Japanese encephalitis virus (JEV) can cause serious encephalitis and Culex mosquitoes are the primary vector. In 2013, a JE outbreak occurred in Shandong Province, China with 407 confirmed cases, including 11 deaths. An investigation on JEV in mosquitoes during the outbreak was conducted. A total of 14,719 mosquitoes were collected at 3 sites. For the 12,695 Culex tritaeniorhynchus mosquitoes, 88/201 pooled samples were positive by RT-PCR for the presence of the pre-membrane or envelope protein coding genes. The maximum likelihood estimates of JEV positive individuals per 1,000 vectors were 12.0, 7.2, and 6.0 in the 3 sites respectively with an overall estimate of 9.1. Phylogenetic analysis on these pre-membrane (n = 72) and envelope (n = 26) sequences with those of reference strains revealed they belonged to genotype I. This study describes the molecular epidemiology of JEV and suggests the high infection rate in mosquitoes is an important factor for the outbreak.

Japanese encephalitis virus (JEV) is a single-stranded RNA virus that belongs to the genus Flavivirus, family Flaviviridae. Most human infections are asymptomatic or result in only mild symptoms. However, a small percentage of infected persons develop acute encephalitis, with a 20%–30% case-fatality rate and neurologic or psychiatric sequelae in 30%–50% of survivors. JEV is endemic in 24 countries, all in Asia and the Western Pacific region with an estimated 67,900 JE cases annually. JEV strains are divided into 5 genotypes, I to V. Most JEV isolates from China belong to genotype I and genotype III. Genotype I JEV has been isolated in China since 1979 and is now recognized as the dominant genotype in many regions, whilst JEV strains isolated before the 1970s belonged to genotype III. In 2009, one strain of genotype V were reported to be isolated from Culex tritaeniorhynchus collected in Tibet. Also, genotype V sequences were detected in one pool of Culex bitaeniorhynchus in ROK in 2011. The re-emergence of this rare genotype after a hiatus of more than a half-century (since 1952 in Malaysia) emphasizes the need for enhanced JE surveillance to monitor the JEV dynamics within the region.

In China, JE was epidemic in most regions in 1950s and was classified as a National Notifiable Infectious Disease in 1951. From 1965 to 1977, 1.4 million JE cases were reported in 26 of China’s 29 provinces (incidence: 7.06–20.09 per 100,000). The JE incidence has remarkably decreased as JE vaccine was included into Expanded Program of Immunization (EPI) in China since 2008, and in recent years about 2500 cases were reported annually. In Shandong Province, the annual reported JE cases ranged from 35 to 249 during 2005 to 2012. However, a JE outbreak was observed in Shandong in 2013 with 407 cases and 11 deaths (Figure 1). The objectives
of this study were to determine which mosquitoes were infected and identify the JEV genotypes circulating during the 2013 outbreak.

Results

Mosquito collection. Mosquitoes were collected at 3 counties of Shandong Province: Junan, Kenli, and Rongcheng (Figure 2). A total of 14,719 mosquitoes were collected at the three sites from July to August in 2013. *Culex tritaeniorhynchus* was the most common species in all the 3 counties with a total number of 12,695 (86.2%) (Table 1). Its constituent ratio ranged from 81.0% to 88.4% in the 3 sites. However, a little difference on species constitution was observed in the three sites. *Aedes albopictus* had a relatively higher constituent ratio in Kenli (8.6%), and *Anopheles sinensis* had a higher constituent ratio in Rongcheng (14.9%).

Infection rate. Of the 201 pools of *Culex tritaeniorhynchus* mosquitoes, 88 pools were JEV positive by RT-PCR amplification of PrM and E genes (Table 2). Different amplification efficiency was observed. 88 pools were positive by RT-PCR targeting PrM gene and only 26 pools—all included in the 88 PrM positive pools—were positive by targeting E gene. No JEV RNA was detected in the 42 pooled samples of other mosquitoes (n = 2,024).

The maximum likelihood estimation (MLE) suggested a high JEV infection rate in *Culex tritaeniorhynchus* with an overall estimate of 9.1 per 1,000 mosquitoes. The highest infection rate occurred in Kenli county with up to 12.00 per 1,000 mosquitoes. A relative low infection rate estimate was observed in Junan county (Table 2).

Sequence analysis on PrM and E genes. The nucleotide sequences of 650-nt PrM (n = 88) and 1500-nt E (n = 26) genes derived from mosquitoes in this study were compared with those of reference strains of different genotypes. All the Shandong strains in this study clustered into genotype I in the phylogenetic tree on PrM sequences (Figure 3). No geographical segregation was observed for the PrM sequences in the four counties with 97.8%–100.0% nucleotide similarities among themselves. However, a relative long genetic distance was observed between Shandong strains and those from the 2010 outbreak in ROK[3]. Homologous comparison

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Table 1 | Numbers of different mosquito species collected at 3 sites in Shandong, China in 2013

| Species                  | Collection sites |
|--------------------------|-----------------|
|                          | Junan Kenli Rongcheng | Total (%) |
| *Aedes albopictus*       | 30 570 28 | 628 [4.3] |
| *Anopheles sinensis*     | 300 230 585 1115 | 7.6 |
| *Armigeres obturans*     | 150 0 46 | 196 [1.3] |
| *Culex pipiens pallens*  | 0 0 56 | 56 [0.4] |
| *Culex tritaeniorhynchus*| 3675 5850 3170 12695 | 86.2 |
| *Mansonia uniformis*     | 0 0 29 | 29 [0.2] |
| **Total**                | 4155 6650 3914 14719 | 100.0 |

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Figure 1 | Clinical cases of Japanese encephalitis in Shandong Province, China, 2005–2013.

Figure 2 | Collection sites of mosquitoes in Shandong Province, China in 2013. Maps were created using Mapinfo software; data are from the National Fundamental Geographic Information System (NFGIS) website (http://ngcc.sbsm.gov.cn/).
revealed up to 2.7% nucleotide divergence between Shandong strains and ROK strains. Also, it is observed that Shandong sequences from different sites may have especially high identities (e.g. 100% PrM nucleotide identity between strains RC6 and VN11 and 100% between KL37 and VN47).

In the phylogenetic tree based on E gene, Shandong strains formed into two main lineages in genotype I (Figure 4). Homologous comparison revealed 98.0%–100.0% nucleotide similarities among themselves and 88.5%–99.7% nucleotide similarities with strains from other regions in genotype I.

**Sequence comparison with vaccine strains used in China.** Compare to vaccine strains P3 (AY243844) and SA14-14-2 (AF315119) currently used in China, JEV strains identified from mosquitoes in this study had 88.0%–88.4% and 87.5%–88.0% nucleotide, and 97.4%–97.8% and 96.8%–97.2% amino acid similarities on the E region, respectively. The PrM region of Shandong JEV strains showed 88.7%–89.6% nucleotide and 94.9%–96.2% amino acid similarities with that of vaccine strain SA14-1-2.

Four amino acid residues of JEV strains identified in mosquitoes were different from both vaccine strains used in China: E129 (Thr → Met), E222 (Arg → Ser), E327 (Ser → Thr), and E366 (Arg → Ser) (Figure 5).

**Discussion**

Since the late 1970s, the immunization with JE vaccine became common in mainland China, and annually number of JE cases decreased gradually. Currently, the highly epidemic provinces include Henan, Chongqing, Sichuan, Guizhou, and Yunnan. They are located in the southwest or the middle area of China, and accounted for more than 50% of the total cases in recent years7. JE was not highly epidemic in the eastern part of China. It has an area of 156,700 km2 and a population of 95.79 million (2010 census data). Mosquitoes were collected in Pigpens and human dwellings in the villages from three counties (Junan, Kenli, and Rongcheng) from July to August 2013. Hand-held aspirators were used to collect mosquitoes about 15

Different amplification efficiencies were observed in comparison between PrM and E coding sequences. The considerably higher positive rate for amplification of PrM coding sequence is supposed to be attributed to the less amplification length of PrM (674 nt) than that of E gene (1,581 nt). E coding sequence has been frequently used for genotyping and molecular epidemiological study of JEV10–14. However, the higher amplification efficiency of PrM gene and its similar phylogenetic appearance with that of E gene reflect that PrM RT-PCR detection may provide a robust, economic and sensitive method for investigating the JEV infections in mosquito vectors.

*Culex tritaeniorhynchus* has been demonstrated to be the primary vector for JEV in China and most other Asia countries15,16,19. In this study, *Culex tritaeniorhynchus* constitutes a dramatic proportion (86.2%) of total mosquitoes collected, and it is hypothesized that the extended rainy season from August to September in 2013 is believed to be responsible for the large amount of *Culex tritaeniorhynchus* populations, as is similar with the situation in ROK in 201017. RT-PCR detection revealed a high rate of JEV infection with 9.1 per 1000 (MLE). Prevalence > 5 per 1000 is considered as ‘epidemic risk’ in the risk assessment model for West Nile virus. So, these data indicate that *Culex tritaeniorhynchus* carried JEV at high prevalence in Shandong Province during the period of the 2013 outbreak and therefore may have contributed to transmission and outbreak of JE. More detailed analysis might be able to provide valuable information on the factors contributing to the high JEV activity in *Culex tritaeniorhynchus* at that time.

In China, genotype III was previously the most common genotype, but, through sequencing of old and new isolates, it has been shown that genotype III has been superseded gradually by genotype I. And the genotype shift was observed in many other regions in Asia as well17–20. In the present study, all detected JEV sequences belonged to genotype I and no other genotypes were observed. These results indicate that genotype I is still the predominant JEV circulating in mosquitoes in Shandong Province in 2013. Phylogenetic analysis and homologous comparison revealed these JEV sequences had close relationship with those from other provinces in China in recent years, indicating the predominant JEV transmission chains circulating in mainland China recently is associated with the 2013 outbreak in Shandong Province. Moreover, it is observed that no geographical segregation was observed for the PrM sequences in the three surveillance sites and some sequences from different sites may have especially high nucleotide identities (up to 100%), suggesting that frequent JEV transmission occurred within these sites.

Currently in China, the vaccine strains P3 and SA14-4-2 both belong to genotype III. However, all JEV strains identified in this study belonged to genotype I and four amino acid residues were identified to be different with both vaccine strains (Figure 5). Although there are evidences of cross-protection by antibodies stimulated by these vaccines21–23, continuous surveillance on JEV should be maintained to understand the genetic characterization of circulating JEV and to avoid potential vaccine breakthrough.

In conclusion, results from this study revealed high infection rate of JEV in *Culex tritaeniorhynchus* during an outbreak in Shandong Province in 2013, described the molecular epidemiology, and demonstrated the importance of mosquito vector investigation in JEV surveillance. Further mosquito surveillance is needed to understand the dynamics of JEV transmission in Shandong and to characterize the role of other potential vectors in the maintenance and human transmission of JEV.

**Methods**

Shandong Province and mosquito collection. Shandong is a coastal province located in the eastern part of China. It has an area of 156,700 km² and a population of 95.79 million (2010 census data). Mosquitoes were collected in Piggens and human dwellings in the villages from three counties (Junan, Kenli, and Rongcheng) from July to August 2013. Hand-held aspirators were used to collect mosquitoes about 15
Figure 3 | Phylogenetic tree on 650-nt PrM gene of JEV strains. Shandong strains from mosquitoes in 2013 all belong to genotype I. Branch names in red, green, and blue indicate strains from Kenli, Rongcheng, and Junan, respectively. Triangles indicate strains from mosquitoes in Shandong in 2010.
Figure 4 | Phylogenetic tree on 1500-nt envelope gene of JEV strains. Shandong strains in 2013 all belong to genotype I. Branch names in red, green, and blue indicate strains from Kenli, Rongcheng, and Junan, respectively. Triangles indicate strains from mosquitoes in Shandong in 2010.
minutes after sunset (18:30–20:00). To clear the intervention from porcine blood, only empty mosquitoes were collected. Mosquitoes were identified according to morphological characteristics, pooled by species, date and site of collection (50–100 individuals per pool), and stored at liquid nitrogen until processed.

RT-PCR. Mosquito pools were homogenized in a mixer mill MM400 (Retsch GmbH, Germany) for 10 min at 20/s after addition of 1 ml of MEM (Gibco, USA) and three 3-mm steel balls to each tube. After centrifugation at 12,000 g for 30 min, the supernatant was sterilized by filtration. Viral RNA was extracted from the supernatant using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA, USA). RT-PCR was performed using a SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen, Carlsbad, CA, USA). Primer pairs JEV-prMf/JEV-prMr and JEV-Ef/JEV-Er were used to amplify the 674-nt PrM and 1,581-nt E protein coding sequences, respectively. To prevent cross-contamination, an RT-PCR using the RNA extracted from MEM served as a blank control, and a negative control containing all the components of the reaction mixture except for the template was also included.

Infection rate. The number of JEV positive mosquitoes per 1,000 individuals was estimated from RT-PCR results by maximum likelihood estimation using PooledInfRate Excel Add-In (version 4.0).

Sequence analysis. PCR products were purified using a QiAquick gel extraction kit (Qiagen, Valencia, CA), and the amplicons were bidirectionally sequenced using an ABI 3130 genetic analyzer (Applied Biosystems, Hitachi, Japan). Homologous comparison was carried out by BioEdit 7.0.5.3 software. Phylogenetic trees were constructed by Mega 4.0 using neighbor-joining method after estimation of genetic distance using the Kimura two-parameter method. A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0.

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**Author contributions**

Z.T., G.L., M.W., Y.S. and A.X. conceived the study and drafted the paper, X.L. (Xiaojuan Lin), L.S., S.W. and H.W. (Hanyan Wang) gathered and analyzed the data, Z.T. and X.L. (Xiaodong Liu) prepared the figures 1–5, and H.W. (Huanyu Wang), W.L. and N.C. helped to interpret results and contributed to the writing. All authors reviewed the manuscript.

**Additional information**

Nucleotide accession numbers PrM and E coding sequences determined in this study were deposited in GenBank under accession numbers KJ190833–KJ190938.

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