Virologica Sinica (2021) 36:33–42
https://doi.org/10.1007/s12250-020-00239-w

www.virosin.org
www.springer.com/12250

RESEARCH ARTICLE

Emergence of Zika Virus in *Culex tritaeniorhynchus* and *Anopheles sinensis* Mosquitoes in China

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Received: 11 October 2019 / Accepted: 8 April 2020 / Published online: 2 July 2020

Abstract

Zika virus (ZIKV) has been isolated from mosquitoes such as *Aedes*, *Mansonia uniformis*, and *Culex perfuscus*; However, the isolation of ZIKV from *Anopheles sinensis* and *Culex tritaeniorhynchus* has not yet been reported. In June and July 2018, 22,985 mosquitoes and 57,500 midges were collected in Jiangxi Province in southeastern China. Among them, six strains of ZIKV were isolated from mosquitoes: four from *An. sinensis* and two from *Cx. tritaeniorhynchus*. Molecular genetic analysis showed that the ZIKV isolated from *An. sinensis* and *Cx. tritaeniorhynchus* belonged to genotype 2 in the Asian evolutionary branch of ZIKV. In addition, the ZIKV strains isolated from *An. sinensis* and *Cx. tritaeniorhynchus* had amino acid substitutions identical to ZIKV strains prevalent in South America since 2015. This study is the first to isolate ZIKV from mosquito specimens collected in the wild of Jiangxi Province, China; This is also the first time that ZIKV has been isolated from *An. sinensis* and *Cx. tritaeniorhynchus*. Given that *An. sinensis* and *Cx. tritaeniorhynchus* have a very wide geographical distribution in China and even in eastern and southern Asia, the isolation of several strains of ZIKV from these two mosquitoes poses new challenges for the prevention and control of ZIKV infection in the mainland of China and countries and regions with the same distribution of mosquitoes.

Keywords *Anopheles sinensis* · *Culex tritaeniorhynchus* · Zika virus (ZIKV)

Introduction

Arboviruses are propagated via sensitive arthropods (e.g., mosquito, tick, sandfly, and midge), the bites of which transmit the viruses to humans and animals (Weaver and Reisen 2010). In 1992, the International Arbovirus Center registered 534 arboviruses, of which 128 can cause human and livestock diseases (Karabatsos 1985). More than 300 arboviruses are mosquito-borne (Karabatsos 1985), including the highly pathogenic dengue virus (DENV) (OMS 2000), Japanese encephalitis virus (JEV) (Erlanger *et al.* 2009), West Nile virus (Mackenzie *et al.* 2004), and Zika virus (ZIKV) (Franca *et al.* 2016). The 2015–2016 ZIKV epidemic in Brazil and other countries in South America spreaded to more than 60 countries, and approximately 2 million people were infected (Franca *et al.* 2016), some of whom developed congenital Zika virus syndrome (Mlakar

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12250-020-00239-w) contains supplementary material, which is available to authorized users.

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et al. 2016), Guillain-Barre syndrome (GBS) (Cao-Lormeau *et al.* 2016), and non-vector-borne stillbirth (e.g., materno-fetal, sexual, and post-transfusion) (Enserink 2015; Ogden *et al.* 2016; Rahman and Huhtaniemi 2017). Additionally, indigenous ZIKV infections occurred in Cambodia (Vireak *et al.* 2012), Thailand (Buathong *et al.* 2015), and Vietnam (Moi *et al.* 2017), occasionally resulting in microcephaly. The huge public health burden of ZIKV infection and the lack of vaccines and effective treatments have necessitated the gathering of information on the vectors and biological characteristics of ZIKV to promote infection prevention and control.

ZIKV was first isolated from the serum samples of rhesus monkeys from the Zika forest in Uganda in 1947; The prototype strain was MR766 (Karabatsos 1985). Since then, ZIKV has been isolated from mosquito specimens many times (Diallo *et al.* 2014; Epelboin *et al.* 2017; Guedes *et al.* 2017). In total, 31 strains of ZIKVs were isolated from mosquito specimens collected in Seychelles, 28 from 10 species of *Aedes* mosquitoes, and 1 each from *Mansonia uniformis*, *Culex perfuscus*, and *Anopheles coustani* (Diallo *et al.* 2014). From 1952 to August 12, 2017, ZIKV was isolated from 16 *Aedes* species (Epelboin *et al.* 2017), and *Ae. aegypti* is the leading vector of ZIKV (Epelboin *et al.* 2017). Thus, mosquito-borne arboviruses, especially zika viruses, are of serious public health concern.

Jiangxi Province encompasses the middle and lower reaches of the Yangtze River and contains many rivers and smaller waterways, as well as an extensive road network, which facilitates exchanges between Jiangxi Province and the surrounding provinces (People's Government of Jiangxi Province 2019). The rapid development of transportation and tourism has increased the risk of transporting viruses transmitted by blood-sucking insects from surrounding areas into the province. For example, outbreaks of DENV occurred in Guangdong Province (south of Jiangxi Province) (Zhang *et al.* 2014; Sun *et al.* 2016), and Akabane virus has been detected in mosquitoes in Hunan Province (Cao *et al.* 2019). To evaluate the situation of the arbovirus species and their distribution in Jiangxi Province, we collected blood-sucking insects in ten counties from June to July 2018. Six ZIKV strains were isolated from *Culex tritaeniorhynchus* and *Anopheles sinensis*. This is the first report of isolation of ZIKV from mosquito specimens in Jiangxi Province from *Cx. tritaeniorhynchus* and *An. sinensis*.

Materials and Methods

Specimen Collection

In June and July 2018, we collected blood-sucking insects (mosquitoes and midges), in Xiajiang, Yongfeng, Anfu, Jing Gangshan, Lichuan, Zixi, Yanshan, Yushan, and Fuliang counties and Long-Hushan Town of Jiangxi Province. The specimen collection sites were selected based on their suitability for the breeding of blood-sucking insects and human and livestock activities (*e.g.*, housing and sheep, chicken, and pig farms). Insect specimens were collected using an ultraviolet mosquito-lured lamp (Wuhan Jixing Environmental Protection Technology Co., Ltd.) from 18:00 to 06:00. The mosquito specimens were classified according to their morphology under ice bath conditions, and numbered and registered according to the collecting environment and species. The specimens were stored in liquid nitrogen and transferred to the laboratory for testing (Song *et al.* 2017).

Cell Culture

BHK-21 (golden hamster kidney cells) and C6/36 (*Aedes albopictus* oocytes) cells were cultured in 90% Eagle's medium (7% fetal bovine serum [FBS; Invitrogen], 1% penicillin–streptomycin [100 U/mL], 1% glutamine [30 g/L], and 1% $NaHCO_3$) and 89% Roswell Park Memorial Institute 1640 medium (Invitrogen) (10% FBS [Invitrogen] and 1% penicillin–streptomycin [100 U/mL]) in a 5% CO_2 incubator at 37 °C and 28 °C, respectively (Fu *et al.* 2017; Song *et al.* 2017).

Virus Isolation

Mosquitoes were combined into pools of 50–100 and ground using a glass grinder. Each pool was washed twice with 1.5 mL of grinding fluid (93% Eagle's medium [laboratory preparation], 5% penicillin–streptomycin [100 U/mL], 1% glutamine [30 g/L], and 1% $NaHCO_3$), then 1.5 mL of grinding fluid was added, and the sample was repeatedly ground in an ice bath. Next, the cells were centrifuged (20,000 $\times g$, 4 °C, 20 min), and 100 μL of the supernatant was collected and inoculated into 80% confluent BHK-21 and C6/36 cells in 24-well plates (Corning Inc.) and cultured in a 5% CO_2 atmosphere at 37 °C and 28 °C, respectively. The cytopathic effect (CPE) was evaluated under a microscope at 12-h intervals. Upon the appearance of CPE, the virus solution was collected and stored at −80 °C. Specimens without CPE were blindly passaged in the above two cell lines for three generations, and those that did not show CPE were discarded (Fu *et al.* 2017; Song *et al.* 2017).

Minimum Infection Rate

The pooled infection rate was calculated as follows: pooled infection rate = positive specimen pool number ÷ total number of pools of processed specimens.

Assuming that each positive pool contains only one infected mosquito, the minimum infection rate of 1000

mosquitoes = number of pools of positive specimens (number of infected mosquitoes) ÷ the total number of treated mosquitoes × 1000 (Feng *et al.* 2012; Ren *et al.* 2017).

Viral RNA Extraction and cDNA Library Preparation

Total RNA was extracted from the specimens using a Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. The extracted RNA sample was immediately placed in a 65 °C water bath for 10 min followed by an ice bath for 2 min. The RNA sample (32 μL) was transferred to a first-strand reaction tube (GE Healthcare, Little Chalfont, Buckinghamshire, UK), allowed to stand for 1 min at room temperature, and 1 μL of random primer (pd(N)6) was added. Finally, the sample was centrifuged, placed in a water bath at 37 °C for 60 min, and stored at −40 °C.

Nucleotide Sequence Determination and Viral Gene Analysis

Polymerase chain reaction (PCR) amplification was performed using cDNA as the template, 2 × GoTaq® Green Master Mix (Promega, Madison, WI, USA), 10 μmol/L upstream and downstream primers for flavivirus genes (Wang *et al.* 2011), and the ZIKV coding region (ORF) gene (Song *et al.* 2017). Next, 5 μL of the PCR products was resolved by electrophoresis in 1% agarose gel and sequenced. The sequence of the coding region of ZIKV was obtained using an amplification primer of the whole ZIKV genome sequence, which was obtained from reference (Fu *et al.* 2017; Song *et al.* 2017).

The nucleotide sequences were aligned using the Basic Local Alignment Search Tool of the National Center for Biotechnology Information. Nucleotide sequence splicing and mass analysis were performed using Seqman software (DNAStar, Madison, WI, USA), and multiple sequence alignments were performed with BioEdit software (ver. 7.0, Thomas). Phylogenetic analysis by the neighbor-joining method with 1000 bootstrap replicates was performed using MEGA ver. 6.0 software. The homology of the nucleotide and amino acid sequences was analyzed using MegAlign (Fu *et al.* 2017; Song *et al.* 2017). The viral gene sequences are shown in the Supplementary file (Supplementary Table S1).

Plaque Assay

BHK-21 cells were transferred to a six-well culture plate (Corning Inc.) and cultured to 80% confluence. Virus suspensions (10^{-1}–10^{-6} dilutions) were added to the six-well culture plates (0.1 mL/well). After adsorption for 1 h at 37 °C in 5% CO_2, 1.3% methylcellulose–MEM semi-solid medium (5 mL/well) containing 2% FBS was added to each well. When plaques were visible under a microscope (3–5 days), the medium was discarded, the cells were stained with crystal violet, and the number of plaque-forming units (PFU) was calculated (Cao *et al.* 2016).

Results

Insect Specimens

In June and July 2018, in total, 22,985 mosquitoes of four genera and four species (*Cx. tritaeniorhynchus*, *An. sinensis*, *Cx. pipiens quinquefasciatus*, and *Armigeres subalbatus*) and 57,500 midges (types to be identified) were collected in ten counties/towns of Jiangxi Province (Fig. 1). There were 16,592 *Cx. tritaeniorhynchus* and 5822 *An. sinensis* specimens (72.2% [16,592/22,985] and 25.3% [5822/22,985] of the total mosquitoes, respectively) (Table 1). *Cx. tritaeniorhynchus* and *An. sinensis* are local dominant mosquitoes.

Virus Isolation and Mosquito Infection of ZIKV

Eleven virus isolates were stably passaged in tissue culture; these included several JEV and DENV strains (results not shown). Six ZIKV isolates, (JXJA1835-1, JXJA1835-2, JXJA1839-1, JXJA1866, JXJA1874-1, and JXLHS1807) (Table 2), showed CPE and were stably passaged in tissue culture. Of these, five were isolated from specimens collected in June, and one from a specimen collected in July. Two of the six ZIKV strains were isolated from *Cx. tritaeniorhynchus* and four from *An. sinensis* (Table 2). The ZIKV isolate, JXLHS1807, from *An. sinensis* showed CPE in BHK-21 cells and formed plaques (Fig. 2).

Four mosquito species (*Cx. tritaeniorhynchus*, *An. sinensis*, *Cx. pipiens quinquefasciatus*, and *Ar. subbalbatus*) were collected in Jiangxi Province. Among these, six ZIKV strains were isolated from *Cx. tritaeniorhynchus* and *An. sinensis* collected in Yongfeng County, Anfu County, and Long-Hushan Town. The minimum ZIKV infection rate of *Cx. tritaeniorhynchus* and *An. sinensis* was 0.76 and 2.05, respectively (Table 3).

Molecular Biological Characteristics of the ZIKV Strains

Nucleotide Sequence Analysis

The number of nucleotide and amino acid sequences of the *E* gene (Li *et al.* 2006; Lin *et al.* 2008) of the six ZIKV strains (JXJA1835-1, JXJA1835-2, JXJA1839-1, JXJA1866, JXJA1874-1, and JXLHS1807) were 1510 nt

Table 1 Specimens of blood-sucking insects collected in Jiangxi Province in 2018.

Period of sample collection	Sampling site (county)	Mosquito species				Total	Midges
		Culex tritaeniorhynchus	*Culex quinquefasciatus*	*Armigeres subalbatus*	*Anopheles sinensis*		
June	Xiajiang	3130 (1530/1600)[a]	0	80	830 (730/100)	3960 (2260/1700)	3000
	Yongfeng	5100 (2100[b]/3000)	0	0	2600 (1250[b]/1350)	7700 (3350/4350)	1100
	Anfu	530[b]	0	0	500[b]	1030	550
	Jing Gangshan	55	39	0	33	127	4550 (1900/2650)
	Subtotal	8815 (4215/4600)	39	80	3963 (2513/1450)	12897 (6847/6050)	9200 (6550/2650)
July	Lichuan	270	0	0	230	500	12400
	Zixi	15	0	6	91	112	7800
	Long-Hushan town	520	0	44	197[b]	761	8900
	Yanshan	5150	0	370	1100	6620	10600
	Yushan	1660	0	12	200	1872	5300
	Fuliang	162	0	20	41	223	3300
	Subtotal	7777	0	452	1859	10088	48300
Total		16592 (11992/4600)	39	532	5822 (4372/1450)	22985 (16935/6050)	57500 (54850/2650)

[a](1530/1600): (number of ground mosquito specimens/number of unground mosquito specimens).

[b]ZIKV isolated from this mosquito specimen.

and 504 aa, and similarity among the six strains was 99.7%–100.0% and 99.2%–100.0%, respectively.

The nucleotide and amino acid sequences (Li *et al.* 2006; Lin *et al.* 2008) of the JXLHS1807 ORF were 10,296 nt and 3423 aa in length, respectively, and encoded a single reading frame. The details of the six ZIKV strains are listed in Table 2.

The nucleotide and amino acid sequence homologies of the ORF of JXLHS1807 and other ZIKV isolates were 88.4% (African type 1 MR766, Uganda), 88.4% (African type 2 ArD7117, Senegal), 96.2% (Africa type 1 MR766, Uganda), and 96.3% (African type 2 ArD7117, Senegal). The corresponding levels of homology with Asian ZIKV strains were 95.3% (Asian type 1 P6-740, Malaysia), 99.7% (Asian type 2 SZ-WIV01, American Samoa), 98.4% (Asian type 1, P6-740, Malaysia), and 99.5% (Asian type 2 SZ-WIV01, American Samoa) (Table 4).

ZIKV Amino Acid Sequence Variation

The amino acid substitutions S139N in the PrM protein; D683E, V763M, and T777M in the E protein; and M/t2634V in the NS5 protein of JXLHS1807 were identical to those of the ZIKV strains prevalent in South America since 2015. The E proteins of the other five ZIKV strains isolated from *Cx. tritaeniorhynchus* and *An. sinensis* (JXJA1835-1, JXJA1835-2, JXJA1839-1, JXJA1866, and JXJA1874-1) had the D683E, V763M, and T777M amino acid substitutions (Table 4).

Molecular Phylogenetic Analysis of the ZIKV Strains

A molecular phylogenetic analysis based on the viral open reading frame nucleotide sequences showed that JXLHS1807 was an Asian type 2 ZIKV, as those isolated from *Cx. pipiens quinquefasciatus* (GZDJ1685) and *Ar. subbalbatus* (GZDJ1666-2) collected in Guizhou Province in 2016. And Asian type 2 ZIKVs were also isolated in Yap Island (ECMN2007, Micronesia), French Polynesia (1_0199_PF, French Polynesia), Brazil (SSABR1, Brazil), Puerto Rico (PRVABC59, Puerto Rico), and Haiti (Haiti 1225/2014, Haiti) since 2007. However, JXLHS1807 was not in the same evolutionary branch as the ZIKV strains prevalent in South America since 2015 (Fig. 3).

Discussion

A variety of *Aedes* species, particularly *Ae. aegypti* and *Ae. Albopictus*, can be infected by ZIKV, which can be detected in their salivary glands, suggesting that they are

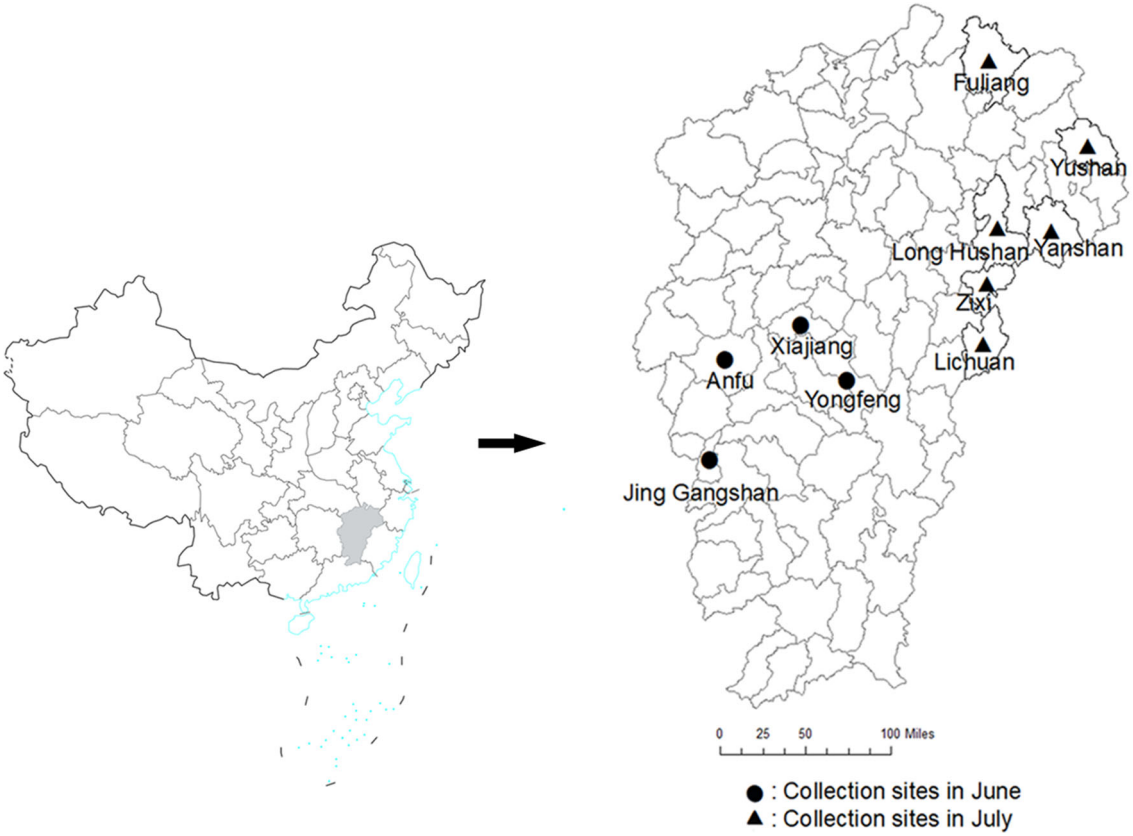

Fig. 1 Locations of blood-sucking insects collected in Jiangxi Province in 2018.

Table 2 ZIKV strains isolated from mosquitoes in Jiangxi Province in 2018.

Sampling date		Sampling site (county)	Strain	Mosquito species	Breeding ground	GenBank Accession No.
June	2018.06.12	Yongfeng	JXJA1835-1	*Anopheles sinensis*	Cowshed	MK696546
	2018.06.12	Yongfeng	JXJA1835-2	*Anopheles sinensis*	Cowshed	MK696547
	2018.06.12	Yongfeng	JXJA1839-1	*Culex tritaeniorhynchus*	Cowshed	MK696548
	2018.06.13	Anfu	JXJA1866	*Anopheles sinensis*	Sheepfold	MK696549
	2018.06.13	Anfu	JXJA1874-1	*Culex tritaeniorhynchus*	Cowshed	MK696550
July	2018.07.10	Long-Hushan town	JXLHS1807	*Anopheles sinensis*	Pigsty	MK696551

vectors for ZIKV (Lourenco-de-Oliveira and Failloux 2017). Indeed, ZIKV can be detected in the salivary glands of *Cx. pipiens quinquefasciatus* after artificial infection (Guo *et al.* 2016; Guedes *et al.* 2017). ZIKV strains have also been isolated from *An. coustani*, *An. gambiae*, *Cx. perfuscus*, and *M. uniformis* (Diallo *et al.* 2014; Epelboin *et al.* 2017), *Cx. pipiens quinquefasciatus* (Guedes *et al.* 2017; Song *et al.* 2017), and *Ar. subbalbatus* (Fu *et al.* 2017), as well as from *Cx. tritaeniorhynchus* and *An. sinensis* in this study (Table 2). Therefore, *Cx. perfuscus*, *An. coustani*, *Ar. subbalbatus*, *M. uniformis, Cx. pipiens quinquefasciatus, Ar. subbalbatus, An. sinensis,* and *Cx. tritaeniorhynchus* also carry ZIKV and are potential secondary vectors (Epelboin *et al.* 2017).

An. sinensis is widely distributed throughout mainland China, except Xinjiang and Qinghai (18°10′–53°33′ N,

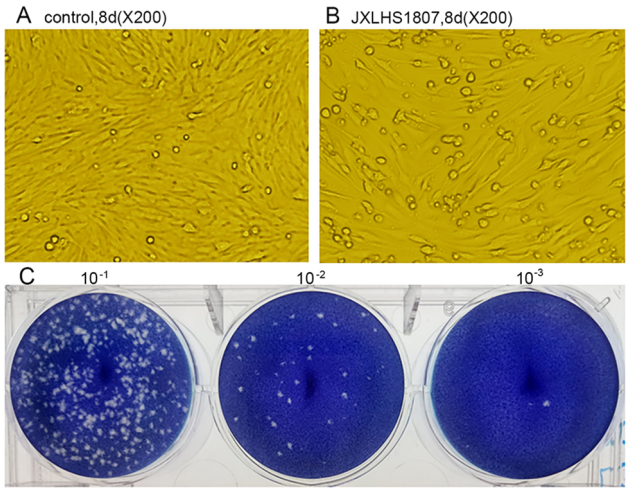

Fig. 2 CPE and plaques caused by ZIKV (JXLHS1807). Normal BHK-21 cells cultured for 8 days grew densely and were neatly arranged (**A**), whereas inoculation with ZIKV strain JXLHS1807 reduced the number of adherent cells and induced rounding and exfoliation (**B**). Manification, 200×. **C** Plaques in monolayers of BHK-21 cells caused by JXLHS1807 with different dilutions.

103°04′–135°2′30″ E). Female *An. sinensis* feed on the blood of humans and livestock, with a preference for the blood of large livestock species such as cattle, horses, and donkeys. Rice fields are the main breeding grounds for *An. sinensis*, but they also proliferate in swamps, reed fields, lakesides, channels, ponds, and depression ponds. *An. sinensis* is the main vector of malaria in China (Ma 1981; Ren *et al.* 2015). *An. sinensis* in China also carries the Getah virus (GETV), Banna virus (BAV, Seadornavirus), Liaoning virus (LNV), Kadipiro virus (KDV), and DNV (Liang *et al.* 2018). The geographical distribution of *Cx. tritaeniorhynchus* in China is similar to that of *An. sinensis*, and is also found in eastern, southern, and southeastern Asia. Female *Cx. tritaeniorhynchus* mosquitoes feed on the blood of humans and animals (typically pigs and cattle) (Miller *et al.* 2012; Gould *et al.* 2017). *Cx. tritaeniorhynchus* is the major vector of JEV (Miller *et al.* 2012; Liang *et al.* 2018). In addition, in China, *Cx. tritaeniorhynchus* carries an alphavirus (GETV), flaviviruses (JEV, Tambosu virus, and mosquito-borne flavivirus), bunyaviruses (AKV and Cat Que virus), BAV (genus *Seadornavirus*, family Reovirus), LNV, KDV, orbivirus (Yunnan orbivirus), parvovirus (DNV), and ten other viruses (Liang *et al.* 2018). *An. sinensis* and *Cx. tritaeniorhynchus* are the dominant mosquito species in mainland China and carry the largest number of arboviruses (Liang *et al.* 2018). We report here the first isolation of ZIKV from *Cx. tritaeniorhynchus* and *An. sinensis*.

The ZIKV strains isolated from *Cx. tritaeniorhynchus* and *An. sinensis* in Jiangxi Province in 2018 were in the same evolutionary branch as those isolated from *Cx. pipiens quinquefasciatus* and *Ar. subbalbatus* in Guizhou Province in 2016, suggesting that despite the geographic (> 1000 km) and temporal (2 years) separation of the ZIKV strains isolated in Jiangxi and Guizhou Provinces, they originate from the same evolutionary population. Indeed, the ZIKV isolated in Guizhou Province in southwestern China in 2016 and the ZIKV isolated in Jiangxi Province in southeastern China in 2018 may have been transmitted to China simultaneously. The phylogenetic analysis suggests that the ZIKV strains isolated in Guizhou Province in 2016 and Jiangxi Province in 2018 are in the same evolutionary cluster as those isolated in Micronesia in 2007 (ECMN2007) and French Polynesia in 2014 (1-0016_PF) (Fig. 3). Therefore, the ZIKV strains isolated from *Cx. pipiens quinquefasciatus*, *Ar. subbalbatus*, *An. sinensis*, and *Cx. tritaeniorhynchus* in China from 2016–2018 entered the country during or before 2016.

One ZIKV strain each was isolated from *Cx. pipiens quinquefasciatus* and *Ar. subbalbatus* specimens collected in

Table 3 ZIKV infection rate of *Cx. tritaeniorhynchus* and *An. sinensis*.

Collection date	Collection site (county)	Mosquito species	No. Individuals/no.pools	No. Positive Pools/no.pools	MIR(/1000)
June	Yongfeng	*Culex tritaeniorhynchus*	2100/14	1/14	0.48
	Yongfeng	*Anopheles sinensis*	1250/8	2/8	1.60
	Anfu	*Culex tritaeniorhynchus*	530/5	1/5	1.89
	Anfu	*Anopheles sinensis*	500/5	1/5	2.00
July	Long-Hushan town	*Anopheles sinensis*	197/4	1/4	5.08
Subtotal		*Culex tritaeniorhynchus*	2630/19	2/19	0.76
		Anopheles sinensis	1947/17	4/17	2.05

Table 4 Variation in the amino acid sequences of ZIKVs isolated from *Cx. tritaeniorhynchus* and *An. sinensis*.

Genotype	Strain	Source			Homology of nucleotide (amino acid) of virus		Divergences of amino acids				
		Country	Year	Host	JXLHS1807(ORF)	JXLHS1807(E)	D683E	V763M	T777M	S139N	M/t2634V
Africa I	MR766	Uganda	1947	Rhesus money	88.4% (96.2%)		D	V	T	S	M
Africa II	ArD7117	Senegal	1968	*Aedes luteocephalus*	88.4% (96.3%)		D	V	T	S	M
Asia I	P6-740	Malaysia	1966	*Aedes aegypti*	95.3% (98.4%)		D	V	T	S	T
Asia I	GZDJ1666-2	China	2016	*Armigeres subalbatus*	99.9% (99.7%)		E	M	M	N	M
Asia II	GZDJ1685	China	2016	*Culex quinquefasciatus*	99.9% (99.7%)		E	M	M	N	M
Asia II	SZ-WIV01	American-Samon	2016	Human	99.7% (99.5%)		E	M	M	N	M
Asia II	ECMN2007	Micronesia	2007	Human	97.8% (98.8%)		E	V	T	S	M
Asia II	H/PF/2013	French-Polynesia	2013	Human	99.5% (99.4%)		E	M	M	N	M
Asia II	1_0035_PF	French-Polynesia	2014	Human	99.4% (99.4%)		E	M	M	N	M
Asia II	ZikaSPH2015	Brazil	2015	Human	99.3% (99.2%)		E	M	M	N	V
Asia II	JXLHS1807[a]	China	2018	*Anopheles sinensis*			E	M	M	N	M
Asia II	JXJA1835-1[a]	China	2018	*Anopheles sinensis*		99.7% (99.2%)	E	M	M		
Asia II	JXJA1835-2[a]	China	2018	*Anopheles sinensis*		99.8% (99.4%)	E	M	M		
Asia II	JXJA1839-1[a]	China	2018	*Culex tritaeniorhynchus*		99.8% (99.4%)	E	M	M		
Asia II	JXJA1866[a]	China	2018	*Anopheles sinensis*		99.8% (99.4%)	E	M	M		
Asia II	JXJA1874-1[a]	China	2018	*Culex tritaeniorhynchus*		99.8% (99.4%)	E	M	M		

[a]ZIKV strains isolated in this study.

Guizhou Province, China, in 2016 (Fu *et al.* 2017; Song *et al.* 2017). In the PCR detection of the ZIKV gene in all the collected mosquitoes, only the pools of mosquito specimens isolated from the virus were positive for ZIKV PCR (Fu *et al.* 2017; Song *et al.* 2017). In this study, six ZIKV strains were isolated from *An. sinensis* (four strains) and *Cx. tritaeniorhynchus* (two strains) specimens collected in Jiangxi Province, China. The infection rate of ZIKV in this survey in Jiangxi Province was 0.76 per 1000 *Cx. tritaeniorhynchus*, and the infection rate of ZIKV per 1000 *An. sinensis* was 2.05. In particular, the infection rate of ZIKV (JXLHS1807) isolated from *An. sinensis* collected in July was 5.08 per 1000 *An. sinensis*, which indicates that the infection rate of ZIKV per 1000 *An. sinensis* may be higher than 2.05 and significantly higher than the ZIKV infection rate of *An. sinensis* in other areas during the same period in 2018 (Table 3). The high infection rate of ZIKV in *An. sinensis* and *Cx. tritaeniorhynchus* raises a new research topic regarding whether ZIKV can infect local animals or humans. ZIKV is an arbovirus circulating between *Aedes* and non-human primates (Epelboin *et al.* 2017; Lourenco-de-Oliveira and Failloux 2017), which raises the following questions: (1) non-human primates are known to be ZIKV hosts in nature, but recent results suggest that pigs and rabbits may be potential hosts for ZIKV (Ragan *et al.* 2017). Since almost all households in rural areas of Jiangxi province raise pigs, and most of the specimen collection points in this study are pigsties and cow enclosures, it is necessary to conduct a study on ZIKV infection in local domestic animals and investigate whether there is natural circulation between mosquitoes and animals such as pigs or cows. (2) Are there any cases of ZIKV infection in humans in the local area? There have been cases of imported ZIKV infection in China, but no local infections have been reported (Li *et al.* 2016). Since most ZIKV infections present only mild manifestations such as fever or fever with rash, these manifestations may also be overlooked or misdiagnosed as DENV or JEV infection. A recent study reported that the frequency of ZIKV co-infection with other classical flaviviruses was higher than that of its single infection (Duong *et al.* 2017). Therefore, it is necessary to strengthen the detection of ZIKV infection in arbovirus-infected patients in Jiangxi province in the summer, such as the detection of ZIKV and DENV co-infections, to determine whether there are ZIKV-infected patients in the local area. Finally, given that ZIKV has been isolated from *Cx. pipiens quinquefasciatus* (Song *et al.* 2017), *Ar. subbalbatus* (Fu *et al.* 2017), *An. sinensis*, and *Cx. tritaeniorhynchus* collected in the wild in China, it is necessary to conduct an artificial infection study on these mosquitoes to clarify whether they can be ZIKV vectors and to assess their public health significance in ZIKV transmission.

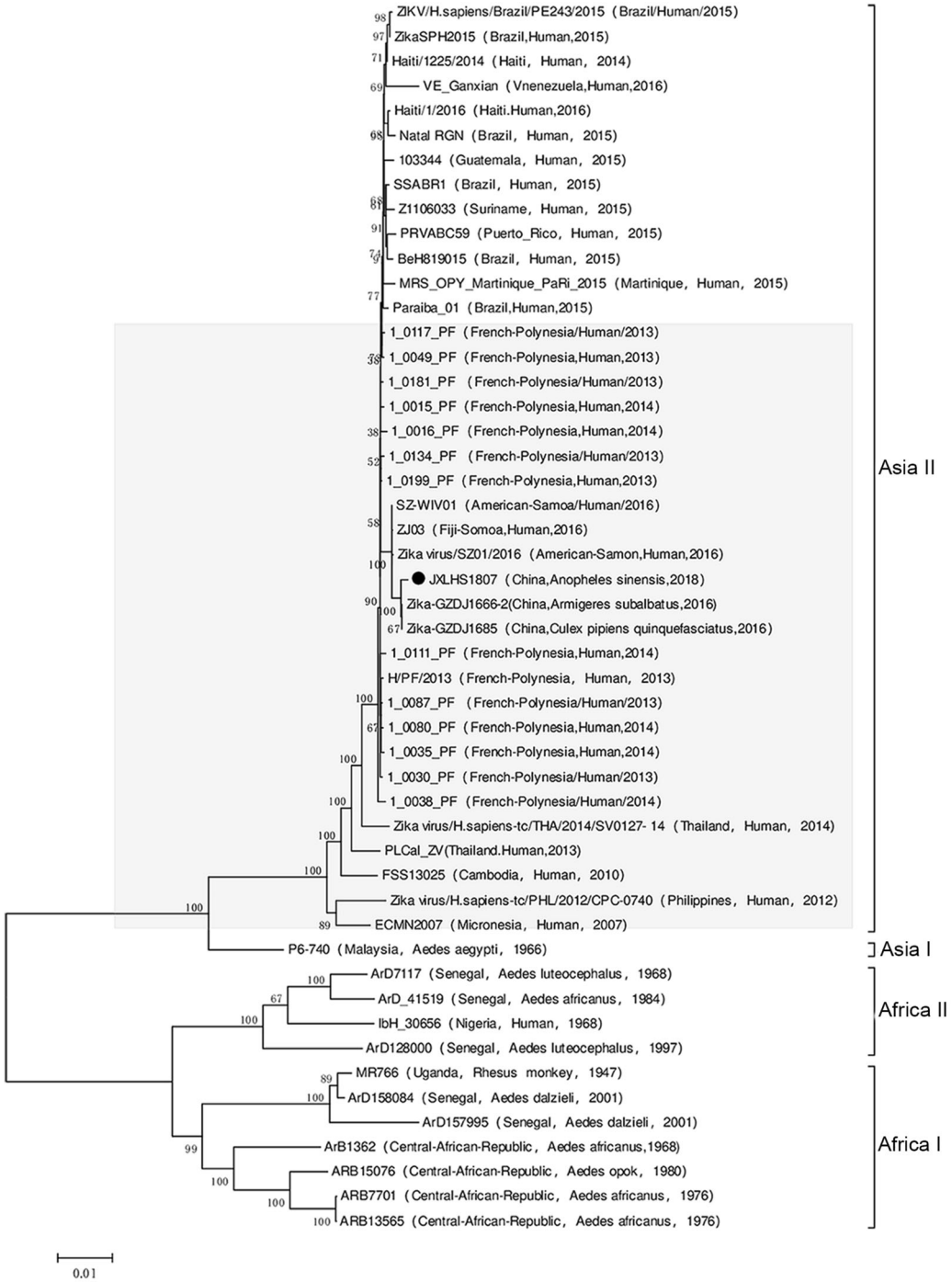

Fig. 3 Molecular phylogenetic analysis based on the coding region of ZIKV (JXLHS1807) isolated from *An. sinensis*. Black spot, ZIKV strain isolated in this study. Each evolutionary branch consists the background information of virus strains (region, host, year).

Acknowledgements This work was supported by grants from the National Science and Technology Major Project (2018ZX10711001, 2018ZX10102001); The National Key Research and Development Program of China (2017YFC1200202); Science and Technology Project of Jiangxi (2014BBG70097) and Development Grant of State Key Laboratory of Infectious Disease Prevention and Control (2014SKLID103, 2015SKLID505). The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the article.

Author Contributions JW, HX, SS and RC collected specimens, performed the experiments, analyzed the data and drafted the paper; NF, SF helped to perform the experiments and analyze the data; SZ, ZX, YH, WL and FL helped to collect specimens and analyze the data; HW, XL, GL conceived and designed the research study,

analyzed the data and finalized the paper. All authors read and approved the final version of the paper.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Animal and Human Rights Statement All animal experiments were conducted in strict compliance with the regulations set by the Animal Ethics Committee of China CDC.

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