Extensive genetic diversity of Rickettsiales bacteria in multiple mosquito species

Wen-Ping Guo1,*, Jun-Hua Tian2,*, Xian-Dan Lin3, Xue-Bing Ni3, Xiao-Ping Chen1, Yong Liao4, Si-Yuan Yang5, J. Stephen Dumler5, Edward C. Holmes1,6 & Yong-Zhen Zhang1

Rickettsiales are important zoonotic pathogens, causing severe disease in humans globally. Although mosquitoes are an important vector for diverse pathogens, with the exception of members of the genus Wolbachia little is known about their role in the transmission of Rickettsiales. Herein, Rickettsiales were identified by PCR in five species of mosquitoes (Anopheles sinensis, Armigeres subalbatus, Aedes albopictus, Culex quinquefasciatus and Cu. tritaeniorhynchus) collected from three Chinese provinces during 2014–2015. Subsequent phylogenetic analyses of the rrs, groEL and gltA genes revealed the presence of Anaplasma, Ehrlichia, Candidatus Neoehrlichia, and Rickettsia bacteria in mosquitoes, comprising nine documented and five tentative species bacteria, as well as three symbionts/endosymbionts. In addition, bacteria were identified in mosquito eggs, larvae, and pupae sampled from aquatic environments. Hence, these data suggest that Rickettsiales circulate widely in mosquitoes in nature. Also of note was that Ehrlichia and Rickettsia bacteria were detected in each life stage of laboratory cultured mosquitoes, suggesting that Rickettsiales may be maintained in mosquitoes through both transstadial and transovarial transmission. In sum, these data indicate that mosquitoes may have played an important role in the transmission and evolution of Rickettsiales in nature.

The order Rickettsiales (Alphaproteobacteria) comprises of a group of obligate intracellular bacteria that are common parasites of eukaryotes. The order contains three documented families (Anaplasmataceae, Holosporaceae, and Rickettsiaceae) as well as one tentative family (Candidatus Midichloriaceae)1,2. Rickettsiales are well known as zoonotic pathogens, causing such severe human diseases as anaplasmosis, ehrlichiosis, rickettsioses, and scrub typhus3, as well as being associated with extensive agricultural losses4,5. The global incidence and geographic range of rickettsial diseases is seemingly experiencing its second pronounced increase in the last 40 years6,7, and the incidence of human monocytotropic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA) have increased steadily since their discovery in the 1980s and 1990s, respectively6,7,14–16. Due to better diagnostic techniques and enhanced surveillance, the identification of new Rickettsiales and/or their associated diseases has increased markedly over the last 10 years7,12–15, and bacteria that had previously been considered nonpathogenic to humans are now associated with disease6,7,14–16. Clearly, Rickettsiales will present a considerable public health challenge for the foreseeable future.

One of the most striking features of Rickettsiales is their diverse host range that includes protists, hydra, annelids, arthropods, vertebrates, and even plants15,17–20. However, only ticks have been found to act as the vectors for bacteria of the genera Anaplasma and Ehrlichia1,4,14,18. In nature, Rickettsia bacteria are spread by either transstadial and transovarial (i.e. vertical) transmission in their arthropod hosts, or by horizontal (co-feeding) transmission through an infected vertebrate15,17,21,22. To date, although Anaplasma and Ehrlichia bacteria are

1State Key Laboratory of Infectious Disease Prevention and Control, Department of Zoonoses, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing; Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, China. 2Wuhan Center for Disease Control and Prevention, Wuhan, Hubei Province, China. 3Wenzhou Center for Disease Control and Prevention, Wenzhou, Zhejiang Province, China. 4Ganzhou Center for Disease Control and Prevention, Ganzhou, Jiangxi Province, China. 5Department of Pathology, Uniformed Services University for the Health Sciences, Bethesda, MD 20814, USA. 6Marie Bashir Institute for Infectious Diseases and Biosecurity, Charles Perkins Centre, School of Life and Environmental Sciences and Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia. 7These authors contributed equally to this work. Correspondence and requests for materials should be addressed to Y.-Z.Z. (email: zhangyongzhen@icdc.cn)
passed transstadially in ticks, definitive evidence for transovarial transmission is not yet available\textsuperscript{3,14}, such that vertebrates (e.g. rodents and ruminants) are required as their main amplifying hosts.

Mosquitoes are members of the family Culicidae and comprise more than 3,500 species. Many species (although only females) feed on blood from various vertebrate hosts, including mammals and birds. Mosquitoes are the most important vector of disease-causing pathogens in humans and animals\textsuperscript{23}, with, for example, more than one-half of the global population at risk for mosquito-borne infections such as dengue and malaria\textsuperscript{24,25}. However, despite their significance in pathogen transmission, with the sole exception of the genus Wolbachia\textsuperscript{24,26}, little is known about the circulation and transmission of Rickettsiales bacteria in mosquitoes. Indeed, there is only a single description of the 16S rRNA (rrs) gene of Anaplasma bacteria in the midgut of Anopheles mosquitoes, although it could not be excluded that the bacteria were from ingested blood\textsuperscript{27}, while a recent experimental study reported the transmission potential of R. felis by Anopheles gambiae mosquitoes\textsuperscript{28}.

To determine whether mosquitoes are indeed hosts of Rickettsiales bacteria, we collected mosquitoes at different life stages – adults, eggs, larvae, and pupae – from a variety of locations in Hubei, Jiangxi, and Zhejiang provinces, China, and examined the prevalence of Rickettsiales bacteria in mosquitoes. Indeed, we conducted a laboratory investigation of the potential transmission of Rickettsiales.

**Results**

**Collection of mosquitoes and detection of Rickettsiales DNA.** During 2014–2015, 971 adult mosquitoes were collected from three regions in China: (i) Wuhan city in Hubei province, (ii) Yudu county in Jiangxi province, and (iii) Cixi city in Zhejiang province (see Supplementary Fig. S1). The numbers, species, and geographic distributions of the adult mosquitoes collected are shown in Table 1. After morphological examination and sequence analysis of the 18S rRNA gene, Anopheles sinensis, Armigeres subalbatus, Aedes albopictus, Culex quinquefasciatus, Cu. tritaeniorhynchus, and Cu. t. Bacterial species are abbreviated as follows: Candidatus Anaplasma boleense, C. A. bo; Anaplasma marginale, A. mar; Anaplasma phagocytophilum, A. pha; Anaplasma platys, A. pla; Candidatus Anaplasma rodocensae, C. A. rod; Ehrlichia chaffensis, E. cha; Ehrlichia sp. EH317, E. eh; Ehrlichia sp. NS101, E. ns; Candidatus Rickettsia sp. Anopheles sinensis, C. R. as; Rickettsia bellii, R. bel; Rickettsia monacensis, R. mon; Rickettsia symbiont of Nephotettix cincticeps, R. nc; Candidatus Neoehrlichia mikurensis, C.N. mik. \textsuperscript{9}PCR positive/mosquitoes collected.

| Species | Location | C.A. bo | A. bov | A.mar | A. pha | A.pla | C.A. rod | E.cha | E.eh | E.ns | C.R.as | R.bo | R.mon | R.nc | C.N.mik | Total◊ |
|---------|----------|---------|--------|--------|--------|--------|---------|--------|------|------|--------|------|------|------|--------|--------|
| Ae.a.   | Hubei    | 0       | 0      | 0      | 0      | 0      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 1      | 1/144  |
|        | Zhejiang | 1       | 0      | 2      | 1      | 0      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 0      | 4/6    |
| An.s.   | Hubei    | 1       | 5      | 25     | 0      | 8      | 2       | 0      | 1    | 0    | 1      | 1    | 0    | 0    | 2      | 46/192 |
|        | Jiangxi  | 0       | 2      | 4      | 0      | 2      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 0      | 8/38   |
| Zhejiang| 0       | 0      | 1      | 0      | 0      | 0      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 1/48   |
| Ar.s.   | Hubei    | 0       | 5      | 4      | 4      | 3      | 1       | 0      | 2    | 1    | 0      | 0    | 0    | 0    | 7      | 27/103 |
|        | Jiangxi  | 2       | 0      | 0      | 4      | 1      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 7/42   |
| Zhejiang| 1       | 0      | 0      | 1      | 5      | 0      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 4/14   |
| Cu.q.   | Hubei    | 0       | 0      | 0      | 0      | 0      | 0       | 2      | 1    | 2    | 0      | 0    | 0    | 1    | 7      | 144/7  |
| Cu.t.   | Hubei    | 0       | 0      | 2      | 1      | 5      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 2    | 10/144 |
| Zhejiang| 0       | 0      | 5      | 0      | 5      | 0      | 0       | 0      | 0    | 0    | 0      | 0    | 0    | 0    | 5/96   |
| Total   |          | 5       | 12     | 43     | 11     | 21     | 5       | 1      | 5    | 1    | 1      | 1    | 1    | 1    | 120    | 971/2   |

Table 1. Prevalence of Rickettsiales bacteria in adult mosquitoes collected in Hubei, Jiangxi, and Zhejiang provinces, China, during 2014–2015. *Mosquito species are abbreviated as follows: Aedes albopictus, Ae.a.; Anopheles sinensis, An.s.; Armigeres subalbatus, Ar.s.; Culex quinquefasciatus, Cu.q.; Culex tritaeniorhynchus, Cu.t. Bacterial species are abbreviated as follows: Candidatus Anaplasma boleense, C. A. bo; Anaplasma marginale, A. mar; Anaplasma phagocytophilum, A. pha; Anaplasma platys, A. pla; Candidatus Anaplasma rodocensae, C. A. rod; Ehrlichia chaffensis, E. cha; Ehrlichia sp. EH317, E. eh; Ehrlichia sp. NS101, E. ns; Candidatus Rickettsia sp. Anopheles sinensis, C. R. as; Rickettsia bellii, R. bel; Rickettsia monacensis, R. mon; Rickettsia symbiont of Nephotettix cincticeps, R. nc; Candidatus Neoehrlichia mikurensis, C.N. mik. \textsuperscript{9}PCR positive/mosquitoes collected.

Phylogenetie analysis of Rickettsiales gene sequences recovered from mosquitoes.

Phylogenetic analysis of the rrs gene sequences revealed that the Chinese mosquitoes collected in this study contained bacteria from the Anaplasma, Ehrlichia, or Candidatus Neoehrlichia genera of the family Anaplasmataceae, and from the genus Rickettsia of the family Rickettsiaceae (Fig. 1). Within the genus Anaplasma, the rrs gene sequences fell into six clusters on the phylogeny (Fig. 1A), corresponding to (i) four documented species: A. bovis, A. marginale, A. phagocytophilum, and A. platys, and (ii) two novel candidate species: Anaplasma sp. Bole...
environments in Wuhan (Hubei province), China, during 2014–2015. Abbreviations are the same as those given in Table 1.

| Species | Anaplasma<sup>a</sup> | Ehrlichia | Rickettsia | C. N. mik | Total (mean ± 95% CI) |
|---------|----------------------|-----------|------------|-----------|----------------------|
| Egg     |                      |           |            |           |                      |
| Ae.a    | 0 0 0 0 0 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 2/9 (10.5 ± 13.8)    |
| An.s    | 0 0 1 1 0 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0/0                 |
| Ar.s    | 0 0 0 0 0 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0/0                 |
| Cu.t    | 0 0 0 0 0 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0/10                |
| Subtotal| 0 0 1 1 0 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 2/19 (10.5 ± 13.8)  |
| Larvae  |                      |           |            |           |                      |
| Ae.a    | 1 4 4 7 1 1 0 6 1 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 27/144 (18.8 ± 6.5)  |
| An.s    | 0 2 5 5 1 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 13/48 (27.1 ± 12.6) |
| Ar.s    | 1 6 6 10 2 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 25/48 (52.1 ± 14.4) |
| Cu.q    | 1 0 2 3 0 2 0 1 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 11/144 (7.6 ± 4.4)  |
| Cu.t    | 0 0 0 0 2 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 4/144 (2.8 ± 2.7)   |
| Subtotal| 3 12 17 25 6 3 1 6 1 1 5| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 80/528 (15.1 ± 3.1) |
| Pupae   |                      |           |            |           |                      |
| Ae.a    | 0 0 1 0 1 1 0 2 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 7/144 (2.8 ± 2.7)   |
| An.s    | 1 0 1 0 1 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 4/88 (4.5 ± 4.4)    |
| Ar.s    | 1 2 2 0 1 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 6/48 (12.5 ± 9.5)   |
| Cu.q    | 0 1 0 0 0 0 0 0 1 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 3/144 (2.1 ± 2.4)   |
| Cu.t    | 0 0 0 0 0 0 0 0 1 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 3/130 (2.3 ± 2.6)   |
| Subtotal| 2 3 4 0 3 1 1 3 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 0 0 0 0 0 0| 23/554 (4.2 ± 1.7)  |

Table 2. Prevalence of Rickettsiales bacteria in eggs, larvae and pupae collected from aquatic environments in Wuhan (Hubei province), China, during 2014–2015. A. bolesense bacteria, which were first identified in ticks sampled from Bole in the Xinjiang Uygur Autonomous Region, China<sup>4</sup>, formed two distinct lineages according to their vector origins. It is notable that the mosquito Candidatus A. rodomose bacteria were closely related to Anaplasma sp. ZJ24 identified in Rattus losea rat from Zhejiang (FJ182047). Within the clusters of A. bovis, and A. platys bacteria, the mosquito Anaplasma bacteria were clearly distinct from those described previously (with 0.6–2.9% and 0.4–2.1% differences, respectively). A. bovis bacteria formed two lineages, one of which was most closely related to an A. bovis isolated from goats in China<sup>28</sup>. Particularly notable was that the A. platys bacteria from mosquitoes were diverse, showing 97.9–99.6% identity with known A. platys, and occupied the basal position in this cluster.

Candidatus Rickettsia bacteria from mosquitoes formed three clusters, compatible with the existence of three species (Fig. 1B). One cluster comprised the mosquito rrs gene sequences WHARSA-128 and HHAEAL-113 and Ehrlichia sp. NS101 identified from deer in Japan. In contrast, the mosquito rrs gene sequence WHCUA-68 was more closely related to those of human E. chaffeensis<sup>31</sup>. The remaining mosquito Rickettsia bacteria formed a distinct cluster most closely related to the tick bacteria Ehrlichia sp. EHH317. Candidatus Neoehrlichia mikurensis bacteria were also identified in mosquitoes, showing a close relationship with those previously identified in rodents and humans<sup>33</sup>. The Ehrlichia bacteria from mosquitoes formed three clusters, compatible with the existence of three species (Fig. 1B). One cluster comprised the mosquito rrs gene sequences WHARSA-128 and HHAEAL-113 and Ehrlichia sp. NS101 identified from deer in Japan. In contrast, the mosquito rrs gene sequence WHCUA-68 was more closely related to those of human E. chaffeensis<sup>31</sup>. The remaining mosquito Rickettsia bacteria formed a distinct cluster most closely related to the tick bacteria Ehrlichia sp. EHH317. Candidatus Neoehrlichia mikurensis bacteria were also identified in mosquitoes, showing a close relationship with those previously identified in rodents and humans<sup>33</sup>.
gene sequences recovered from the *Ehrlichia* positive samples were also closely related each other and formed a distinct cluster. Finally, those mosquito sequences recovered from *Rickettsia* positive samples were closely related to those from *R. bellii* and *R. monacensis* (Fig. 2B).

In the gltA gene tree, all *Anaplasma* sequences formed three clusters (Fig. 3A). Within the *Candidatus A. boleense* cluster, the bacteria formed a cluster clearly distinct from that previously documented in ticks. Strikingly, although the mosquito *Anaplasma* bacteria (ZJARSA-8, WHANSL-27–1, JXARSA-29, WHAEAL-17-2, JXANSA-19, WHARSL-30, WHAEAP-26) were most closely related to *A. platys* bacteria in both the *rrs* and *groEL* trees, in the gltA tree they grouped with strain *Anaplasma* sp. clone SY124 previously identified in ticks from Shenyang of China. Further studies are needed to determine whether these bacteria belong to *A. platys* or represent co-infection (and/or recombinant event). Within the genus *Rickettsia*, the gltA gene sequences from mosquitoes were closely related to those from *R. bellii* and *R. monacensis*, respectively, while sequences WHCUTA-121 and WHCUTA-130 formed a distinct lineage (Fig. 3B). As no *gltA* sequences from the *Rickettsia* symbiont of *Nephotettix cincticeps* were available, we could not determine their phylogenetic relationship.

**Rickettsiales in different life stages of mosquitoes sampled in nature.** The detection rate of Rickettsiales in adult mosquitoes from our three sampling sites was 19.78% in *An. sinensis*, 23.90% in *Ar. subalbatus*, 3.33% in *Ae. albopictus*, 4.86% in *Cu. quinquefasciatus*, and 6.25% in *Cu. tritaeniorhynchus*. Overall, *Anaplasma* bacteria showed the highest prevalence (10.2%), exhibiting species, geographic and annual variation (Table 1, Supplementary Table S2). In contrast, the detection rates of bacteria from the *Ehrlichia*, *Candidatus Neoehrlichia*, and *Rickettsia* genera were lower in adult mosquitoes (0.72%, 0.51%, and 1.13%, respectively), possibly caused by bias in the PCR assay. Finally, among all the Rickettsiales identified in mosquitoes, *A. marginale* had the highest detection rate (4.43%), followed by *A. phagocytophilum* (2.16%).

To better understand the mosquito circulation of Rickettsiales, eggs, larvae, and pupae were collected from aquatic environments in Wuhan. Although only *A. marginale* and *A. phagocytophilum* were identified in two pools of *An. sinensis* eggs, more Rickettsiales were found in these five mosquito species at the larval and pupal stages (Table 2, see Supplementary Table S3). In similar pattern to that observed in adult mosquitoes, *Anaplasma* bacteria were at higher prevalence in larvae and pupae than *Ehrlichia*, *Candidatus Neoehrlichia*, and *Rickettsia*. Notably, *A. phagocytophilum* and *A. marginale* were highly prevalent in larvae. As with the adults, three species of *Ehrlichia* (*E. chaffeensis*, Candidate *Ehrlichia sp. EHH317, and *Candidateus Ehrlichia* sp. NS101) were detected in the larvae and pupae stages. With the exception of *Ar. subalbatus*, *Candidateus N. mikurensis* were identified in four other species of mosquitoes. Finally, a novel candidate species (or symbiont/endosymbiont) (*Candidateus Rickettsia* sp. *Culex tritaeniorhynchus*) was identified in *Cu. tritaeniorhynchus* larvae.
In juvenile mosquitoes, the prevalence of these bacteria was high in *Ar. subalbatus* (52.08% larvae and 12.50% in pupae), *An. sinensis* (27.08% in larvae and 4.55% in pupae), and *Ae. albopictus* (18.75% in larvae and 4.86% in pupae), but relatively low in *Cu. quinquefasciatus* (7.64% in larvae and 2.08% in pupae) and *Cu. tritaeniorhynchus* (2.78% in larvae and 2.31% in pupae). In sum, these data suggest that Rickettsiales may be transmitted through the transstadial and/or transovarian transmission routes in mosquitoes, although the number of observations in eggs was small (95% CI 0–24.8%).

More than one species of *Anaplasma* bacteria was detected in some individuals of both adult and juvenile mosquitoes (Table 4), indicative of co-infection. In adult mosquitoes, the most common combinations were *A. marginale* and *A. bovis*, and *A. marginale* with *A. platys*. The co-infection of three bacteria (*A. bovis, A. marginale, and A. platys*) was detected in one *An. sinensis* individual. Interestingly, co-infection with *A. phagocytophilum* and *Ehrlichia* sp. EHh317 was also detected in *Ar. subalbatus*. Notably, co-infection was relatively more common in *An. sinensis* mosquitoes, but absent in *Cu. quinquefasciatus* and *Cu. tritaeniorhynchus* mosquitoes. For juvenile
mosquitoes, the co-infection of *Anaplasma* bacteria was observed within larvae and pupae of *An. sinensis* and *Ar. subalbatus* and *Ae. albopictus* mosquitoes, but not in *Cu. quinquefasciatus* and *Cu. tritaeniorhynchus* (Table 4). The most common combination was also *A. marginale* or *A. phagocytophilum* with other bacteria. Finally, co-infection with bacteria from the *Ehrlichia* and *Anaplasma* genera was also detected in juvenile mosquitoes.

**Rickettsiales in different life stages of laboratory reared mosquitoes.** To confirm the transstadial and transovarian transmission of Rickettsiales, adult (and/or larvae for *Cu. tritaeniorhynchus*) mosquitoes collected in the field from Wuhan were reared in the laboratory for an entire life cycle (adult-egg-larva-pupae-adult) and tested for the presence of bacteria. From the adult (parent) mosquitoes collected in field, *Ehrlichia* and *Rickettsia* bacteria were identified; *Ehrlichia* sp. EHH317 in *Ae. albopictus*, *R. japonica* in all five species of mosquitoes, and *R. monacensis* and *R. sibirica* in *Cu. tritaeniorhynchus* (Table 3). Accordingly, these bacteria were also identified in their offspring. Notably, *R. japonica* was detected in all life stages of *Ar. subalbatus* and *Cu. quinquefasciatus*, and in three stages of the other three mosquito species. Additionally, both *R. japonica* and *R. monacensis* were successfully identified at each life stage in *Cu. tritaeniorhynchus*, again supporting the transstadial and transovarian transmission of Rickettsiales. As *Anaplasma* and *Candidatus* N. mikurensis were not identified in adult mosquitoes sampled in the field for this experiment, they were similarly not found in laboratory reared eggs, larvae, pupae and adult mosquitoes.

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**Figure 3.** Phylogenetic trees of the citrate synthase gene (gltA) of bacteria of the genera *Anaplasma* (A) and *Rickettsia* (B). The figure description follows that in Fig. 1.
Discussion

Rickettsiales are associated with a wide range of animals including diverse arthropods, mammals and birds. High levels of genetic diversity in Rickettsiales have been identified in both ticks and vertebrates, including multiple infection by distinct bacteria in a single tick species. Our phylogenetic analysis revealed the co-circulation of nine documented and five tentative species bacteria, as well as three symbionts/endosymbionts in five species of mosquitoes. Of particular note in this context was that two distinct *Rickettsia* species (or symbionts/endosymbionts) were identified in mosquitoes and that a single mosquito can harbor two or more species from the genera *Anaplasma* and *Ehrlichia*. As more than 3,500 species of mosquitoes are distributed worldwide, it is likely that additional (and/or novel) mosquito-associated Rickettsiales (or symbiont/endosymbionts) will be discovered in the future.

Ticks are considered the primary vectors for Rickettsiales, especially in the case of *Anaplasma* and *Ehrlichia*. Although mosquitoes are both diverse and abundant, with the exception of the genus *Wolbachia*, there is little evidence for mosquitoes serving as competent vectors or hosts of Rickettsiales bacteria. Previous studies showed that mosquito cell lines (i.e. *Ae. albopictus* and *An. gambiae* cells) could be used to propagate *A. marginale*, *R. felis*, *R. montanensis*, and *R. peacockii* bacteria. Lindh and colleagues also reported the detection of the *rrs* gene of *A. platys* and *A. ovis* in the *Anopheles* midgut, but could not exclude that the *Anaplasma* bacteria were derived from ingested blood. Recently, Dieme and colleagues reported the transmission potential of *R. felis* infection by *An. gambiae* mosquitoes. Herein, we document the presence of diverse bacteria of the genera *Anaplasma*, *Ehrlichia*, *Candidatus Neoehrlichia* and *Rickettsia* in five species of adult and juvenile mosquitoes sampled from three Chinese provinces. In addition, Rickettsiales bacteria were identified in each mosquito life stage (egg, larvae, pupae, and adult). Hence, these data clearly show that diverse Rickettsiales are present in mosquitoes in nature, such that mosquitoes may have played an important role in the transmission, and likely evolution, of Rickettsiales bacteria. In addition, it was noteworthy that some of the Rickettsiales sequences (for example, WHCUTL-65, WHCUTA-121, WHCUTA-130 and WHANSA-146) recovered from mosquitoes were closely related to those of symbionts/endosymbionts. Finally, as only PCR was used here, which may result in some bias, it is possible that the diversity and the prevalence of Rickettsiales bacteria in mosquitoes may be higher than we report.

Intracellular parasites are transmitted by either transstadial and/or transovarial mechanisms (i.e. vertical transmission) in arthropods or by horizontal transmission via infected vertebrates. As horizontal transmission is dependent on the density of susceptible hosts and their intervals of patent infection, the transovarial and/or
transstadial pathways may be more reliable routes to transmit intracellular parasites\(^\text{40,41}\). Although transovarial and/or transstadial transmission has been documented in members of the *Rickettsiaceae*\(^\text{4,14,45}\), this process is not thought to occur to a significant degree in those *Anaplasmataceae* and *Ehrlichia* bacteria documented to date, which may in part be due to lack of the aldolase/adding domain protein\(^\text{44}\). Hence, the known *Anaplasmataceae* and *Ehrlichia* bacteria (excluding endosymbionts such as *Midichloria*) transmitted by ticks require feeding on an infected vertebrate\(^\text{14}\). Notably, we identified *Anaplasmataceae* and *Ehrlichia* bacteria in eggs, larvae, and pupae, as well as adult mosquitoes collected in the field. Additionally, *Ehrlichia* and *Rickettsia* bacteria were identified in eggs, larvae, pupae, and adults reared in the laboratory. Together, these data suggest that Rickettsiaceae, including *Anaplasmataceae* and *Ehrlichia* spp., may be transmitted transovarially and transstadially in mosquitoes in nature. However, as the detection rates of *Ehrlichia* and *Rickettsia* bacteria in laboratory reared mosquitoes were relatively low, further studies are needed to determine the efficiency of transovarial transmission.

The prevalence of *Anaplasmataceae*, *Ehrlichia*, and *Rickettsia* bacteria in nature varies substantially with respect to vectors, hosts and geographic regions\(^\text{4,14,46}\). Similarly, ticks collected at different time points in the same locality can display different infection rates\(^\text{44}\). Such variation can be attributed to several factors, including host susceptibility and competence, the availability of different reservoir hosts, and geo-ecologic factors\(^\text{4,14,16,45–47}\). In addition, adult ticks have an additional blood meal compared to nymphs, such that the infection rates of *A. phagocytophilum* in adult ticks are higher than in nymphs\(^\text{36}\), and because of a lack of transovarial transmission, tick larvae are considered free of *Anaplasmataceae* bacteria\(^\text{4}\). Our analysis of mosquitoes also revealed variation in infection rate according to geographic location, mosquito species and their life stage, and sampling times. For example, infection rates were higher in adult mosquitoes of *An. sinensis* (20.50%) and *Ar. subalbatus* (23.90%), but lower in adult *Ae. Albopictus* (3.33%), *Cu. quinquefasciatus* (4.86%), and *Cu. ttaeniorhynchus* (6.25%), perhaps reflecting further differences in susceptibility. Furthermore, compared with *Ehrlichia*, *Candidatus* *N. mikurensis*, and *Rickettsia*, *Anaplasmataceae* bacteria showed the highest infection rates in adult and juvenile mosquitoes collected in the field.

In all the gene trees inferred here, *A. marginale* from mosquitoes were closely related to strains sampled from cattle\(^\text{41}\). For *Candidatus A. rodomosense*, a close relationship between mosquito- and rodent-associated bacteria is observed in the *rs* gene tree. Hence, these data are compatible with the inter-species transmission of bacteria among mosquitoes and mammals, such that mosquitoes may act as transmission vectors. However, for other *Anaplasmataceae* bacteria (*Candidatus A. boleense*, *A. bovis*, and *A. platys*), the strains identified here were phylogenetically distinct in all three gene trees, such that additional studies are needed to determine whether they are specifically adapted to mosquitoes.

### Table 4. Co-infection of Rickettsiaceae in adult, larva and pupae mosquitoes collected in Hubei, Jiangxi, and Zhejiang provinces, China, during 2014–2015.

| Species       | Location  | Life stage | Bacteria                                      | PCR positive/Mosquitoes collected (%) |
|---------------|-----------|------------|-----------------------------------------------|---------------------------------------|
| *An. sinensis*| Hubei     | Larvae     | *A. marginale, A. phagocytophilum*             | 1/144 (0.69)                          |
|               |           |            | *A. bovis, A. marginale*                       | 1/144 (0.69)                          |
|               |           |            | *A. marginale, Ehrlichia sp. EHh317*           | 1/144 (0.69)                          |
|               |           |            | *A. bovis, A. phagocytophilum, Ehrlichia sp. EHh317* | 1/144 (0.69)                          |
|               |           | Adult      | *A. bovis, A. marginale*                       | 3/192 (1.56)                          |
|               |           |            | *A. marginale, A. platys*                      | 3/192 (1.56)                          |
|               |           | Pupae      | *A. bovis, A. marginale*                       | 1/192 (0.52)                          |
| *Ar. subalbatus*| Hubei   | Larvae     | *A. marginale, A. phagocytophilum*             | 1/48 (2.1)                            |
|                |           |            | *A. marginale, A. platys*                      | 1/48 (2.1)                            |
| *An. albopictus*| Hubei  | Larvae     | *A. marginale, A. phagocytophilum*             | 2/103 (1.94)                          |
|                |           |            | *A. marginale, A. platys*                      | 2/103 (1.94)                          |
|                |           | Adult      | *A. marginale, A. platys*                      | 3/192 (1.56)                          |
|                |           |            | *A. bovis, A. marginale*                       | 2/103 (1.94)                          |
|                |           | Pupae      | *A. bovis, A. marginale*                       | 1/192 (0.52)                          |

For example, infection rates were higher in adult mosquitoes of *An. sinensis* (20.50%) and *Ar. subalbatus* (23.90%), but lower in adult *Ae. Albopictus* (3.33%), *Cu. quinquefasciatus* (4.86%), and *Cu. ttaeniorhynchus* (6.25%), perhaps reflecting further differences in susceptibility. Furthermore, compared with *Ehrlichia*, *Candidatus* *N. mikurensis*, and *Rickettsia*, *Anaplasmataceae* bacteria showed the highest infection rates in adult and juvenile mosquitoes collected in the field.
Bovine anaplasmosis caused by *A. marginale* is widely distributed in tropical and subtropical regions globally, and responsible for substantial economic losses\(^1\). Indeed, cattle can develop persistent infections and serve as reservoirs of *A. marginale*\(^4\). It was therefore notable that the *A. marginale* bacteria identified here were more common than other bacteria in *An. sinensis* and *Ar. subalbatus* mosquitoes, and that these strains are closely related to widely distributed ruminant strains\(^31,49\). Additionally, *An. sinensis* and *Ar. subalbatus* mosquitoes prefer to feed on large animals including cattle, and the geographic distribution of a variety of mosquito species (*An. sinensis*, *Ar. subalbatus*, * Ae. albopictus*, and *Cu. quinquefasciatus*) overlaps with the distribution of bovine anaplasmosis in China. Consequently, it is possible that mosquitoes are involved in the spread of bovine anaplasmosis, beyond serving as mechanical fomites.

The identification of extensive genetic diversity of Rickettsiales (especially *Anaplasma*) in adult and juvenile mosquitoes indicates that mosquitoes may have played an important role in the transmission of Rickettsiales bacteria, and that their role as vectors needs to be investigated further. These data are also compatible with the notion that Rickettsiales can be maintained in mosquitoes through transstadial and transovarial transmission. Due to the global distribution of these mosquitoes, greater efforts are clearly needed to determine their role in the evolution and natural transmission of Rickettsiales.

**Material and methods**

**Mosquito collection and identification.** During the summer (June to August) in 2014 and 2015, 971 adult mosquitoes were collected by ultraviolet light traps from sheep folds, cattle pens, ponds, creeks, and indoors at night in three regions of China: (i) Wuhan city in Hubei province, (ii) Yudu county in Jiangxi province, and (iii) Cixi city in Zhejiang province (see Supplementary Fig. S1). Similarly, mosquito eggs, larvae and pupae were collected from aquatic environments in Wuhan. All mosquitoes were identified to the species level and life stage based on morphologic criteria\(^50\) and further by molecular differentiation as described previously\(^51\). The main morphological characters distinguishing mosquito pupae from larvae are that the latter are comma-shaped in their lateral aspect and the head and thorax are merged into a cephalothorax. All samples collected were stored at \(-80 \degree C\) until DNA extraction.

**Mosquito culture.** For mosquito culture, adult mosquitoes (fed or unfed, Table 3), including *Ae. albopictus*, *An. sinensis*, *Ar. subalbatus* and *Cu. tritaeniorhynchus* collected from the natural aquatic environments in Wuhan were reared for a complete life cycle (adult-egg-larvae-pupae-adult) as described previously\(^50,52\). Larvae and adult *Cu. quinquefasciatus* were also collected for a complete life circle (adult-egg-larvae-pupae-adult or larvae-pupae-egg-larvae-pupae-adult). Samples were collected from adult/larvae mosquitoes caught in the field and at each life stage of cultured mosquitoes.

**DNA extraction, PCR amplification, and sequencing.** After washing twice with PBS, adult mosquitoes, larva and pupae were individually homogenized with a mortar and pestle. Approximately 450 *An. sinensis* and 500 *Cu. tritaeniorhynchus* eggs were pooled (a total of 19 pools) and homogenized. After homogenization, the suspension was incubated at 4 \degree C for 1 h and centrifuged at 2,500 g for 5 min, and the upper fraction collected. DNA was extracted from individual mosquitoes or mosquito pools with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions, and then subjected to PCR for amplification of both bacterial gene sequences (*rrs*, *groEL* and *gltA* genes) and mosquito 18S rRNA genes\(^51\). Rickettsial DNA was detected using nested PCR targeting a conserved sequence of the Rickettsiales *rrs* gene using the primers Eh-out1/Eh-out2 (outer primers) and Eh-gs1/Eh-gs2 (inner primers)\(^53\). Primers designed in-house were also used to amplify complete *rrs* gene sequences and partial *groEL* and *gltA* gene sequences by nested PCR. All primer sequences are described in Supplementary Table S4.

DNA samples of *E. chaffeensis* strain Arkansas were used as positive controls, with distilled water used as a negative control. Amplified DNA was purified by electrophoresis in low-melting point agarose and ligated into the cloning vector pMD19-T. Subsequently, each vector was transformed into *E. coli* and plated onto agarose culture dishes. Twenty clones were picked from each 20th dish and sent to the Sangon Biotechnology Company (Shanghai, China) for sequencing. To prevent contamination, the pre-PCR mix was prepared in a separate room and template DNA was added using dedicated pipets and tips.

**Sequence data and genetic analyses.** DNA sequences of the three bacterial genes obtained here were aligned with existing reference sequences in GenBank using ClustalW (default parameters) as implemented in the MEGA program, version 5.2\(^24\). Nucleotide and amino acid sequence identities were calculated using DNAStar (DNASTAR, Inc., Madison, WI). Data sets of the following sizes were then used in an evolutionary analysis: (i) a 1425 bp *rrs* alignment (N = 131 sequences); (ii) a 1656 bp *groEL* gene alignment (N = 82); and (iii) a 1227 bp *gltA* gene alignment (N = 59). The sequences recovered in this study were named according to their relatedness to known bacteria, geographic origins, and sample numbers. All sequences obtained here have been submitted to GenBank and assigned accession numbers KU585921-KU586334.

**Phylogenetic analyses.** The best-fit evolutionary model for all sequence alignments was determined using iModelTest\(^59\) and found to be the General Time Reversible (GTR) nucleotide substitution model with a gamma (\(\Gamma\))-distribution model of among-site rate variation and a proportion of invariant sites (i.e. GTR + \(\Gamma\) + I). Phylogenetic trees using this model were then estimated using the Maximum Likelihood (ML) method implemented in PhyML (version 3)\(^56\). All trees were mid-point rooted for clarity only.
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Author Contributions
Y.Z.Z. conceived the research project; J.H.T., X.D.L. and Y.L. collected the samples, W.P.G., X.B.N., X.P.C., and S.Y.Y. performed research; W.P.G. and Y.Z.Z. analyzed the data; Y.Z.Z., W.P.G., J.X., J.S.D. and E.C.H. wrote the manuscript.

Additional Information
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