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Short communication

Molecular detection of novel circoviruses in ticks in northeastern China

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ABSTRACT

Novel circular single-stranded DNA (ssDNA) genomes have been found in various animals using high-throughput sequencing techniques. In this study, two circular ssDNA genomes were detected in adult ticks from northeastern China by Solexa sequencing and PCR. The two sequences shared a similar genomic organization to circoviruses, with genomes of 1936 bp (TiCV-1) and 1812 bp (TiCV-2), each including two major open read frames (ORFs), ORF1 and ORF2, encoding putative replicase and capsid proteins, respectively. The potential stem-loop structure of a circovirus was predicted in the intergenic region between the two ORFs. Sequence comparison showed that the genome of TiCV-2 was almost the same as that of TiCV-1, except for two deletions and several mutations, and they had a high identity of 71.3–72.9% with Raven circovirus. The infection rates of circoviruses were calculated by the maximum likelihood estimation as 3.2% (95% CI, 1.9–5.2%) for TiCV-1 in the investigated *Haemaphysalis longicornis*, and 1.2% (95% CI, 0.2–4.0%) for TiCV-2 in *Ixodes crenulatus* from Yichun of Heilongjiang Province. These results indicate that the two sequences are distantly related to known circovirus genomes and may represent novel species in the family Circoviridae.

1. Introduction

Members of the Circoviridae family, including Gyrovirus and Circovirus, are small non-enveloped single-stranded DNA viruses with circular genomes of ~2 kb (Todd et al., 2001). The genus Gyrovirus has only one member, chicken anemia virus (CAV), which can cause clinical disease and subclinical immunosuppression in chickens worldwide (Todd, 2004). In contrast, circoviruses have been considered causative pathogens of pigs, dogs, and minks (Segales et al., 2005; Li et al., 2013; Lian et al., 2014), as well as a broad range of avian species, including canaries, geese, pigeons, and parrots (Schoemaker et al., 2000; Phenix et al., 2001; Smyth et al., 2001). Porcine circovirus (PCV) has a huge impact on swine production worldwide, and can cause post-weaning multi-systemic wasting syndrome, dermatitis, nephropathy syndrome, and reproductive disorders (Zhai et al., 2014). Dog circovirus is considered to be a cause of alimentary syndromes, such as vomiting, diarrhea, and hemorrhagic enteritis (Li et al., 2013; Zaccaria et al., 2016; Dowgier et al., 2017). Mink circovirus can cause refractory diarrhea (Hai et al., 2014). Avian circoviruses have been associated with symptoms of immunosuppression, lymphoid depletion, and developmental abnormalities, including feathering disorders, beak/claw deformity, and growth retardation (Raue et al., 2005; Shearer et al., 2008).

As most circoviruses cannot readily be grown in cell culture, the high-throughput sequencing techniques have enabled the discovery of novel circoviruses in wild animals and insects (Ge et al., 2011; Marton et al., 2015). Here, we report on the detection of circovirus-like genomes in ticks from northeastern China, which are distantly related to known circoviruses and may represent novel species in the family Circoviridae.

2. Materials and methods

2.1. Tick collection and viral metagenomic analysis

Unfed adult ticks were collected by flagging vegetation in the

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Heilongjiang and Jilin provinces of northeastern China from April–May 2015. The sampling sites included Jingxin (42°25′ N,130°38′ E) and Dunhua (42°27′ N,129°50′ E) of Jilin, and Shuangyashan (46°27′ N, 131°12′ E), Tongjiang (47°43′ N, 133°29′ E), Sufenhe (44°23′ N, 131°9′ E), Yichun (47°24′ N, 128°23′ E), Jiamusi (46°43′ N, 133°12′ E) of Heilongjing (Supplementary Table S1). There are Changbai, Daxinganling, and Xiaoxinganling mountains in the northeast region, with a forestry area accounting for 14.7% of China, and a forest coverage rate reaching 39.6%; thus, there are abundant wild animal and plant resources.

The tick species were identified following morphological criteria as described previously (Chen et al., 2010).The sampled ticks were pooled (approximately 15 ticks per pool) according to the tick species and sampling sites (Supplementary Table S1). Each group was homogenized in a magnesium salt (SM) buffer (50 mM Tris, 10 mM $MgSO_4$, 0.1 M NaCl, pH 7.5), and viral RNA extraction, Solexa sequencing, and data analysis were conducted as previously described (Gong et al., 2015; Liu et al., 2016). Briefly, the viral RNAs and DNAs were extracted, and random primers were used to reverse transcribe the RNA to obtain single-stranded cDNA. Double-stranded cDNA (dscDNA) was synthesized using a Klenow fragment, and sequence-independent single-primer amplification (SISPA) was employed to obtain sufficient viral nucleic acid. The purified PCR products of the 6 groups were pooled together and then subjected to Solexa sequencing in one lane by the Beijing Genome Institute (BGI, Shenzhen, China).

2.2. PCR assays

The precipitate of each tick pool was used for DNA extraction using a TIANamp Genomic DNA Purification System (Tiangen, Beijing, China), and the viral metagenomic results were confirmed by amplifying the partial gene of the circovirus with PCR using the primers Circo-F1 and Circo-R1 (Supplementary Table S2). To obtain the full-length sequence of the circovirus, the primers used to amplify the complete genome were designed according to the viral metagenomic results (Supplementary Table S2). The PCR reaction used 1 μL of genomic DNA as a template in a 25-μL reaction mixture that contained 2.5 μL of 10 × PCR reaction buffer, 1.6 μL of 2.5 mM dNTP mixture, 0.2 U Taq polymerase (Takara Biomedical Technology Co., Ltd, Beijing, China), and 1 μL of each primer (10 mM). The reaction was conducted at 94 °C for 5 min followed by 35 cycles of denaturation for 40 s at 94 °C, annealing for 30–60 s at 52–56 °C, extension for 60–90 s at 72 °C, and a final incubation for 10 min at 72 °C. All the PCR products were analyzed by electrophoresis in 1% agarose gel and sequenced.

2.3. Phylogenetic analysis

Phylogenetic analyses of the whole genome of circoviruses were conducted using the MEGA 5.2 software (http://www.megasoftware.net/). The neighbor-joining method was employed to construct a phylogenetic tree. The reliability of the branches of the tree was assessed using a bootstrap analysis with 1000 replicates.

3. Results and discussion

In this study, we collected 1840 unfed adult ticks from the Jilin and Heilongjiang provinces and found six tick species from 3 genera, with the most abundant being *Haemaphysalis longicornis* (32.4%), followed by *Ixodes persulcatus* (21.0%), *Ixodes crenulatus* (19.2%), *Haemaphysalis concinna* (14.4%), *Dermacentor nuttalli* (7.5%), and *Dermacentor silvarum* (5.5%). Each tick pool (see Supplementary Table S1) was homogenized in SM buffer for viral metagenomic analysis. Among the sequences annotated as mammalian viruses, 30 contigs were found to target the genome of Raven circovirus (GenBank® access number DQ146997), with 76–85% identity (Supplementary Table S3).

The results of the Solexa sequencing were confirmed by amplifying

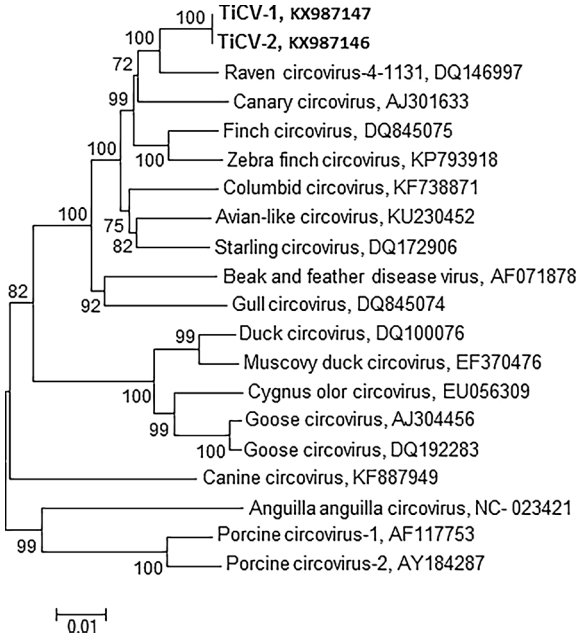

Fig. 1. Phylogenetic analysis of the tick circovirus complete sequences. The phylogenetic tree was generated using MEGA version 5.2 software. Bootstrap testing (1000 replicates) was performed and the bootstrap values are indicated. Sequences are identified by virus species and their GenBank accession numbers. Tick circoviruses identified in this study are marked in black.

the partial fragment of the circovirus genome via PCR. The two species of ticks, *H. longicornis* and *I. crenulatus,* were found to be Circovirus-positive, while the other investigated 4 tick species were negative. The full-length sequences of circoviruses were obtained by PCR using the overlapping primer sets based on the results of Solexa sequencing combined with inverse PCR (Supplementary Table S2), revealing that the circular genome was 1936 nucleotides (nt) for circovirus in *H. longicornis* (GenBank® accession number KX987147) and 1812 nt in *I. crenulatus* (GenBank® accession number KX987146), tentatively designated TiCV-1 and TiCV-2, respectively.

Sequence analysis revealed two large ORFs in the genome, ORF-V1 (ORF 1) and ORF-C1 (ORF 2), which encode the replication-associated proteins (Rep) and the viral capsid protein (Cap). The intergenic region between the two larger ORFs could form a potential stem-loop structure that includes a conserved non-nucleotide (5′-TAGTATTAC-3′) at the apex of the stem-loop structure within the circoviruses, where rolling circle replication of the viral DNA is initiated (Fig. 1). The inverted repeat 5′-cggccacttggagccacgg-3′ is a potential binding site of the circovirus replicase, which was found to overlap the stem-loop nonamer, and the hexamer 5′-GGAGCC-3′ was found two times in or adjacent to this inverted repeat.

Comparison of the two full-length sequences of circoviruses showed that the genome of TiCV-2 was almost the same as that of TiCV-1, except for two deletions at positions 935–1032 and 1360–1385, and five substitutions at positions 436, 1266, 1287, 1480, and 1904 of the genome of TiCV-1. There was a deletion of 26 nucleotides in the coding region of the TiCV-2 Cap compared with that of TiCV-1. Thus, a low identity of 69.0% was found between the Cap amino acid sequences of the two tick circoviruses, while the percentage of Rep amino acid sequence identity was 99.7%.

Comparison of the TiCV-1 and TiCV-2 genome with other circoviruses showed a high identity of 72.9% and 71.3% with the genome of Raven circovirus (RaCV), and a low identity (41.0% to 65.9%) with the canary circovirus (CaCV), Finch circovirus (FiCV), Starling circovirus (StCV), Beak and feather disease virus (BFDV), Gull circovirus (GuCV), Duck circovirus (DuCV), and Goose circovirus (GoCV). The putative Rep protein of TiCV-1 and TiCV-1 was 296 aa, and it had a high identity