SCIENCE CHINA
Life Sciences

SPECIAL TOPIC: Emerging and re-emerging viruses
• RESEARCH PAPER •

December 2017 Vol.60 No.12: 1372–1382
https://doi.org/10.1007/s11427-017-9190-6

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

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Received July 22, 2017; accepted September 16, 2017; published online October 26, 2017

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 belongs 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

Citation: Song, S., Li, Y., Fu, S., Lei, W., Guo, X., Feng, Y., Gao, X., Li, X., Yang, Z., Xu, Z., He, Y., Wang, H., Zhou, H., Wang, B., Lu, X., and Liang, G. (2017). Genome sequencing and phylogenetic analysis of Banna virus (genus *Seadornavirus*, family Reoviridae) isolated from Culicoides. Sci China Life Sci 60, 1372–1382. https://doi.org/10.1007/s11427-017-9190-6

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

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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 hematophagic 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).

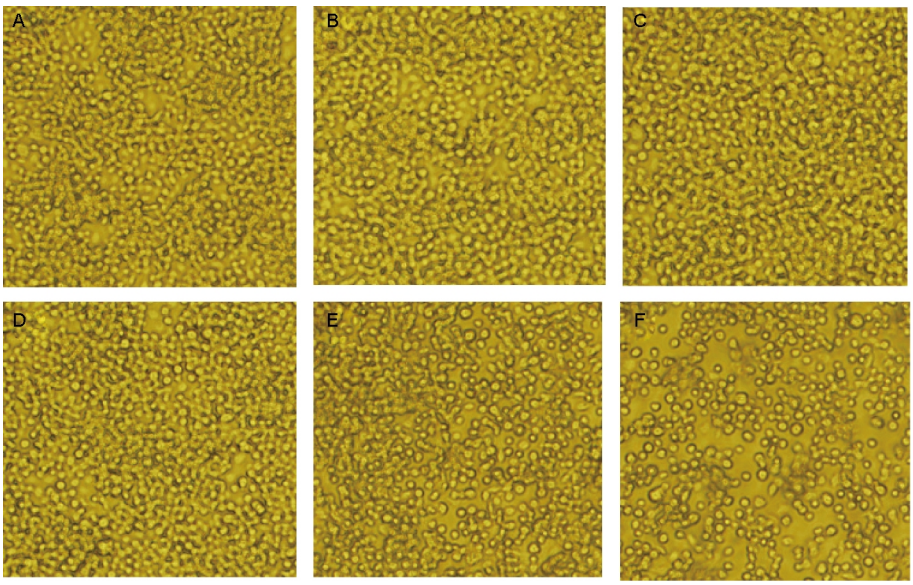

Figure 1 (Color online) Cytopathic effect of YN12243 on C6/36 (×200). A–C, Uninfected C6/36 control cells 24 48 and 72 h, respectively. D, Infected C6/36 cells, 24 hours post-infection. E, Infected C6/36 cells, 48 hours post-infection, showing cell aggregation. F, Infected C6/36 cells, 72 hours post-infection, showing cell aggregation and detachment of infected cells.

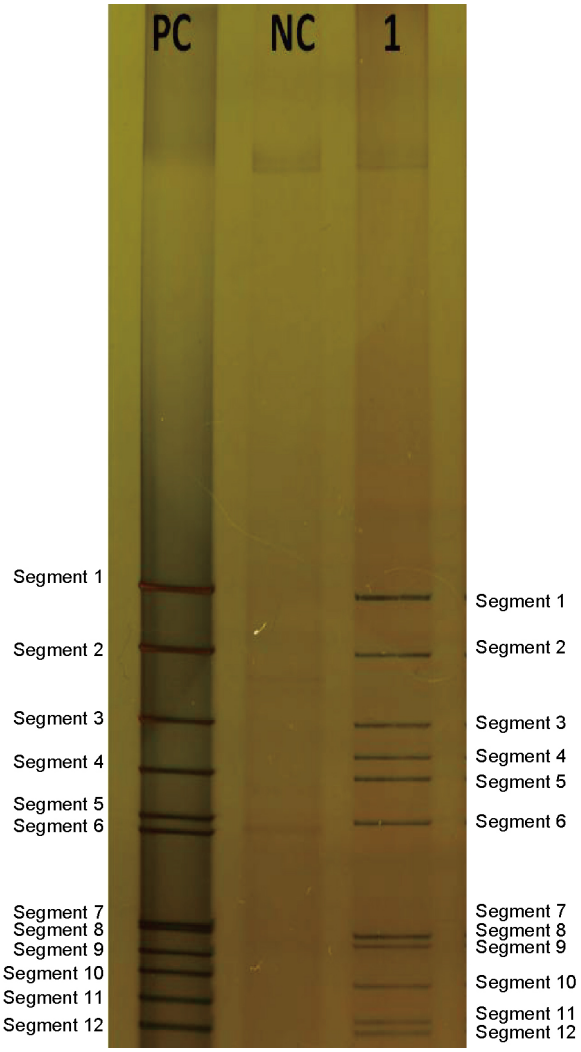

Figure 2 (Color online) Electrophoretic migration patterns of the dsRNA of YN12243 virus as determined by polyacrylamide gel electrophoresis. (PC), positive control:BAV (HB35) which was a distribution pattern of 6-6 and belonged to BAV by molecular evolution analysis (unpublished). NC, negtive control; 1, YN12243 virus.

Gene amplification

RT-PCR amplification and sequencing were performed using the cDNA template of the YN12243 virus and a primer specific to segment 12 (850 bp). The sequence analysis revealed that YN12243 was a BAV.

Genomic amplification of the YN12243 virus was carried out using published BAV genome sequence primers (Liu et al., 2016a). The results showed that only 12 of the 24 pairs of primers yielded a PCR product for the corresponding gene segments (Figure 3A); To obtain the entire nucleotide sequence of the YN12243 genome, 17 pairs of primers (Table 1) were redesigned and positive amplification products of YN12243 virus sequences were obtained (Figure 3B).

Analysis of nucleotide and amino acid homology

The whole genome sequence analysis indicated that the genome of YN12243 was 17,803 bp long (excluding non-coding regions), encoding 5,925 amino acids. The YN12243 genome nucleotide and amino acid information are shown in Table 2.

Comparative analyses of the nucleotide and amino acid sequences of each gene segment, between YN12243 and other BAVs transmitted by different vectors, revealed that YN12243 had RNA nucleotide and amino acid sequences (excluding non-coding regions) that were the same length as SC043 (a BAV strain isolated from Culicoides and registered in GenBank, but on which no article has been published), 02VN018b, 02VN078b, and JKT-6423 (isolated from mosquitoes). Nucleotide and amino acid homology analysis 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. (Table 2)

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 Liaoning 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 Anopheles (*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

Table 1 Primers used in this study[a)]

Segment	Number	Primer	Sequence (5′→3′)	Size (bp)	Ta (annealing temperature)
Seg-1	1.1*	BAV1.1F(13-1155)	ATCAACAAGGAATGGACGTTCA	1,142	50°C
		BAV1.1R(13-1155)	TATGTAGACCCTCGTTGCGAAG		
	1.2*	BAV1.2F(876-1857)	CGAAAGAAGTGATGAATGAG	981	50°C
		BAV1.2R(876-1857)	CCGTGATATGTAGTCCCAAT		
	1.3*	BAV1.3F(1712-2736)	GCAAAGCGTATAATAACTCC	1,024	52°C
		BAV1.3R(1712-2736)	CTTAAGCATATCTCAGGTGTA		
	1.4*	BAV1.4F(2478-3750)	AAAGGCTATTTAGCGTTAGACGT	1,272	52°C
		BAV1.4R(2478-3750)	CTATCCAAGGCATTCAAGAGTTC		
Seg-2	2.4	BAV2.4F(1849-2998)	TCCGTAATGTATCAGGTAAC	1,149	49°C
		BAV2.4R(1849-2998)	GCGTAAGATAGTATTCCACC		
	2.1*	BAV2.1F(41-1176)	TCACATCCCCGTTCTTAAA	1,135	50°C
		BAV2.1R(41-1176)	CGTAACCATGCGTATGCTC		
	2.2*	BAV2.2F(1050-2020)	TCACACGCTATTTTTACTTAC	970	49°C
		BAV2.2R(1050-2020)	TACTCACCGCTGATGTTTCTA		
	2.3*	BAV2.3F(2370-2996)	AATTATCCAGTATATGTTGAGCC	626	49°C
		BAV2.3R(2370-2996)	AGATAGTATTCCACCACATTTCA		
Seg-3	3.3	BAV3.3F(1564-2227)	AATAGTTGGCGGTGGTCAG	663	52°C
		BAV3.3R(1564-2227)	ACGAGAATCCCCACACAGG		
	3.1*	BAV3.1F(39-840)	GACAGTGGAAGTATAGTAGAAAAC	801	52°C
		BAV3.1R(39-840)	TTGAACTTATGGTCAGTACGCCTC		
	3.2*	BAV3.2F(763-1638)	TCGTGTTAATTGGTGAGAGTC	875	49°C
		BAV3.2R(763-1638)	CTTGATCAGTGATAATGGCAT		
Seg-4	4.1	BAV4.1F(13-1121)	TTTTGCATCATGGCGTGGGT	1,108	52°C
		BAV4.1R(13-1121)	TCTTTCAACGCCATCGTCAGC		
	4.3	BAV4.3F(1104-1773)	GACGATGGCGTTGAAAGA	669	49°C
		BAV4.3R(1104-1773)	AACTGACAACGAATCTTTTACG		
	4.1*	BAV4.1F(15-264)	TTGCATCATGGCGTGGGTT	249	50°C
		BAV4.1R(15-264)	TGCGTTGTAATCTTCAGCC		
	4.2*	BAV4.2F(762-1334)	TTTGCAGGCAATATATAGTAGGC	572	50°C
		BAV4.2R(762-1334)	ATCGCATCATAGTTCGTAGTCTT		
	4.3*	BAV4.3F(1438-1782)	ATTGCTGGATATACAGTGTACGA	344	50°C
		BAV4.3R(1438-1782)	GTGATTTCATTAAACATCTTGGT		
Seg-5	5.2	BAV5.2F(116-1484)	TTGTGACTGCTTCGGACT	1,368	49°C
		BAV5.2R(116-1484)	ATGACCCTATCAACCACC		
	5.1*	BAV5.1F(11-843)	TTACAAGAACCTAACATCGC	832	50°C
		BAV5.1R(11-843)	AGCCGTTTCACAAATCCGTC		
	5.2*	BAV5.2F(690-1494)	CAATTTTTGGAAAGACGTACA	804	52°C
		BAV5.2R(690-1494)	ACAATGAGGATAAACCTGAGC		
Seg-6	6.1	BAV6.1F(41-1644)	CTCTGCTGTGTGGTGGGA	1,603	55°C
		BAV6.1R(41-1644)	CATTCCCTGCTACCCCAC		
Seg-7	7.1*	BAV7.1F(59-956)	GTCTCAATGAACAATGGACAAGC	897	52°C
		BAV7.1R(59-956)	CGGTAACTAAATCCTTCACATCA		
	7.2*	BAV7.2F(870-1023)	CACCGACCGATGATGCCTGAG	153	55°C
		BAV7.2R(870-1023)	CCCAACGCCTTACCACGATAC		
Seg-8	8.2	BAV8.2F(23-979)	CCAACAATGGCAAATAGAG	956	50°C
		BAV8.2R(23-979)	ACGAGCCACTCACGATTAC		
	8.1	BAV8.1F(39-1011)	AATAGAGCAACTTCAGCCT	972	50°C
		BAV8.1R(39-1011)	CCACACCTTGATCTCACAT		
Seg-9	9	BAV9F(167-1081)	GATGTTTGATGAATTGGCTC	914	49°C
		BAV9R(167-1081)	TACTACTCTCAGGGGGTGGC		
	9.1*	BAV9.1F(29-890)	ATCGGAGACTGAGTTGAGGGC	861	55°C
		BAV9.1R(29-890)	TAGCACATCCAAATCTTAAGG		
Seg-10	10	BAV10F(31-944)	GGATGTACTGAGTAAGGGTTC	913	53°C
		BAV10R(31-944)	CCGTTGGTAGAGGGTGGT		
Seg-11	11	BAV11F(77-619)	AAAAATCAAGATGACGATCC	542	53°C
		BAV11R(77-619)	ACACCATTAGCACACGAAGA		
Seg-12	12	BAV12-854-S	AAATTGATAGYGYTTGCGTAAGAG	850	53°C
		BAV12-B2-R	GTTCTAAATTGGATACGGCGTGC		

a) Genomic amplification of the YN12243 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 YN12243 virus

Table 2 Genome comparison of Banna viruses isolated from culicoides, mosquitoes and odonata[a)]

	Culicoides				Mosquitoes						Odonata	
	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 (%)
Seg-1	3,660	1,219	3,660 (96.6%)	1,219 (98.8%)	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	955	2,866 (98.2%)	956 (99.2%)	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	720	2,163 (98.5%)	720 (98.8%)	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	577	1,731 (92.0%)	577 (98.1%)	1,731 (85.2%)	577 (93.9%)	1,731 (86.2%)	577 (95.7%)	1,731 (83.5%)	577 (95.5%)	1,887 (77.6%)	629 (86.7%)
Seg-5	1,506	501	1,506 (82.5%)	501 (91.3%)	1,506 (83.3%)	501 (93.1%)	1,506 (83.0%)	501 (92.4%)	1,506 (81.9%)	501 (90.8%)	1,446 (72.7%)	482 (82.0%)
Seg-6	1,278	425	1,278 (88.7%)	425 (96.7%)	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	306	921 (89.7%)	306 (95.8%)	921 (85.6%)	306 (86.9%)	921 (84.9%)	306 (89.2%)	921 (83.9%)	306 (89.9%)	921 (74.9%)	306 (74.2%)
Seg-8	909	302	909 (99.7%)	302 (99.7%)	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	283	852 (97.2%)	283 (97.2%)	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	249	750 (99.1%)	249 (98.4%)	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	180	543 (93.4%)	180 (97.2%)	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	207	624 (98.1%)	207 (96.6%)	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	5,925	17,803 (82.5%–99.7%)	5,925 (91.3%–99.7%)	17,803 (81.0%–90.7%)	5,925 (84.2%–94.8%)	17,803 (80.2%–97.6%)	5,925 (87.0%–97.1%)	17,803 (80.1%–89.9%)	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%

a) The identity of nucleotide and amino acid sequence of genome (excluding noncoding region) between the Banna virus (YN12243) isolated from culicoides and other Banna virus isolated from culicoides, mosquitoes, odonata which has been published in Genbank. The table involves the genome of the virus strains SC043 (GenBank accession number KC954611 to KC954622), 02VN078b (GenBank accession number EU265695 to EU265705, EU312980), 02VN018b (GenBank accession number EU265683 to EU265694), JKT-6423 (GenBank accession number NC_004211, NC_004217 to NC_004221, AF019908, AF052014 to AF052018) and QTM104536 (KX884638 to KX884639).

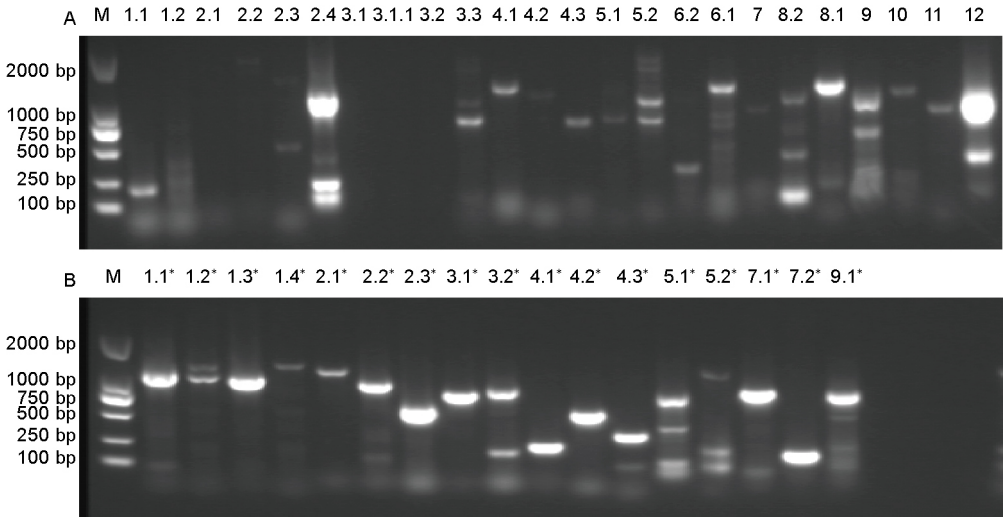

Figure 3 Genomic amplification of the YN12243 virus. A, PCR identification of YN12243 virus in the culture supernatant of C6/36 cells using published primers for amplifying and sequencing the whole genome of Banna virus by 1% agarose gel electrophoresis (AGE). The primer number of 2.4, 3.3, 4.1, 4.3, 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 Reoviridaevirus 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	AAC58684
Seadornavirus	Banna virus	YN12243	
Seadornavirus	Banna virus	SC043	AHF50195
Seadornavirus	Banna virus	02VN078	ACA50122
Seadornavirus	Banna virus	02VN018	ACA50110
Seadornavirus	Banna virus	JKT-6423	AAF78849
Seadornavirus	Banna virus	QTM104536	APG79118
Seadornavirus	Kadipiro virus	JKT-7075	AAF78848
Seadornavirus	Liao ning virus	LNSV-NE9731	AAQ83562
Cardoreovirus	Eriocheir sinensis reovirus	905	AAT11887
Mimoreovirus	Micromonas pusilla reovirus	MPRV	AAZ94041
Aquareovirus	Aquareovirus A (Chum salmon reovirus)	CSRV	AAL31497
Cypovirus	Dendrlymus punctatus cytoplas-mic polyhedrosis virus-1	DsCPV-1	AAN46860
Coltivirus	Colorado tick fever virus	Florio	AAK00595
Dinovernavirus	Aedes pseudoscutellaris revirus	APRV	AAZ49068
Fijivirus	Nilaparvata lugens reovirus	Izumo	BAA08542
Mycoreovirus	Mycoreovirus1 (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

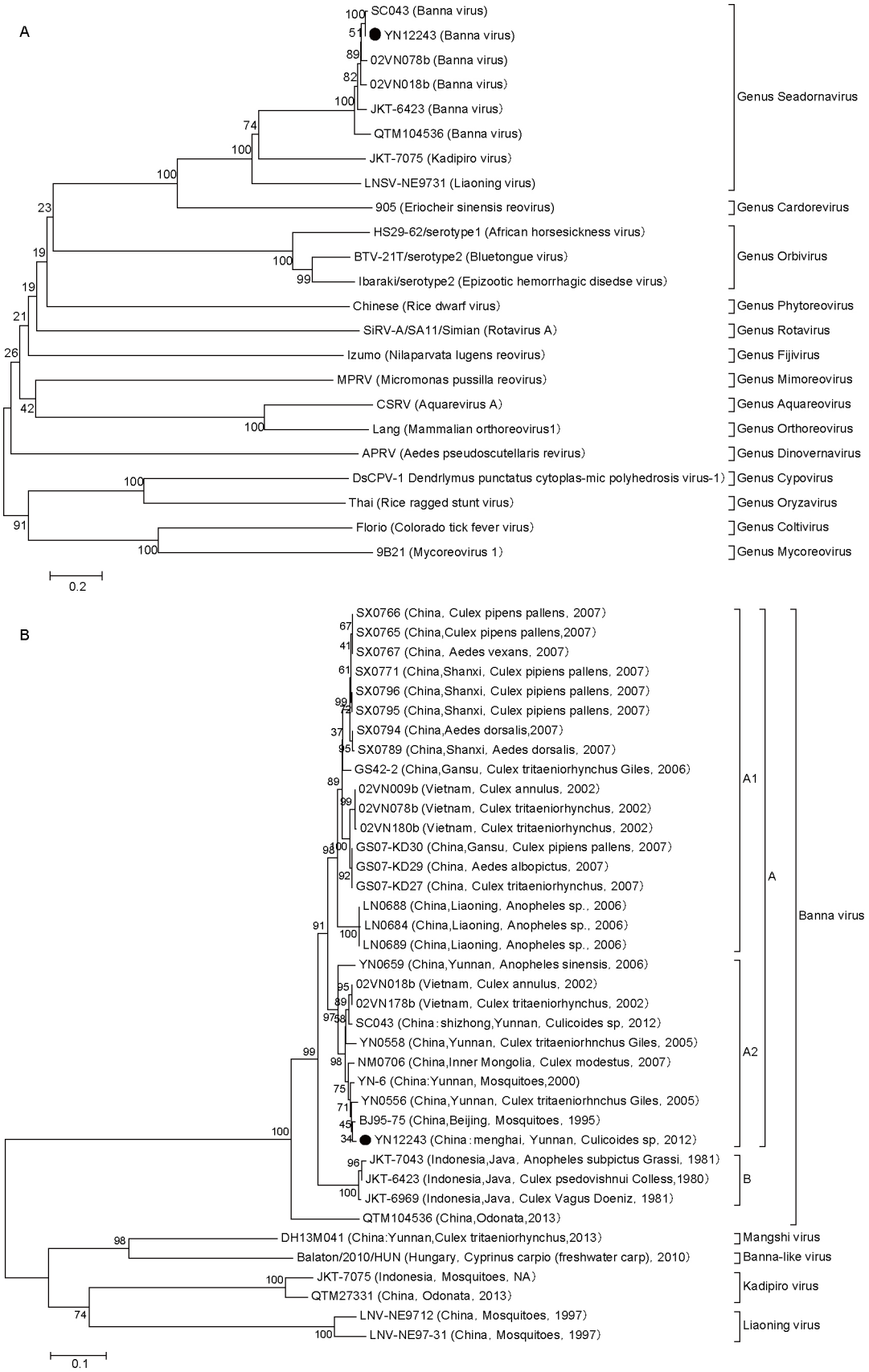

Figure 4 Phylogenetic analysis of YN12243. A, Phylogenetic tree constructed using VP1 amino acid sequences of YN12243 and other Reoviridae virus strains. B, Phylogenetic tree constructed using 12th segment coding region nucleotide sequence of YN12243 and that of other *Seadornavirus* strains.

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
02VN009b	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 psedovishnui Colless*	AF019908
QTM104536	2013	China	*Odonata*	KX884648
LNV-NE9712	1997	Liaoning, China	Mosquitoes	NC_007747
LNV-NE97-31	1997	Liaoning, China	Mosquitoes	AY317110
KDV-JKT-7075	NA	Java, Indonesia	Mosquitoes	AF019909
QTM-27331	2013	China	*Odonata*	KX884661
SX0794	2007	China	*Aedes dorsalis*	GQ331970
SX0765	2007	China	*Culex pipens pallens*	GQ331963
SX0766	2007	China	*Culex pipens 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 tritaeniorhnchus Giles*	FJ161966
YN0558	2005	Yunnan, China	*Culex tritaeniorhnchus 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 tritaeniorhynchus*	KR349198.1
Balaton/2010/HUN	2010	Hungary	*Cyprinuscarpio* (freshwater carp)	JX947850.1

ary 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 form 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 encephalitisor viral 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 2.467×10^{-2} 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 subcloned 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.*

Acknowledgements *This work was supported by the National Natural Science Foundation of China (81290342, 81501757), the Special National Project on Research and Development of Key Biosafety Technologies (2016YFC1201904), the National Key Plan for Scientific Research and Development of China (2016YFD0500300) and the development grants of State Key Laboratory of Infectious Disease Prevention and Control (2014 SKLID103, 2015SKLID505).*

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