Fumagalli, M. J., de Souza, W. M., de Araujo, J., Modha, S., Queiroz, L. H., Durigon, E. L., Murcia, P. R. and Figueiredo, L. T. M. (2019) Krykféie dicistrovirus: a novel dicistrovirus in velvety free-tailed bats from Brazil. Infection, Genetics and Evolution, 75, 104036.

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Deposited on: 27 November 2019

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Title: Krykféie dicistrovirus: a novel dicistrovirus in velvety free-tailed bats from Brazil

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23 **Abstract**

24 The *Dicistroviridae* family comprises positive single-stranded RNA viruses that are
25 classified into *Picornavirales* order. These viruses are identified in arthropod hosts,
26 including some having devastating economic consequences. Here, we described and
27 characterized a novel nearly complete dicistrovirus genome identified in liver samples
28 of velvety free-tailed bats (*Molossus molossus*) collected in June 2010 in Araçatuba
29 city, São Paulo State, Brazil. This novel virus presents a genome of 9,262 nucleotides
30 in length and a typical dicistrovirus genome organization. Based on our phylogenetic
31 analysis and ICTV criteria, we propose this virus as a novel species into the *Triatovirus*
32 genus. Attempts of viral propagation in Vero E6 and C6/36 cell lines were unsuccessful.
33 The novel dicistrovirus was detected only in one out of nine liver bat samples,
34 representing for the first time an internal organ detection from a representative of this
35 virus family.

36

37 **Keywords:** *Dicistroviridae*, *Triatovirus*, *Picornavirales*, picornavirus.

38

39 **1. Introduction**

40 *Dicistroviridae* is a family of small non-enveloped viruses that belong to
41 *Picornavirales* order. Members of this family possess linear, positive-sense RNA
42 genome with ~8.5 to 10 kb in length (Valles et al., 2017). The *Discistroviridae* genomes
43 exhibit a typical organization containing two open reading frames (ORFs) that encodes
44 the non-structural (ORF1) and structural (ORF 2) proteins. The ORF1 includes domains
45 2C-like helicase, 3C-like protease, and 3D-like RNA-dependent RNA polymerase, and
46 the ORF2 contains the capsid domain. Also, the dicistroviruses have two internal
47 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 faeces 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 *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.

73 Samples were separated into eight pools clustered by species and sample type. The
74 sample pools were homogenized using a 5 mm stainless steel bead on Tissuelyser II
75 equipment (Qiagen, Hilden, Germany) during 10 minutes at 30 Hz.

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

85

86 **2.2. Genome characterization and phylogenetic analysis**

87 Viral genomes were assessed for genome size and ORFs prediction using
88 Geneious 9.1.2 (Biomatters, New Zealand). The ORFs were also confirmed using the
89 BLASTX database. Also, protein domains were screened using Pfam (Finn et al.,
90 2014). The nucleotide sequence determined in this study was deposited in GenBank
91 under the accession number MH370347.

92 Maximum likelihood (ML) phylogenetic trees were inferred using a protein
93 alignment of 316 amino acids of the RNA-dependent RNA polymerase region – RdRP
94 (ORF1: 4,952 to ORF1: 5,377, numbered according to the virus identified in this study)
95 and 673 amino acids of Capsid protein. The final alignment contained sequences of the
96 viruses identified in the present study and sequences obtained from representative
97 members of the *Picornavirales* order. The multiple sequence alignments (MSAs) were

98 carried out using PROMALS3D (Pei et al., 2008) and the ML trees were inferred using
99 IQ-TREE version 1.6.0 software using LG+I+G4 to RdRp region and LG+F+I+G4
100 substitution model with 1,000 ultrafast bootstraps (Nguyen et al., 2015). The best-fit
101 models were selected based on 168 reversible amino acids substitution models based
102 on Bayesian Information Criterion (Kalyaanamoorthy et al., 2017). Statistical support
103 for individual nodes of the phylogenetic tree was estimated using the bootstrap value.
104 The phylogenetic tree was visualized using FigTree v.1.4.2.
105

106 **2.4. RT-PCR to new dicistrovirus**

107 To determine the authenticity and frequency of viral genomes of the new
108 dicistrovirus, we designed primer sets to specifically amplify an 1,294 bp long sequence
109 located in ORF 1 gene, based on the virus identified in this study (forward primer: 5′-
110 GCGTTTAGGCGGTGTCCCGC-3′ - Position 4,578 to 4,597; reverse primer: 5′ -
111 CAATATGGGCGACCACACGTCCC -3′ - Position 5,849 to 5,871). Then, the viral
112 RNA of individual bats samples were extracted using the QIAamp viral RNA extraction
113 kit (Qiagen, Hilden, Germany) and converted to cDNA using Superscript III
114 (Invitrogen, Carlsbad, USA) with random hexamers (Invitrogen, Carlsbad, USA),
115 following the manufacturer’s instructions. Subsequently, PCR was performed using
116 Platinum Taq DNA polymerase High Fidelity (Thermo Fisher Scientific, Waltham,
117 USA), according to the manufacturer's instructions. The cycling conditions were: 98°C
118 for 30 seconds followed by 35 cycles at 98°C for 15 seconds, 63ºC for 30 seconds and
119 68ºC for 2 minutes, followed by a final extension of 68°C for 5 minutes. Amplicons
120 were visualized by 2% agarose gel electrophoresis. All PCR products were verified by
121 dideoxy sequencing using ABI 3730 genetic analyzer (Applied Biosystems, Foster
122 City, USA).

123

124 **2.5 Viral propagation in culture cell**

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

137

138 **3. Results and Discussion**

139 We have identified a nearly complete genome of a novel dicistrovirus, which
140 has been tentatively designated as Krykféie dicistrovirus (KRYV), which is a tribute to
141 the indigenous tribe of the Kaingang that inhabited the region of Araçatuba. Krykféie
142 means bat in Kaingang language. The KRYV genome was obtained by 30,504 reads
143 with a mean coverage of 1,190x. This virus was detected in a liver sample pool
144 composed of nine velvety free-tailed bats (*Molossus molossus*) captured in June 2010
145 in Araçatuba city, São Paulo State, Brazil. The genome of 9,262 nt in length, presents
146 a typical genome organization of dicistroviruses with two putative ORFs, which
147 encodes a non-structural protein with 2C-like helicase, 3C-like peptidase, and 3D-like

148 RdRp domains, and in another ORF encodes a structural protein, which contains the
149 capsid domain (**Figure 1a**).

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

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

173 identified a novel dicistrovirus detected for the first time in a liver sample of a velvety
174 free-tailed bat, suggesting an evidence that dicistrovirus may infect bats.
175
176 **5. Conclusions**
177 In summary, we have identified and characterized a new dicistrovirus. Our
178 phylogenetic analysis showed that KRYV represents a novel species to be classified
179 into genus *Triatovirus* within *Dicistroviridae* family. Further studies will be required
180 to confirm if bats can be infected by dicistroviruses.
181
182 **Acknowledgments**
183 This study was supported by the Fundação de Amparo à Pesquisa do Estado de
184 São Paulo, Brazil (Grant number No. 13/14929-1, and Scholarships No. 12/24150-9;
185 15/05778-5; 17/13981-0, 14/20851-8; 16/01414-1; 06/00572-0). PRM was supported
186 by the Medical Research Council of the United Kingdom (Grant MC_UU_120/14/9).
187
188 **Declarations of Interest**
189 None.
190

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240

241 Figure Legend

242

243 **Figure 1.** (a) Genome organization of the nearly complete genome of Krykféie
244 dicistrovirus. The length of the determined nucleotide viral sequence is shown at the
245 top. Solid-lined boxes indicate complete sequence of ORFs, respectively. ORF1
246 encodes a non-structural polyprotein including putative functional domains. The
247 helicase; 3C peptidase protease domain; and RdRp: RNA-dependent RNA polymerase.
248 ORF2 encodes a capsid protein domain. (b) Maximum likelihood phylogenetic tree is
249 showing the evolutionary relationships of virus identified in our study with
250 representatives of the *Picornavirales* order using the alignment of RdRp protein. (c)
251 Maximum likelihood phylogenetic tree is showing the evolutionary relationships of
252 virus identified in our study with representatives of the *Dicistroviridae* family using the
253 alignment of the capsid protein. Phylogenies are midpoint rooted for clarity of
254 presentation. The scale bar indicates evolutionary distance in numbers of substitutions
255 per amino acid site. Bootstrap values of 1,000 replicates are shown in principal nodes.
256 Krykféie dicistrovirus sequence generated in this study is shown in bold and bat
257 silhouette.