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Metagenomic analysis of Sichuan takin fecal sample viromes reveals novel enterovirus and astrovirus

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ABSTRACT

The Sichuan takin inhabits the bamboo forests in the Eastern Himalayas and is considered as a national treasure of China with the highest legal protection and conservation status considered as vulnerable according to The IUCN Red List of Threatened Species. In this study, fecal samples of 71 Sichuan takins were pooled and deep sequenced. Among the 103,553 viral sequences, 21,961 were assigned to mammalian viruses. *De novo* assembly revealed genomes of an enterovirus and an astrovirus and contigs of circoviruses and genogroup I picobirnaviruses. Complete genome sequencing and phylogenetic analysis showed that Sichuan takin enterovirus is a novel serotype/genotype of the species *Enterovirus G*, with evidence of recombination. Sichuan takin astrovirus is a new subtype of bovine astrovirus, probably belonging to a new genogroup in the genus *Mamastrovirus*. Further studies will reveal whether these viruses can also be found in Mishmi takin and Shaanxi takin and their pathogenic potentials.

1. Introduction

Traditionally, viral diversities in different animals were studied through isolation and characterization of the viruses. In the last 20 years, amplification of viral nucleic acids directly from clinical samples of animals by PCR/RT-PCR and sequencing the amplified products has led to the discovery of numerous novel viruses (Bodewes et al., 2013; Delwart, 2012; Katano et al., 2011; Snijders et al., 1990; Whiley et al., 2009). In the last few years, the use of deep sequencing and metagenomics studies has further expanded our knowledge on the diversity of viruses (Ao et al., 2017; Ge et al., 2012; Ng et al., 2009; Tang and Chiu, 2010). For example, we recently described the spectrum of viruses found in dromedary camels using the metagenomics approach, which have led to the discovery of a number of previously unknown viruses (Woo et al., 2014). However, these metagenomics studies have so far been mainly concentrating on the commonly encountered animals, such as cats, dogs, pigs, cattle, rodents and horses (Bodewes et al., 2014; Drewes et al., 2017; Li et al., 2015; Mihalov-Kovacs et al., 2015; Mitra et al., 2016; Phan et al., 2011; Shan et al., 2011), or specific groups of animals with epidemiological interests, such as dromedaries and bats (Ge et al., 2012; Woo et al., 2014; Wu et al., 2012). The viromes of the less commonly encountered and threatened species are relatively understudied.

Takins (*Budorcas taxicolor*) are animals found in the eastern Himalayas. They are classified into three subspecies: *B. taxicolor taxicolor* (Mishmi takin), *B. taxicolor bedfordi* (Shaanxi or golden takin) and *B. taxicolor tibetana* (Tibetan or Sichuan takin). The Sichuan takin is native to Tibet, Sichuan, Gansu and Xinjiang of China. Similar to the giant panda, of which the fecal viromes have recently been analyzed (Zhang et al., 2017), the Sichuan takin inhabits the dense bamboo forests and is considered as a national treasure of China with the highest legal protection. Since 2008, the Sichuan takin's conservation status was considered as vulnerable according to The IUCN Red List of Threatened Species (IUCN, 2017). In 1975, there were around 7000 Sichuan takins in the wild (State Forestry Administration, 2009). We hypothesized that there could also be previously undescribed viruses in

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this threatened species and characterizing these viruses will improve our understandings on the evolution of the corresponding groups of viruses. To test the hypothesis, we analyzed the viromes of the fecal samples of wild Sichuan takins, which is the first metagenomic study on the takins.

2. Materials and methods

2.1. Ethical statement

Collection of fecal samples from the wild Sichuan takins was approved by the administration of Dongyanggou Nature Reserve (DYG NR) and Tangjiahe Nature Reserve (TJH NR).

2.2. Sample collection

All fecal samples of wild Sichuan takins were collected from DYG NR and partly from the conjunction area between DYG NR and TJH NR over a seven-month (January–July 2013) period (Fig. 1A-C). A total of 71 adult wild takins have been tested in this study. DYG NR and TJH NR are located in northern Minshan where inhabits various wildlife, including giant panda (*Ailuropoda melanoleuca*) and golden monkey (*Rhinopithecus roxellanae*). These areas are isolated from human disturbances, such as herb collection and livestock grazing. PCR amplification and sequencing of the mitochondrial cytochrome-b gene was performed to validate that the 71 Sichuan takins were of the species *Budorcas taxicolor* (data not shown).

2.3. Sample preparation for Illumina sequencing

The viral transport medium containing the 71 fecal samples were pooled, 200 µl (about 200 mg of feces per 2 ml viral transport medium) each, and centrifuged at 10000 × *g* for 10 min. The supernatant was then filtered through a 0.45-µm filter (Millipore, Massachusetts, USA) to remove eukaryotic and bacterial cell-sized particles. The filtrate was treated with a cocktail of DNase enzymes, including 14 U of turbo DNase (Ambion, Austin, TX, USA), 20 U of benzonase (Novagen, Madison, WI, USA) and 20 U of RNase One (Promega, Wisconsin, USA) at 37 °C for 60 min in 1x DNase buffer (Ambion, Austin, TX, USA) to digest unprotected nucleic acids. Total RNA from the sample was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA) and a random primer containing a 20-base arbitrary sequence at the 5′ end followed by a randomized octamer (8 N) at the 3′ end. A single round priming and extension was performed using Klenow fragment polymerase (New England Biolabs, Massachusetts, USA). PCR amplification with primer consisting of 20-base arbitrary sequence of the random primer was performed in 20 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 1 min and a final extension at 68 °C for 7 min in an automated thermal cycler (Applied Biosystems, Foster City, CA, USA). Standard precautions were taken to avoid PCR contamination and no amplified PCR product was observed in negative control. The PCR product was purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol with slight modification. The purified DNA was eluted in 15 µl of EB buffer and used as the template for library construction.

2.4. Library construction for illumina sequencing

The metagenomic library was prepared using Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and Nextera XT Index kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, 1 ng of input DNA was tagmented by the Nextera XT transposome at 55 °C for 5 min. This transposome simultaneously fragmented the input DNA and added adapter sequence to the ends, allowing amplification by PCR in subsequent steps. The sequencing library with tagmented DNA was then amplified in 12 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 5 min in an automated thermal cycler (Applied Biosystems, Foster City, CA, USA). Amplified DNA library was purified using 1.8x AMPure XP beads (Beckman Coulter, Danvers, MA, USA) to remove

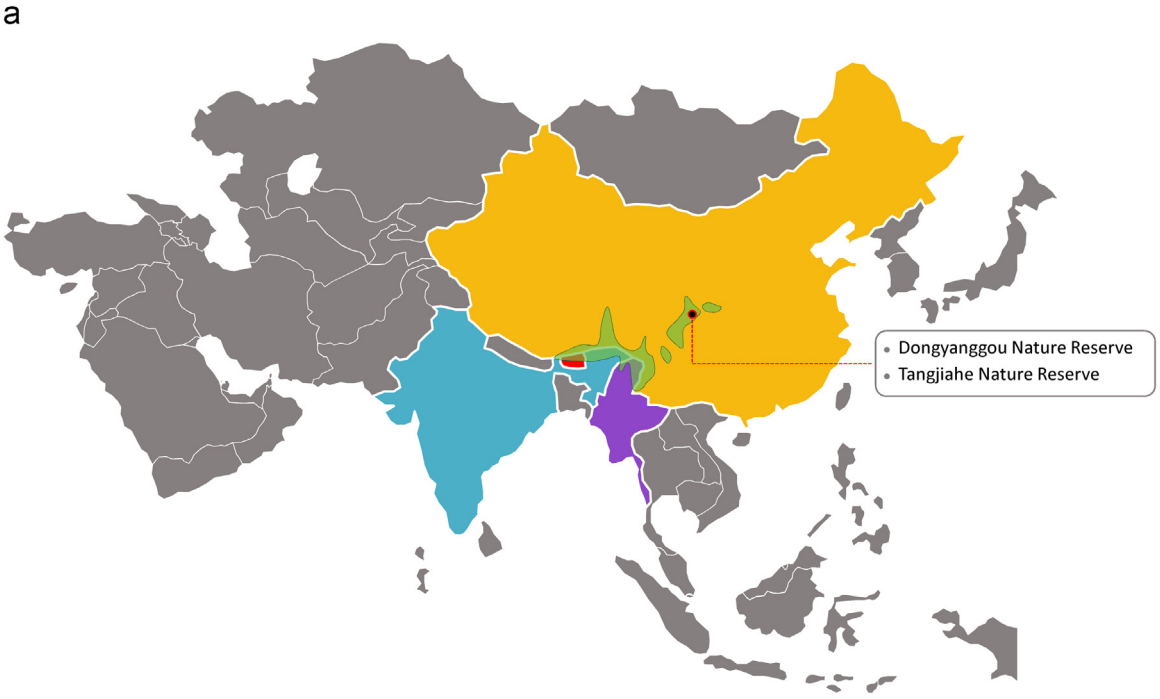

Fig. 1. (A) Map showing the location of the Dongyanggou Nature Reserve and Tangjiahe Nature Reserve in Sichuan Province in China. Green region represents the geographical distribution of takin population in the world according to the IUCN, ranging from Bhutan (red), China (yellow), northeastern India (blue) to northern Myanmar (purple). (B) and (C) Wild takins in the Dongyanggou Nature Reserve.

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