Genome sequencing and phylogenetic analysis of Banna virus (genus Seadornavirus, family Reoviridae) isolated from Culicoides

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In an investigation of blood-sucking insects and arboviruses, a virus (YN12243) was isolated from Culicoides samples collected in the Sino-Burmese border region of Yunnan Province, China. The virus caused cytopathic effect (CPE) in C6/36 cells and passaged stably. Polyacrylamide gel analysis showed that the genome of YN12243 was composed of 12 segments of double-stranded RNA (dsRNA), with a distribution pattern of 6-6. The nucleotide and amino acid sequences of the coding region (1-12 segments) were 17,803 bp and 5,925 amino acids in length, respectively. The phylogenetic analysis of VP1 protein (RdRp) revealed that YN12243 belonged to genus Seadornavirus of family Reoviridae, and further analysis indicated that YN12243 belong to the Banna virus (BAV) genotype A2. Additionally, YN12243 was located in the same evolutionary cluster as BAV strains isolated from different mosquito species, suggesting that the BAV isolated from Culicoides does not have species barriers. These results indicate that Culicoides can also be a vector for BAV. In view of the hematophagous habits of Culicoides on cattle, horses, deer, and other large animals, as well as the possibility of spreading and causing a variety of animal arboviral diseases, it is important to improve infection detection and monitor the BAV in large livestock.

Banna virus, Seadornavirus, Reoviridae, mosquito, Culicoides

INTRODUCTION

BAV was first isolated from the cerebrospinal fluid of viral cerebrospinal encephalitis patients, and from serum samples of fever patients collected from Xishuangbanna, Yunnan Province, China (Xu et al., 1990a; Xu et al., 1990b) and was named after the location in which it was first isolated. Since then, BAV has been isolated from various mosquito specimens (Liu et al., 2010), suggesting that mosquitoes are the natural vector for BAV. To date, BAV has been isolated from mosquito specimens collected in Indonesia (Brown et
al., 1993), Vietnam (Nabeshima et al., 2008), South Korea (Kim et al., 2016), as well as from many subtropical or temperate provinces of China (Gansu, Shanxi, Inner Mongolia, Liaoning, Beijing, and Yunnan) (Liu et al., 2010a; Liu et al., 2010b; Liu et al., 2011). Moreover, it was recently reported that BAV has been isolated in Hungary, which is in the northern, more temperate zone of Europe (Reuter et al., 2013). Because BAV has been isolated from a patient, and can be carried or transmitted by a variety of hematophagous vectors and vertebrates, it is considered as a “newly discovered virus” with considerable public health significance (Liu et al., 2010a; Attoui et al., 2005a).

A new virus genus, Seadornavirus genus (Attoui et al., 2005b), was established in the Reoviridae, as published in Virus Classification-International Virus Classification Committee, 8th report (issued in September 2005). The group contains three members: BAV, Liaoning virus (LNV), and Kadipiro virus (KDV). Among them, BAV can be taken as the representative virus of the group (Attoui et al., 2005b; Mohd Jaafar et al., 2005). The BAV genome is composed of 12 segments of dsRNA. Virus RNA can be isolated into 1–12 bands with different molecular weights on polyacrylamide gel electrophoresis (PAGE). BAV segment 1 encodes the virus RNA-dependent RNA polymerase (RdRp). The VP1 is a highly conserved and an important marker for species identification within the family Reoviridae. Segment 2 encodes the T2 protein, forming the innermost nucleus layer. The segment 12 is conserved and abundant sequences of it can be used to analyze (39 sequences registered in GenBank). Besides, the length of segment 12 is relatively short (670 bp), which makes it easy to obtain a full length sequence by PCR. Due to these factors mentioned above, segment 12 is commonly used for identification and evolutionary analysis of BAV (Attoui et al., 2005b; Mohd Jaafar et al., 2005).

Although BAV has been isolated from various mosquito specimens and is widely distributed in a number of countries, there has been no detailed report on the isolation of BAV from Culicoides. In this study, a virus strain (YN12243) was isolated from Culicoides samples collected in the Sino-Burmese border region of Yunnan Province, China, in 2012, and was identified as BAV according to genome sequencing and a phylogenetic analysis. The article was focused on the analysis of the biological phenotype and viral genomic characteristics of BAV isolated from Culicoides.

RESULTS

CPE

C6/36 cells were inoculated with YN12243 virus and CPE were evaluated daily. The results showed that, at 48 h after inoculation, CPE occurred, manifesting as cell aggregation and shedding. The lesion reached to 75% of CPE at 72 h (Figure 1). The YN12243 virus could be stably transferred into C6/36 cells.

Molecular identification

RNA-PAGE results indicated that YN12243 had 12 segments with a distribution pattern of 6-6. However, compared with the BAV positive control, the 5th and 6th bands of YN12243 were further apart, whereas the 7th and 8th bands were closer to the 9th band (Figure 2).

Figure 1 (Color online) Cytopathic effect of YN12243 on C6/36 (>200). A–C, Uninfected C6/36 control cells 24, 48 and 72 h, respectively. D, Infected C6/36 cells, 24 hours post-infection. E, Infected C6/36 cells, 48 hours post-infection, showing cell aggregation. F, Infected C6/36 cells, 72 hours post-infection, showing cell aggregation and detachment of infected cells.
that indicated the sequence of the whole analysis of acid amplification of BAVs (Figure B). Figure virus obtained redesigned products amplification (B). The results showed that the homology values of the YN12243 and SC043 strains were from 82.5% to 99.7% and from 91.3% to 99.7%, respectively. The homology values of the nucleotide and amino acid sequences of the YN12243 strain with multiple isolates from mosquitoes (including A1, A2, and B genotypes) were from 80.2% to 97.6% (with type A2 being the highest) and from 87.5% to 97.7% (with type B being the highest), respectively. Additionally, the homology of the genome nucleotide and amino acid sequences of YN12243 and BAVs isolated from Odonata (QTM104536) were from 67.4% to 84.3% and from 65.4% to 92.0%, respectively.

Phylogenetic analysis

To clarify the taxonomic status of YN12243, the VP1 protein (RdRp) amino acid sequences of YN12243 and 14 other viruses of the family Reoviridae were subjected to a phylogenetic analysis. The results show that YN12243 was located in the same evolutionary branch as the previously isolated BAVs, belonging to the genus Seadornavirus within the family Reoviridae, along with the Liaoan virus and the Kadipiro virus (KDV virus), thereby confirming that YN12243 was a BAV (Figure 4A). Further analysis of the molecular genetic evolution of YN12243 showed that YN12243 was an A2 genotype BAV (Figure 4B). The viruses used to construct the phylogenetic tree are shown in Tables 3 and 4.

DISCUSSION

Since the first isolation in the 1980s, BAV has been isolated from 10 mosquito species among three genera: Culex (Culex tritaeniorhynchus, Cx. pipiens pallens, Cx. modestus, Cx. annulus, and Cx. pseudovishnui), Aedes (Aedes albopictus, Ae. vexans, and Ae. dorsalis), and Anoph eles (Anopheles sinensis and An. vagus) (Liu et al., 2010a). Additionally, BAV has been isolated from mosquitoes collected in Indonesia (Brown et al., 1993), Vietnam (Nabeshima et al., 2008), and many provinces of China (from the southern Yunnan
| Segment | Number | Primer | Sequence (5’→3’) | Size (bp) | Ta (annealing temperature) |
|---------|--------|--------|------------------|----------|--------------------------|
| Seg-1   | 1.1*   | BAV1.1F(13-1155) | AATCACAAGGAATGGACGGTTCAG | 1,142 | 50°C |
|         | 1.2*   | BAV1.2F(876-1857) | TATGTAAGACCTCTGGTGAAG | 981 | 50°C |
|         | 1.3*   | BAV1.3R(1712-2736) | CGGTAATGTTACTACATC | 1,024 | 52°C |
|         | 1.4*   | BAV1.4R(2478-3750) | CTATCCAGGCTTTCAAGGACGT | 1,272 | 52°C |
|         | 2.4    | BAV2.4F(1849-2998) | TCCTGAATGTCAGGTCGTAAC | 1,149 | 49°C |
|         | 2.1*   | BAV2.1F(41-1176) | GCACAGTGCTAGTTCTTAA | 1,135 | 50°C |
|         | 2.2*   | BAV2.2F(1050-2020) | TAACACCATGCTATATCACC | 970 | 49°C |
|         | 2.3*   | BAV2.3F(2370-2996) | AATATATCAATAGTGTAGC | 626 | 49°C |
|         |        | BAV2.3R(2370-2996) | AGATATATGCACACTGACCATTCACA | | |
| Seg-2   | 3.3    | BAV3.3F(1564-2227) | AATAGGGCGGCGGTGTCGAG | 663 | 52°C |
|         | 3.1*   | BAV3.1F(39-840) | AGCACAAGTCACACCGAG | 801 | 52°C |
|         | 3.2*   | BAV3.2F(763-1638) | TCCTGTTGAATCTTCAAGGACGT | 875 | 49°C |
|         |        | BAV3.2R(763-1638) | CGTGCATGACCATATGCGCAT | | |
| Seg-3   | 4.1    | BAV4.1F(13-1121) | TTTTGCATACGCGCCGTGGT | 1,108 | 52°C |
|         | 4.3    | BAV4.3F(1104-1773) | TTCTTCAAGCCATGTCAGC | 669 | 49°C |
|         | 4.1*   | BAV4.1R(15-264) | AGCCTACAGGTGTTTAAAG | 249 | 50°C |
|         | 4.2*   | BAV4.2F(762-1334) | TTGACAGGCAATATATAGTGACGC | 572 | 50°C |
|         | 4.3*   | BAV4.3R(1438-1782) | ATGCAGTCAAATTGAATTACATGACG | 344 | 50°C |
| Seg-4   | 5.2    | BAV5.2F(116-1484) | TTGGTGACGTCTCCGACT | 1,368 | 49°C |
|         | 5.1*   | BAV5.1F(11-843) | ATGCCATCTCAACACCG | 832 | 50°C |
|         | 5.2*   | BAV5.2F(690-1494) | CAAATTCTAGGAAAGACCTGACA | 804 | 52°C |
|         |        | BAV5.2R(690-1494) | ACAATGAGGATATAACCTGAGC | | |
| Seg-5   | 6.1    | BAV6.1F(41-1644) | CTCCTGCTGTTTGTTGGAG | 1,603 | 55°C |
|         |        | BAV6.1R(41-1644) | CATTCCCCGCTTACCCCA | | |
| Seg-6   | 7.1*   | BAV7.1F(59-956) | GTCTAAAGCTAATTAGACG | 897 | 52°C |
|         | 7.2*   | BAV7.2F(870-1023) | AAGGCGCCGATGCTTCAAC | 153 | 55°C |
| Seg-7   | 8.2    | BAV8.2F(23-979) | CCAACAATGGCAATTAGAG | 956 | 50°C |
|         | 8.1    | BAV8.1R(39-1011) | ACCGATggCACTCGATTAC | 972 | 50°C |
|         |        | BAV8.1R(39-1011) | AAATGGGAGCACCCTCCTGAC | | |
| Seg-8   | 9      | BAV9F(167-1081) | GATGGTTATTGACATGGG | 914 | 49°C |
|         | 9.1*   | BAV9R(167-1081) | TCACTTCTCAAGGAGCGG | 861 | 55°C |
|         | 10     | BAV10F(31-944) | GGAGTGACTCGTTCCGACT | 913 | 53°C |
|         | 11     | BAV11F(77-619) | ATGGACCCGCTTACCCGATAC | 542 | 53°C |
|         | 12     | BAV12-854-S | AAATGGGAGCACCCTCCTGAC | 850 | 53°C |
|         |        | BAV12-854-R | TCACTTCTCAAGGAGCGG | | |

a) Genomic amplification of the YNI2243 virus was carried out using the published BAV genome sequence primers. The table shows 12 pairs of published primers which gains positive amplification products and 17 pairs of new primers (plus*) which designed for amplifying and sequencing the genome of YNI2243 virus.
|                  | Culicoides |       |       |                  |       |       |       |       |       |       |       |      |       |
|------------------|------------|-------|-------|------------------|-------|-------|-------|-------|-------|-------|-------|------|-------|
|                  | YN12243(BAV A2) | SC043(BAV A2) | 02VN078b(BAV A1) | 02VN018b(BAV A2) | JKT-6423(BAV-B) | QTM104536 |       |       |       |       |       |      |       |
|                  | bp (%)     | aa (%) | bp (%) | aa (%)           | bp (%) | aa (%) | bp (%) | aa (%) | bp (%) | aa (%) | bp (%) | aa (%) | bp (%) | aa (%) |
| Seg-1            | 3,660 (84.2%)  | 1,219 (94.8%) | 3,660 (83.5%)  | 1,219 (95.32%) | 3,660 (82.3%)  | 1,219 (95.5%) | 3,660 (76.4%)  | 1,219 (89.8%) |
| Seg-2            | 2,865 (82.8%)  | 955 (93.4%)  | 2,865 (81.1%)  | 955 (90.5%)  | 2,865 (80.9%)  | 955 (92.2%)  | 2,865 (77.0%)  | 955 (87.6%) |
| Seg-3            | 2,163 (81.3%)  | 720 (90.7%)  | 2,163 (80.2%)  | 720 (88.9%)  | 2,163 (80.1%)  | 720 (87.5%)  | 2,163 (72.8%)  | 720 (77.9%) |
| Seg-4            | 1,731 (85.2%)  | 577 (93.9%)  | 1,731 (86.2%)  | 577 (95.7%)  | 1,731 (83.5%)  | 577 (95.5%)  | 1,731 (77.6%)  | 629 (86.7%) |
| Seg-5            | 1,506 (83.3%)  | 501 (93.1%)  | 1,506 (83.0%)  | 501 (92.4%)  | 1,506 (81.9%)  | 501 (90.8%)  | 1,506 (72.7%)  | 482 (82.0%) |
| Seg-6            | 1,278 (84.7%)  | 425 (93.9%)  | 1,278 (82.8%)  | 425 (93.0%)  | 1,278 (83.3%)  | 425 (92.7%)  | 1,278 (75.6%)  | 425 (83.8%) |
| Seg-7            | 921 (85.6%)   | 306 (86.9%)  | 921 (83.9%)   | 306 (89.2%)  | 921 (83.9%)   | 306 (89.9%)  | 921 (74.9%)   | 306 (74.2%) |
| Seg-8            | 909 (83.2%)   | 302 (87.8%)  | 909 (89.4%)   | 302 (89.2%)  | 909 (88.9%)   | 302 (97.7%)  | 909 (80.1%)   | 302 (88.4%) |
| Seg-9            | 852 (81.0%)   | 283 (84.2%)  | 852 (81.7%)   | 283 (87.0%)  | 852 (83.0%)   | 283 (88.0%)  | 852 (67.4%)   | 283 (65.4%) |
| Seg-10           | 750 (83.1%)   | 249 (85.6%)  | 750 (84.4%)   | 249 (90.0%)  | 750 (80.9%)   | 249 (87.2%)  | 387 (68.4%)   | 129 (68.9%) |
| Seg-11           | 543 (87.7%)   | 180 (92.8%)  | 543 (93.6%)   | 180 (96.1%)  | 543 (89.9%)   | 180 (95.0%)  | 543 (84.3%)   | 180 (92%)  |
| Seg-12           | 624 (90.7%)   | 207 (91.8%)  | 624 (97.6%)   | 207 (97.1%)  | 624 (88.1%)   | 207 (88.0%)  | 624 (80.3%)   | 207 (82.2%) |
| Total length of genome | 17,803 (82.5%–99.7%) | 5,925 (91.3%–99.7%) | 17,803 (81.0%–98.0%) | 5,925 (80.7%–97.6%) | 17,803 (80.1%–99.1%) | 5,925 (87.5%–97.7%) | 17,535 (67.4%–84.3%) | 5,837 (65.4%–92%) |
| Average          | 94.5%       | 97.3%       | 84.4%/ (83.9%) | 90.7/ (89.5%) | 85.7%       | 92.1%       | 83.9%       | 92.5%       | 75.6%       | 81.6% |
Figure 3  Genomic amplification of the YN12243 virus. A, PCR identification of YN12243 virus in the culture supernatant of C6/36 cells using published primers for amplifying and sequencing the whole genome of Banna virus by 1% agarose gel electrophoresis (AGE). The primer number of 2.4, 3.3, 4.1, 4.3, 5.2, 6.1, 8.2, 8.1, 9, 10, 11, 12 gain positive amplification products. B, PCR identification of YN12243 virus in the culture supernatant of C6/36 cells using 17 new pairs of primers (Table 1 plus*) which designed for amplifying and sequencing the genome of Banna virus by 1% agarose gel electrophoresis (AGE).

Table 3 Details of Reoviridaeaviruses strains used in this study

| Genus        | Species                          | Strain/Serotype          | GenBank accession No.(VP1) |
|--------------|---------------------------------|--------------------------|----------------------------|
| Orbivirus    | African horsesickness virus      | HS29-62/serotype1        | FJ183364                   |
| Orbivirus    | Bluetongue virus                | BTV-21T/serotype2        | JN255862                   |
| Orbivirus    | Epizootic hemorrhagic disease virus | Ibaraki/serotype2   | AM745077                   |
| Phytoreovirus| Rice dwarf virus                | Chinese                  | AAB18743                   |
| Rotavirus    | Rotavirus (Bovine rotavirus A)   | Simian                   | AAC58864                   |
| Seadornavirus| Banna virus                     | YN12243                  |                            |
| Seadornavirus| Banna virus                     | SC043                    | AIF50195                   |
| Seadornavirus| Banna virus                     | 02VN078                  | ACA50122                   |
| Seadornavirus| Banna virus                     | 02VN018                  | ACA50110                   |
| Seadornavirus| Banna virus                     | JKT-6423                 | AAF78849                   |
| Seadornavirus| Banna virus                     | QTM104536                | AFG79118                   |
| Seadornavirus| Kadipiro virus                  | JKT-7075                 | AAF78848                   |
| Seadornavirus| Liaoning virus                  | LNSV-NE9731              | AAF83562                   |
| Cardoreovirus| Eriocheir sinensis reovirus     | 905                      | AAT11887                   |
| Mimoreovirus | Micromonas pusilla reovirus     | MPRV                     | AAZ49041                   |
| Aquareovirus | Aquareovirus A (Chum salmon reovirus) | CSRV                | AAL31497                   |
| Cypovirus    | Dendrlymus punctatus cytoplas-mic polyhedrosis virus-1 | DsCPV-1 | AAAN46860                  |
| Coltivirus   | Colorado tick fever virus       | Florio                   | AAK00595                   |
| Dinovernavirus| Aedes pseudocutellaris reovirus | APRV                    | AAZ49068                   |
| Fijivirus    | Nilaparvata lugens reovirus     | Izumo                    | BAA08542                   |
| Mycoreovirus | Mycoroovirus1 (Cryphonectria parasitica reovirus) | 9B21               | AAP45577                   |
| Orthoreovirus| Mammalian orthoreovirus 1       | Lang                     | AAA47234                   |
| Oryzavirus   | Rice ragged stunt virus         | Thai                     | AAC36456                   |

Province to the northern Inner Mongolia Autonomous Region) (Liu et al., 2010a; Liu et al., 2011; Liu et al., 2010b). The BAVs isolated from different genera and different species of mosquitoes can apparently be clustered together, with no species barrier being apparent (Liu et al., 2010a; Liu et al., 2016b) between mosquitoes. In this paper, evolutionary
Figure 4  Phylogenetic analysis of YN12243.  A, Phylogenetic tree constructed using VP1 amino acid sequences of YN12243 and other Reoviridae virus strains. B, Phylogenetic tree constructed using 12th segment coding region nucleotide sequence of YN12243 and that of other Seadornavirus strains.
Table 4  Details of Seadornavirus strains used in this study

| Strain/Serotype | Year | Country     | Host                     | GenBank accession No. (Seg-12) |
|-----------------|------|-------------|--------------------------|--------------------------------|
| SC043           | 2012 | Yunnan, China | Culicoides sp.           | KC954622                       |
| 02VN180b        | 2002 | Vietnam     | Culex triaeniorhynchus   | EU265727                       |
| 02VN178b        | 2002 | Vietnam     | Culex triaeniorhynchus   | EU265715                       |
| 02VN099b        | 2002 | Vietnam     | Culex annulus            | EU265682                       |
| 02VN078b        | 2002 | Vietnam     | Culex triaeniorhynchus   | EU265705                       |
| 02VN018b        | 2002 | Vietnam     | Culex annulus            | EU265694                       |
| JKT-7043        | 1981 | Java, Indonesia | Anopheles subpictus Grassia | AF052024                       |
| JKT-6969        | 1981 | Java, Indonesia | Culex Vagus Doenitz    | AF052008                       |
| JKT-6423        | 1980 | Java, Indonesia | Culex pseudovishnui Colless | AF019908                       |
| QTM104536       | 2013 | China       | Odonata                  | KX884648                       |
| LNV-NE9712      | 1997 | Liaoning, China | Mosquitoes               | NC_007747                      |
| LNV-NE97-31     | 1997 | Liaoning, China | Mosquitoes               | YJ317110                       |
| KDV-JKT-7075    | NA   | Java, Indonesia | Mosquitoes               | AF019909                       |
| QTM-27331       | 2013 | China       | Odonata                  | KX884661                       |
| SX0794          | 2007 | China       | Aedes dorsalis           | GQ331970                       |
| SX0765          | 2007 | China       | Culex pipiens pallens   | GQ331963                       |
| SX0766          | 2007 | China       | Culex pipiens pallens   | GQ331964                       |
| SX0767          | 2007 | China       | Aedes vexans             | GQ331965                       |
| SX0771          | 2007 | Shanxi, China | Culex pipiens pallens   | GQ331966                       |
| SX0789          | 2007 | Shanxi, China | Aedes dorsalis           | GQ331967                       |
| SX0795          | 2007 | Shanxi, China | Culex pipiens pallens   | GQ331971                       |
| SX0796          | 2007 | Shanxi, China | Culex pipiens pallens   | GQ331972                       |
| GS42-2          | 2006 | Gansu, China | Culex tritaeniorhynchus Giles | FJ160414                      |
| GS07-KD27       | 2007 | China       | Culex tritaeniorhynchus | GQ331958                       |
| GS07-KD29       | 2007 | China       | Aedes albopictus         | GQ331959                       |
| GS07-KD30       | 2007 | Gansu, China | Culex pipiens pallens   | GQ331960                       |
| LN0684          | 2006 | Liaoning, China | Anopheles sp.           | FJ217989                       |
| LN0688          | 2006 | Liaoning, China | Anopheles sp.           | FJ217990                       |
| LN0689          | 2006 | Liaoning, China | Anopheles sp.           | FJ217991                       |
| YN0659          | 2006 | Yunnan, China | Anopheles sinensis      | FJ161965                       |
| YN0556          | 2005 | Yunnan, China | Culex tritaeniorhynchus Giles | FJ161966                      |
| YN0558          | 2005 | Yunnan, China | Culex tritaeniorhynchus Giles | FJ161964                      |
| YN-6            | 2000 | Yunnan, China | Mosquitoes               | AY568290                       |
| YN12243         | 2012 | Yunnan, China | Culicoides sp.           | MF141023                       |
| NM0706          | 2007 | Inner Mongolia, China | Culex modestus     | GQ331973                       |
| BJ95-75         | 1995 | Beijing, China | Mosquitoes               | AY568289                       |
| DH13M041        | 2013 | Yunnan, China | Culex triaeniorhynchus  | KR349198.1                     |
| Balaton/2010/HUN| 2010 | Hungary     | Cyprinaceps (freshwater carp) | JX947850.1                     |

Phylogenetic analyses of the BAV genome isolated from Culicoides (YN12243) and other mosquito samples were performed for the first time. It was found that the virus was an A2 genotype BAV. Furthermore, the genome sequence of the BAV isolated from Culicoides (SC043), which was previously registered in GenBank, was in the same evolutionary branch as the BAVs isolated from various mosquitoes. The YN12243 and SC043 viruses which belonged to A2 genotype BAV constituted a cluster based on their evolutionary characteristics (Figure 4B). However, The YN12243 and SC043 viruses were located in the different branch on the phylogenetic tree. Whether a species barrier between Culicoides and
various mosquitoes existed is difficult to determine at this stage. More samplings of BAV from Culicoides and in vivo experiments are needed.

The BAV genome (Shi et al., 2016) found in Odonata not only had different nucleotide and amino acid sequence lengths from those of the strains isolated from Culicoides and mosquitoes (Table 2), but also had different molecular genetic evolution characteristics that were intermediate between those of the Culicoides and mosquito viruses. QTM104536 occupies an independent evolutionary branch between BAVs and Banna-like viruses. Whether these results indicate that BAV isolated from Odonata is a new type of BAV or a unique genus needs further study.

Despite the common evolutionary branch of the BAV isolated from Culicoides with that isolated from various mosquitoes, there is no species barrier with respect to the vectors. Moreover, the YN12243 isolated from Culicoides has the same nucleotide and amino acid sequence lengths as the BAVs isolated from mosquitoes, and closer nucleotide and amino acid homology (Table 2). However, we were only able to obtain 50% of the amplified products of YN12243 using the whole genome amplification primers published for BAVs isolated from mosquitoes (Liu et al., 2016a), suggesting that while the nucleotide length is the same but the sequence length differs. Additionally, PAGE results showed that the distance between the segments 5 and 6 of YN12243 was significantly greater than that for mosquito isolates (Figure 2), and the distance from segments 7 and 8 to segment 9 for YN12243 was shorter than that for HB35 isolated from mosquitoes; this indicates that compared with the mosquito isolates, the Culicoides isolate not only has a difference in terms of genomic nucleotide sequence conservation, but also a different secondary or tertiary genomic structure.

BAV was first isolated from patients with fever and viral encephalitis, suggesting that it is associated with a human disease (Xu et al., 1990a; Xu et al., 1990a; Attoui et al., 2005a). Subsequent monitoring showed that serum samples of suspected Japanese encephalitis virus encephalitis cases collected from hospitals were BAV immunoglobulin M (IgM) antibody-positive at a rate of 11.4% (130/1141) (Tao and Chen, 2005). Another study showed that of 63 serum samples of patients diagnosed with viral encephalitis in the acute phase, 11 cases were positive for BAV IgM antibody and 37 cases were positive for Japanese encephalitis virus (JEV) IgM antibody; seven patients were simultaneously positive for both of the IgM antibodies (Yang et al., 1996). These findings further suggest that BAV may be associated with infection in patients with viral encephalitis. Recently, a microneutralization test was used to detect BAV-neutralizing antibody in 200 bovine serum samples and 535 pig serum samples collected from Yunnan Province, which was the initial BAV discovery site (Kou et al., 2016). The results showed that 10 pig serum samples were positive for BAV-neutralizing antibody, with antibody titers of 1:20–1:320, and 1 bovine serum sample was positive (with an antibody titer of 1:160). This was the first time that neutralization test methods were used to test animal serum samples for BAV-neutralizing antibodies (Kou et al., 2016). The results suggest that BAV not only affects humans, but can also infect pigs and cattle. Previous studies involving surveillance of insect-borne viruses in the Sino-Burmese border area over a period of more than 10 years suggested that BAV was only isolated from mosquito specimens (Wang et al., 2011; Sun et al., 2009) and not from Culicoides, it was thought that Culicoides might not carry BAV. However, BAV was isolated from Culicoides in the present study, suggesting the possibility that Culicoides carry BAV. However, whether the ability of Culicoides to carry BAV is innate, or represents a new cross-transfer ability, needs further research.

Molecular genetic evolution analysis identified BAV as a new base virus in the 1900s, with a mean nucleotide substitution rate of $2.467 \times 10^{-4}$ substitutions per site per year (s/s/y); the rate was significantly higher in higher-versus lower-latitude BAV populations (Liu et al., 2016b). The results also suggested that although BAV is widely distributed in the tropics, it has evolved such that it can now survive not only in different regions of the tropics, but also in temperate regions and at higher latitudes (Liu et al., 2016b). The isolation of BAV from Culicoides indicates that Culicoides can be a vector for BAV, thereby expanding the vector range of BAV. Culicoides are blood-sucking insects distributed widely in temperate regions that have hematophagous feeding habits (on cattle, horses, deer, and other large animals). In addition, they can spread various insect-borne viruses, such as blue tongue virus and hemorrhagic fever virus, which caused an epidemic in deer. Thus, it is of great importance to improve the detection and monitoring of animal diseases caused by the spread of BAVs by Culicoides.

**MATERIALS AND METHODS**

**Cell culture**

C6/36 *Aedes albopictus* cells were stored in our laboratory. The cells were cultured in complete medium composed of 45% RMPI 1640 (Invitrogen, USA), 45% Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS; Invitrogen) and 100 U mL$^{-1}$ of penicillin and streptomycin. They were propagated and maintained at 28°C (Wang et al., 2015).

**Virus**

YN12243 (GenBank accession No:MF124330, MF134894, MF134895, MF141015-MF141023) was isolated from Culicoides samples collected in the Sino-Burmese border region
of Yunnan Province, China in 2012. The C6/36 cells were inoculated with YN12243 virus and cultured at 28°C. The cytopathic effect (CPE) was evaluated daily.

**Virus RNA extraction and viral genomic cDNA preparation**

Total RNA was extracted from 140 μL virus-infected C6/36 cell supernatant using the QIAamp Viral RNA Mini Kit (Qiagen Inc., USA) according to the manufacturer’s protocol. 32 μL RNA was added to the first-strand reaction tube provided in the Ready-To-Go You-Prime First Strand Beads kit (GE Healthcare, UK). Then, 1 μL of the random primer pd(N)6 (50 g μL−1) (TaKaRa, Japan) was added into the tube with a final volume of 33 μL. cDNA was synthesized (Wang et al., 2015; Sun et al., 2009).

**dsRNA-PAGE**

A 10% acrylamide (acrylamide/bisacrylamide 29:1; Bio-Rad Laboratories, USA) slab gel (Hoefer Pharmacia Biotech Inc., USA) was prepared together with a 3.5% acrylamide stacking gel (25 mmol L−1 Tris, 192 mmol L−1 glycine, pH 8.3) (Bio-Rad Laboratories). RNA was electrophoresed at room temperature and the viral dsRNA stained with silver nitrate. Virus RNA extracted as described above was subjected to dsRNA-PAGE (Wang et al., 2015).

**Virus gene amplification and sequencing**

Firstly, 24 pairs of primers (Liu et al., 2016) was used to amplify each segment of YN12243 virus genome. Only 12 pairs of primers gain positive amplification products. Then, the amplified sequence (YN12243) was used for primer design by the “Bridging” method, done with Primer Premier 5.0 software (Premier Biosoft International, USA), which followed the principle of primer design (The content of G+C is about 50%). The annealing temperature is about 55°C. Considering the fragment length and annealing temperature of the primer, the optimum reaction conditions for the primer were explored (Table 1) (Wang et al., 2015; Liu et al., 2016).

A 25-μL reaction was prepared using cDNA as a template, upstream and downstream primers (Tsingke, Beijing), GoTaq® Green Master Mix, 2× (Promega, USA). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a Mastercycler instrument (Eppendorf, Germany). The amplification conditions were as follows: 95°C pre-denaturation for 5 min, and 35 cycles of 94°C for 30 s, the annealing temperature (Table 1) for 30 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min. After the PCR reaction was complete, 5 μL of the PCR products was used to detect the amplification products by 1% agarose gel electrophoresis. Products were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and sub-cloned into the pGEM-Teasy vector (Promega) for sequencing analysis. The sequence was checked by BLAST search against the National Center for Biotechnology Information (NCBI) database and virus genes were identified (Wang et al., 2015; Liu et al., 2016a).

**Sequence analysis**

The nucleotide sequences of the BAV genome-protein-encoding regions in GenBank, which were isolated from different countries, vectors and host animals, and the nucleotide sequence information (Table 2) of the BAV (YN12243) obtained in this study were used for a phylogenetic analysis of the BAV genome. The SeqMan software (DNASTar, USA) was used for assembly and quality analysis of the sequencing results of the YN12243 virus strain. BioEdit (ver. 7.0.5.3; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used for multiple sequence alignment. The MEGA 6.0 was used to perform a system evolution analysis, based on the neighbor-joining (NJ) method and with the bootstrap value set to 1,000 (Li et al., 2015). Differential alignment and homology analyses of nucleotide and amino acid sequences were done using GeneDoc and MegAlign software (DNAStar) (Liu et al., 2016a; Attoui et al., 2000; Lu et al., 2016; Deng et al., 2016).

**Compliance and ethics** The author(s) declare that they have no conflict of interest.

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