtained by 30,504 reads with a mean coverage of 1190×. This virus was detected in a liver sample pool composed of nine velvety free-tailed bats (*Molossus molossus*) captured in June 2010 in Araçatuba city, São Paulo State, Brazil. The genome of 9262 nt in length, presents a typical genome organization of dicistroviruses with two putative ORFs, which encodes a non-structural protein with 2C-like helicase, 3C-like peptidase, and 3D-like RdRp domains, and in another ORF encodes a structural protein, which contains the capsid domain (Fig. 1a).

Phylogenetic analysis using the RdRp and Capsid proteins showed that KRYV grouped into the genus *Triatovirus* from *Dicistroviridae* family (Fig. 1b and c) and shared the same common ancestor with Bat cripavirus identified in feces of straw-coloured fruit bat (*Eidolon helvum*) in Cameroon (Yinda et al., 2017). Note that Bat cripavirus and Cripavirus NB-1 incorrectly have 'Cripavirus' in their names, but they are classified into Triatovirus genus. Based on BLASTX analysis, we observed that KRYV shared 36 to 49% of an amino acid identity of the non-structural polyprotein and capsid region with other representative species of *Triatovirus* genus. Based on our analysis and in the species demarcation criteria established by ICTV (Culley and Suttle, 2012), which states that a new species must have ≤90% at amino acid identity, we propose that KRYV should constitute a novel species into *Triatovirus* genus within *Dicistroviridae* family.

To confirm the metagenomic results and to determine the prevalence of KRYV in individual samples, we have screened these samples by RT-PCR. The genome of KRYV was detected only in one individual liver sample of a velvety free-tailed bat. Previously studies described two other dicistroviruses from bat samples, the Cripavirus NB-1 identified in an insectivorous bat (*Pipistrellus pipistrellus*) in Hungary (Reuter et al., 2014), and Bat cripavirus described in a fruit bat (*Eidolon helvum*) in Cameroon (Yinda et al., 2017), both viruses were detected from fecal samples, which suggests a possible contaminant from the bats dietary. The attempts of viral propagation from the positive bat liver sample in vertebrate (Vero E6) and invertebrate (C6/36) cell lines were unsuccessful. The viral genome could be detected by specific RT-PCR only in the first blind passage probably due to inoculum contamination detection. Here, we have identified a novel dicistrovirus detected for the first time in a liver sample of a velvety free-tailed bat, suggesting an evidence that dicistrovirus may infect bats.

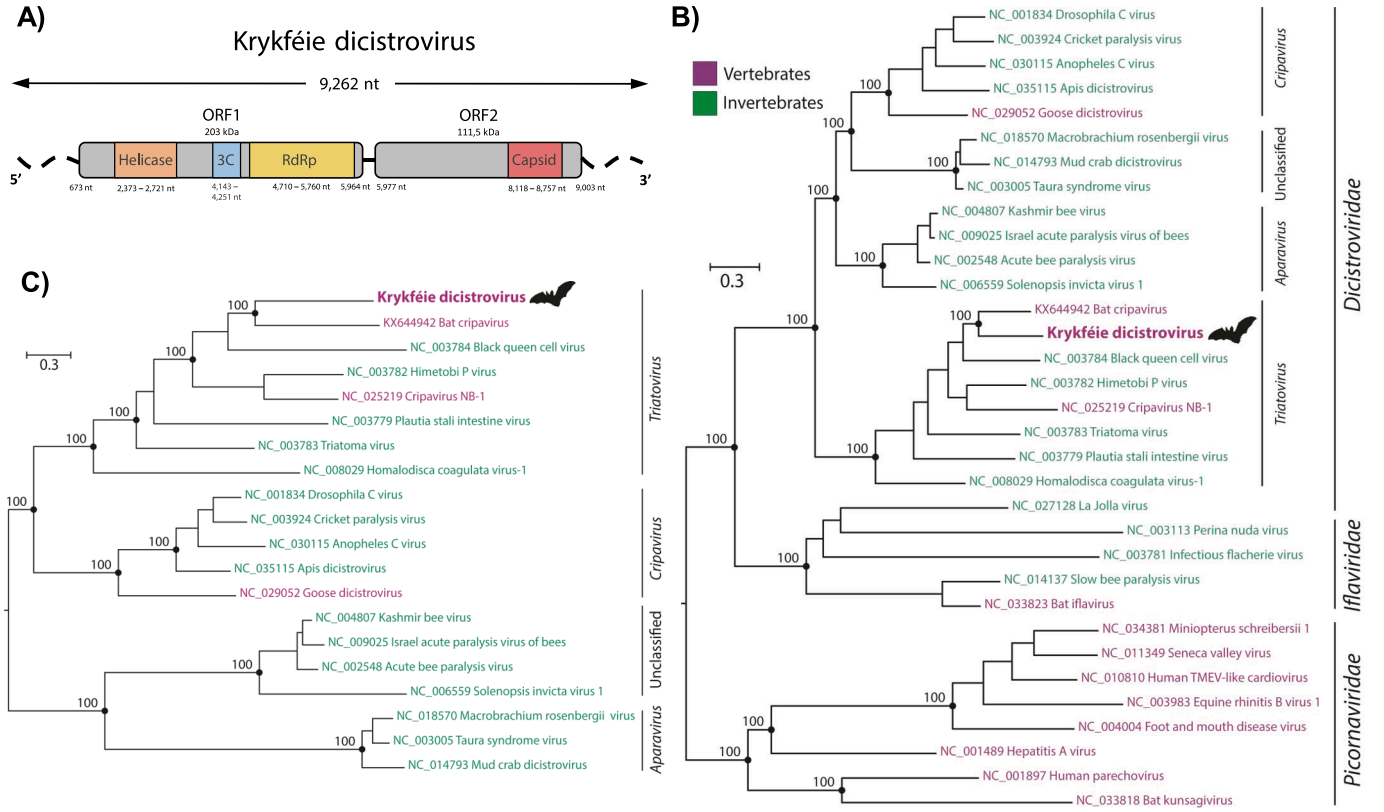


**Fig. 1.** (a) Genome organization of the nearly complete genome of Krykféie dicistrovirus. The length of the determined nucleotide viral sequence is shown at the top. Solid-lined boxes indicate complete sequence of ORFs, respectively. ORF1 encodes a non-structural polyprotein including putative functional domains. The helicase; 3C peptidase protease domain; and RdRp: RNA-dependent RNA polymerase. ORF2 encodes a capsid protein domain. (b) Maximum likelihood phylogenetic tree is showing the evolutionary relationships of virus identified in our study with representatives of the *Picornavirales* order using the alignment of RdRp protein. (c) Maximum likelihood phylogenetic tree is showing the evolutionary relationships of virus identified in our study with representatives of the *Dicistroviridae* family using the alignment of the capsid protein. Phylogenies are midpoint rooted for clarity of presentation. The scale bar indicates evolutionary distance in numbers of substitutions per amino acid site. Bootstrap values of 1000 replicates are shown in principal nodes. Krykféie dicistrovirus sequence generated in this study is shown in bold and bat silhouette.

## 4. Conclusions

In summary, we have identified and characterized a new dicistrovirus. Our phylogenetic analysis showed that KRYV represents a novel species to be classified into genus *Triatovirus* within *Dicistroviridae* family. Further studies will be required to confirm if bats can be infected by dicistroviruses.

## Declaration of Competing interest

None.

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