INTRODUCTION

The symbiotic relationship between aphids and endosymbionts is ubiquitous. To date, the endosymbionts harbored by aphids are divided into obligate (or primary) and facultative (or secondary) symbionts. Obligate symbiont *Buchnera aphidicola* is indispensable for aphids since it can offer essential amino acids that the aphid host cannot synthesize themselves or obtain from the phloem of plants (Baumann, 2005; Douglas, 1998) while facultative symbionts are not strictly required for host survival and reproduction (Oliver, Degnan, Burke, & Moran, 2010). However, recent research found that one strain of *Serratia symbiotica* in *Cinara tujafilina* has been undergoing the transformation from facultative symbiont to become an obligate intracellular one (Manzano-Marín & Latorre, 2014) and *Wolbachia*...
has evolved to become a co-obligatory symbiont in the banana aphid *Pentalonia nigronervosa* (De Clerck et al., 2015). Nevertheless, the facultative symbionts do confer traits which impact on host aphid fitness (Guo et al., 2017). A key trait conferred by symbionts is resistance, this "symbiont-mediated resistance" concept was first proposed by Oliver, Moran, and Hunter (2005) and states that secondary symbionts can confer host aphid defense toward adverse situations. For instance, *S. symbiotica* can confer heat resistance for host aphid (Gómez-Valero et al., 2004; Montllor, Maxmen, & Purcell, 2002) and *Hamiltonella defensa* can protect host aphids such as pea aphid *Acyrthosiphon pisum* (Łukasik, van Asch, Guo, Ferrari, & Godfray, 2013; Oliver, Campos, Moran, & Hunter, 2008; Oliver, Russell, Moran, & Hunter, 2003; Oliver et al., 2005). *Sitobion avenae* (Łukasik, Dawid, Ferrari, & Godfray, 2013), *Rhopalosiphum padi* (Linnaeus) (Leybourne, Bos, Valentine, & Karley, 2018), and *Aphis craccivora* (Asplen et al., 2014) against parasitoid wasps. Moreover, the impacts of nine facultative symbionts on aphids were described one by one (Guo et al., 2017) and the global geographic distribution of eight facultative symbionts in aphids tested so far was summarized by Zytynska and Weisser (2016).

Facultative symbionts are generally inherited maternally with high frequencies (Luan, Sun, Fei, & Douglas, 2018; Luan et al., 2016), however, horizontal transmission of facultative symbionts occurs occasionally (Russell, Latorre, Sabater-Muñoz, Moya, & Moran, 2003; Sandström, Russell, White, & Moran, 2001). Despite the horizontal transmission and substantial benefits conferred by facultative symbionts, the bacteria are still maintained at intermediate level in nature (Castañeda, Sandrock, & Vorburger, 2010; Henry, Maiden, Ferrari, & Godfray, 2015; Unckless & Jaenike, 2012; Watts, Haselkorn, Moran, & Markow, 2009; Zytynska & Weisser, 2016). Also, the infection frequencies are dynamic, differing across temporal and spatial gradients, and food-plant associations (Oliver, Smith, & Russell, 2014). Most researchers agree with the idea that there exist costs for hosts to harbor the facultative symbionts (Oliver et al., 2008; Scarborough, Ferrari, & Godfray, 2005) and fitness reduction in aphids containing the facultative symbionts have been found in some cases (Laughton, Fan, & Gerardo, 2013; Vorburger & Gouskov, 2011) such as the infection of *H. defensa* could reduce aphid longevity (Vorburger & Gouskov, 2011). However, multiple infections of facultative symbionts are common in nature (Ferrari, West, Via, & Godfray, 2012; Oliver et al., 2014; Russell et al., 2013). The interactions between different symbionts conferring the host are complex. Some symbionts exhibit additive effects to the host: coinfection of *S. symbiotica* and *H. defensa* in *A. pismum* resulted in higher resistance to parasitism of *Aphidius ervi* (Oliver, Moran, & Hunter, 2006). However, inhibiting effects were found in another case: *A. pismum* coinfected with *Rickettsiella viridis* and *H. defensa* were more exposed to predation (Polin, Le Gallic, Simon, Tsuchida, & Outreman, 2015).

Both *Rhopalosiphum maidis* (Fitch) and *R. padi* are two important pest species on maize especially during the later growth stage, sharing the same niche, feeding on leaves, leaf sheath, husks of maize. What’s more, both *Rhopalosiphum* species can transmit viruses including Maize dwarf mosaic virus and Barley yellow dwarf virus (Chen et al., 1996; Parry, Macfadyen, & Kriticos, 2012; Saksema, Singh, & Sill, 1964; Smyrnioudis, Harrington, Clark, & Katis, 2001) which may cause serious economic damages to their host plants. Recent research showed the importance of facultative symbionts for host aphids such as *A. pismum* (Łukasik, van Asch, et al., 2013). *A. craccivora* (Wagner et al., 2015), *Aphis fabae* (Castañeda et al., 2010), and *R. padi* (Leybourne et al., 2018). Several studies have assessed endosymbiont infections in *R. padi* to date. For instance, *H. defensa*-infected nymphs of *R. padi* collected from UK showed fivefold higher resistance to the parasitoid wasp *Aphidius colemani* (Viereck) than uninfected nymphs (Leybourne et al., 2018). De la Peña, Vandomme, and Frago (2014) found that *R. padi* collected from northwest of Belgium was only associated with *S. symbiotica*, whereas research showed five *R. padi* individuals collected from wheat harbored SMLS (*Sitobion micanthi* L. type symbiont) but no *Ricketttsia* (Li, Xiao, Xu, Murphy, & Huang, 2011) and an absence of targeted facultative symbionts was found in *R. padi* collected in Chile (Zepeda-Paulo, Ortiz-Martinez, Silva, & Lavandero, 2018). However, few research described the infection situation of symbionts in a particular region for *R. maidis* except one which reported that no facultative symbionts were detected from 25 *R. maidis* collected in Morocco (Fakhour et al., 2018). In this study, we conducted an extensive survey of seven facultative symbionts in hosts *R. maidis* and *R. padi* collected from the maize (*Zea mays* L.) in China and four European countries to assess geographic infection patterns of these facultative symbionts.

### 2 MATERIALS AND METHODS

#### 2.1 Sample collection

We collected a total of 882 *R. maidis* from 37 geographical populations and 585 *R. padi* from 32 geographical populations. All aphids were collected from maize and the distance between each two samples was at least 10 m. All these collection sites (except four European populations) were selected to cover the comprehensive maize cultivating areas in China as much as possible and the collection work was done via random generation of co-ordinates within each site. More than 20 aphids per population were collected for most populations, although some populations may have fewer samples. All samples were identified by COI (mitochondrial cytochrome oxidase I) gene (Primers: LCO1490: 5′-GGTCAACAAA TCATAAGATATTGG-3′; HCO2198: 5′-TAAACTTCAGGGTGACC AAAAAATCA-3′) (Foissy, Black, Hoeh, Lutz, & Vrijenhoek, 1994) and the information of aphid samples used in this study was listed in Tables A1 and A2 and the collecting locations were labeled on the maps (Figures 1 and 2). All collected aphids were preserved in absolute ethanol and stored at −20°C before molecular analysis.

#### 2.2 DNA preparation

Total DNA was extracted from single aphid using TEN buffer (10 mM Tris–HCl pH = 8, 2 mM EDTA pH = 8, 0.4 M NaCl), 20% SDS and...
5 M NaCl solution according to the salting-out method (Sunnucks & Hales, 1996). 20–30 μl TE buffer was used to dissolve the DNA precipitate and the DNA quality was assessed with a Nanodrop 2000/2000C instrument. Then the DNA samples were kept at −20°C for further use.

2.3 | Symbionts detection

All 1,467 samples of the two aphid species were screened for the presence of seven facultative symbionts. Diagnostic PCR analysis was conducted using the specific primers listed in Table A3 to detect respective endosymbionts. PCR reactions of 20 μl volume for each sample were carried out under the following conditions: an initial denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. DNA from aphids in laboratory of functional and evolutionary entomology (University of Liège) known to harbor a specific symbiont was used as a positive control and solution without DNA template was used as a negative control. The PCR products were detected by 1.5% agarose gel electrophoresis.

2.4 | Sequencing and analysis of H. defensa

PCR reactions were performed again in a 50 μl volume with the DNA samples positive with H. defensa (n = 63 for R. padi, n = 141 for R. maidis), then PCR products were purified using PCR Clean-up kit (Sangon) and sent for sequencing without cloning. Obtained sequences were verified via BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and assembled in DNAMAN v6. H. defensa sequences downloaded from NCBI (http://www.ncbi.nlm.nih.gov/)

**FIGURE 1** Collecting locations of *Rhopalosiphum padi* in China and Europe. Numbers on the map correspond to locality numbers in Table A1

**FIGURE 2** Collecting locations of *Rhopalosiphum maidis* in China. Numbers on the map correspond to locality numbers in Table A2
of other species were the source for multiple sequence alignment by DNAMAN and MEGA. The phylogenetic analyses were conducted using the Maximum likelihood methods with MEGA 4 software. Clade support was assessed with 1,000 bootstrap replicates (Stamatakis, Hoover, & Rougemont, 2008).

2.5 | Statistical analysis

Differences in the infection frequency of detected symbionts and the proportion of symbiont number per aphid between R. padi and R. maidis from 19 common locations (Figures 4 and 5) and between R. padi populations from China and Europe (Figures 1 and 6) were determined using two-tailed Fisher’s exact tests implemented in the software SPSS (SPSS v16.0). The linear correlation analysis was accomplished using Pearson distribution with the software SPSS to assess whether the infection frequencies of the symbionts were correlated with the latitudes of collecting sites.

3 | RESULTS

3.1 | Seven facultative symbionts were detected in R. padi and R. maidis

Both R. maidis (n = 882) and R. padi (n = 585) were frequently infected with various symbionts (Table 1). The infection frequencies for the seven targeted symbionts varied from 0.2% to 60.9% (Table 1) and only 20.2% of R. maidis and 17.1% of R. padi were not infected with any of the seven symbionts screened for (Table 2). Rickettsia ranked the highest frequency in the two aphid species (51.6% in R. maidis; 60.9% in R. padi) followed by R. insecticola (34.1% in R. maidis; 40.7% in R. padi) and Spiroplasma (35.8% in R. maidis; 26.3% in R. padi), whereas both aphids had the lowest infection rate of S. symbiotica that only nine samples of R. maidis and one sample of R. padi were infected.

The trends of symbiont diversity per aphid were similar in both species (Table 2). Aphids infected with only one symbiont ranked the highest proportion of 29.0% in R. maidis and 32.8% in R. padi, respectively. Totally, around half of the tested aphids were infected with multiple symbionts (50.8% of R. maidis and 50.1% of R. padi). The double infected samples occupied 25.1% in R. maidis and 30.4% in R. padi. In addition, two samples of R. maidis harbored as many as six facultative symbionts simultaneously and no R. padi was infected with six symbionts in a single aphid.

3.2 | Frequencies of seven facultative symbionts in each population of R. padi and R. maidis

Two heatmaps displaying the infection frequencies of the symbionts in each population of R. padi and R. maidis were generated (Figure 3), from which we can find that Rickettsia, R. insecticola and Spiroplasma were found in high densities in both aphid species, whereas S. symbiotica was rarely detected. Among all the symbionts, only one population of R. padi (GY—5.0% of individuals) and five populations of R. maidis (TL—5.3% of individuals, LG—4.2% of individuals, XD—4.2% of individuals, DY—12.5% of individuals, and CS—21.4% of individuals) contained S. symbiotica. For H. defensa, the highest frequency in R. padi was 37.5% of HBD population, whereas this bacterium was detected in most populations of R. maidis and the highest frequency was 58.3% of XX population. All samples of R. padi collected from ZJK were infected with R. insecticola and the highest frequency of this bacterium in R. maidis was 79.2% of XX population. As for Rickettsia, all samples of HEB, KS, DN, and SB populations in R. padi were infected, whereas JN population of R. maidis had highest infection frequency of 83.3%. The highest frequencies of Spiroplasma in R. padi and R. maidis were 75.0% of AZ and 83.3% of MZ, respectively. There were nine populations (HEB, SY, TL, ZY, LF, XX, YN, KS, and QT) of R. padi with frequencies from 5.0% to 20.8% and 11 populations (HEB, GZL, SY, TL, ZY, TS, LF, DZ, LY, LH, and BJ) of R. maidis with

| TABLE 1 | Total frequency of detection of each symbiont in the two aphid species |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | Proportion of infected aphids (%) |
|                  | Serratia symbiotica | Hamiltonella defensa | Regiella insecticola | Rickettsia sp. | Spiroplasma sp. | Wolbachia sp. | Arsenophonus sp. |
| Rhopalosiphum maidis | 1.0               | 16.0               | 34.1               | 51.6           | 35.8           | 2.8            | 26.0            |
| Rhopalosiphum padi | 0.2               | 10.8               | 40.7               | 60.9           | 26.3           | 3.3            | 18.0            |

| TABLE 2 | Total frequency of symbiont numbers infected in a single aphid |
|------------------|------------------|------------------|------------------|------------------|
|                  | Proportion of infected aphids (%) |
|                  | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Rhopalosiphum maidis | 20.2 | 29.0 | 25.1 | 17.2 | 6.2 | 2.0 | 0.2 |
| Rhopalosiphum padi | 17.1 | 32.8 | 30.4 | 13.7 | 4.6 | 1.4 | 0   |
frequencies from 3.8% to 47.4% had been detected with the infection of Wolbachia. Regarding to Arsenophonus, the highest frequency in *R. padi* was 85.7% of DN population and there was only one population (DZ) free of Arsenophonus in *R. maidis*, the highest frequency in *R. maidis* was 85.7% of CS population.

### 3.3 Comparison of symbiont infection between *R. maidis* and *R. padi* from 19 common locations

The infection frequencies of each symbiont within 456 samples of *R. maidis* and 370 samples of *R. padi* from 19 common locations (Figure 4) were compared using the method of Fisher’s exact test (Table 3).

Frequencies of *H. defensa* (16.0%), *Spiroplasma* (41.4%), and *Arsenophonus* (24.8%) in *R. maidis* exhibited higher prevalence than in *R. padi* (5.9%, 23.5%, and 12.2%, respectively). Conversely, *R. padi* harbored more *R. insecticola* (42.7%) and *Rickettsia* (59.7%) compared with *R. maidis* (34.2% and 51.1%). There was no significant difference of *S. symbiotica* and *Wolbachia* between the two aphid species from 19 common locations.

The aphids infected with only one symbiont occupied the highest proportion from the 19 sites for both species (Figure 5). However, the proportion of *R. padi* infected with single symbiont (37.6%) was significantly higher than that of *R. maidis* (30.0%) (*p* = 0.026). Significant higher proportions of *R. maidis* harbored three (20.0%) (*p* = 0.001) and four (6.4%) (*p* = 0.033) symbions per aphid than that of *R. padi*.

**FIGURE 3** Heatmaps showing proportion of symbiont occurrence in each population. (a) *Rhopalosiphum padi*, (b) *R. maidis*. The infection frequencies of seven facultative symbionts were represented by the values in the heatmaps. Numbers on figure (a) correspond to locality numbers in Table A1 and numbers on figure (b) correspond to locality numbers in Table A2.
No significant difference ($p > 0.05$) was observed between *R. maidis* and *R. padi* of the aphid free of detected symbionts or infected with two, five and six kinds of the symbionts per aphid.

### 3.4 Symbiont infection difference between China and Europe of *R. padi*

The infection frequencies of each symbiont in *R. padi* between 516 samples from China and 69 samples from four European countries were compared using the method of Fisher's exact test (Table 4). The proportions of *H. defensa* (30.4%), *Rickettsia* (76.8%), and *Arsenophonus* (47.8%) in samples collected from Europe were significantly higher than from China (8.1%, 58.7%, and 14.0%, respectively). As for the other symbionts detected in this study, no significant difference was found between Chinese and European samples.

*R. padi* infected with single symbiont occupied the highest proportion of 34.7% from China and was significantly higher than the proportion from Europe of 18.8% ($p = 0.009$), however, double-infected *R. padi* numbers ranked the first among European samples that reached 27.5% (Figure 6). Also, significant higher proportion of European samples harbored 3 (26.1%) ($p = 0.004$), 4 (11.6%) ($p = 0.009$), and 5 (5.8%) ($p = 0.009$) symbionts simultaneously in a single *R. padi* than Chinese samples (12.0%, 3.7%, and 0.8%, respectively). In total 71.0% of European samples were multi-infected, which was higher than Chinese populations of 47.3%. There was no significant difference between Chinese and European samples that were free of symbionts or double infected ($p > 0.05$).

### 3.5 Geographic distribution of facultative symbionts

*H. defensa* was more widely distributed in *R. maidis* (34 of 37 populations were infected) than in *R. padi* (16 of 32 populations were infected). Also, all *H. defensa*-infected populations with the infection frequencies

### Table 3: Significance of difference of symbiont frequencies between *Rhopalosiphum maidis* and *Rhopalosiphum padi* from 19 common locations

| Aphid species pairwise comparison | Symbiont                  | Fisher's exact test two-tailed $p$-values |
|----------------------------------|---------------------------|-------------------------------------------|
| *R. maidis*/*R. padi*            | Serratia symbiotica       | 1.000                                     |
| *R. maidis*/*R. padi*            | Hamiltonella defensa       | <0.001*                                   |
| *R. padi*/*R. maidis*            | Regiella insecticola      | 0.014*                                    |
| *R. padi*/*R. maidis*            | Rickettsia                | 0.014*                                    |
| *R. maidis*/*R. padi*            | Spiroplasma               | <0.001*                                   |
| *R. padi*/*R. maidis*            | Wolbachia                 | 0.861                                     |
| *R. maidis*/*R. padi*            | Arsenophonus              | <0.001*                                   |

Notes. These are the results of the statistical analysis which was carried out.

*Means that there is significant difference of the symbiont frequencies between two aphid species. The aphid species with higher average frequency is listed in the front.
various insect species. The sequences from the hosts belonging to Aphididae assembled in one cluster, whereas from Aleurodidae gathered into another cluster. Interestingly, *R. maidis* and *R. padi* are two affinities species that both of them belong to *Rhopalosiphum* genus, with same niche in maize plant in late development stage of maize, however, phylogenetic tree verified that the two haplotypes of *H. defensa* sequences from *R. maidis* fell into group A with the highest homology to *A. pisum* and *Uroleucon rubedoeckiae* whereas the haplotype from *R. padi* fell into group B closest to *A. craccivora* (Figure 7).

### 4 | Discussion

#### 4.1 Frequency of seven facultative symbionts in *R. padi* and *R. maidis*

In this study, we surveyed the infection status of seven facultative symbionts within *R. maidis* and *R. padi* populations collected from maize host. Both *Rhopalosiphum* species exhibited broad symbiotic associations with several facultative symbionts and almost half of the samples (50.8% of *R. maidis* and 50.1% of *R. padi*) were infected with two or more symbionts. In addition, we detected two samples from a number of 882 of *R. maidis* which were super-infected with six facultative symbionts, whereas previous study found that one sample from a number of 318 of *A. pisum* which harbored four facultative symbionts simultaneously (Russell et al., 2013). The infection frequencies of detected symbionts in this study ranged from 0.2% to 60.9%, these differences may result from the benefit-cost balance associated with harboring symbionts (Simon et al., 2007; Vorburger, Ganesanandamoorthy, & Kwiatkowski, 2013). Furthermore, non-selective factors such as transmission rates, migration, and drift may also affect the frequency and distribution of the symbionts (Oliver et al., 2014). Interestingly, both *R. maidis* and *R. padi* were rarely infected with *S. symbiotica* (Table 1), whereas this bacterium was frequently detected in *A. pisum* (Sepúlveda, Zepeda-Paulo, Ramírez, Lavandero, & Figueroa, 2017; Tsuchida, Koga, Shibao, Matsumoto, & Fukatsu, 2002) and *Aphis craccivora* (Brady et al., 2014), which supports the result that symbiont combinations are mainly host specific (Fakhour et al., 2018).

### 3.6 Phylogenetic relationships

A 1,272 bp length fragment of the 16S rDNA sequence of *H. defensa* was obtained after removing the inaccurate terminal sequences. We got one haplotype from 63 infected *R. padi* and two haplotypes from 141 infected *R. maidis* among which, only one *R. maidis* sample of XX population was amplified with the distinct haplotype. The three sequences were deposited in GenBank with accession numbers of KY550361–KY550363. Three haplotype sequences showed 99.8% similarity to the 16S rDNA sequences of *H. defensa* isolated from

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**TABLE 4** Significance of difference of symbiont frequencies of *Rhopalosiphum padi* between China and Europe

| Aphid groups pairwise comparison | Symbiont              | Fisher’s exact test two-tailed p-values |
|---------------------------------|-----------------------|----------------------------------------|
| China/Europe                    | Serratia symbiotica   | 1.000                                  |
| Europe/China                    | Hamiltonella defensa   | <0.001*                                |
| Europe/China                    | Regiella insecticola   | 0.605                                  |
| Europe/China                    | Rickettsia            | 0.004*                                 |
| Europe/China                    | Spiroplasma           | 0.109                                  |
| China/Europe                    | Wolbachia             | 0.150                                  |
| Europe/China                    | Arsenophonus          | <0.001*                                |

Notes. These are the results of the statistical analysis which was carried out.

*Means there is significant difference of the symbiont frequencies between two aphid groups. The group with higher average frequency is listed in the front.
Both *R. maidis* and *R. padi* were frequently infected with *Rickettsia* and *R. insecticola*, whereas previous study demonstrated that *A. pism* both from pea and alfalfa were rarely infected with *R. insecticola* (Sepúlveda et al., 2017) and both symbionts showed a low frequency in *A. craccivora* from several host plants (Brady et al., 2014). In addition, European samples exhibited significantly higher frequencies of *H. defensa* than Chinese ones although Henry et al. (2015) found *R. padi* collected from UK harbored none symbionts of *R. insecticola*, *H. defensa* as well as *S. symbiotica*. Furthermore, *R. padi* collected from Western Europe were free-infected with four targeted facultative symbionts (Desneux et al., 2018) whereas in other cases, European *R. padi* lines were found infections with *S. symbiotica* (De la Peña et al., 2014) and *H. defensa* (Leybourne et al., 2018). Also, research showed that Spiroplasma in *A. pism* was rarely coinfected with other symbionts (Rock et al., 2017) whereas in our study, this bacterium was relative prevalent in both *R. maidis* and *R. padi* commonly coexisted with other symbionts. Moreover, infection frequencies of symbionts can also differ among host plants species. For instance, *H. defensa* was exclusively detected in *A. craccivora* collected from alfalfa (Brady et al., 2014) and there existed great diversity for the symbionts like *R. insecticola* in *A. pism* collected from different host plants (Russell et al., 2013).

It is widely accepted that infection frequency and retention of an endosymbiont in insect are determined mainly by three aspects: first, the fidelity of maternal transmission (Luan et al., 2016, 2018);
second, influences on the fitness of the insect host; third, the frequency of horizontal transmission (Fukatsu, Nikoh, Kawai, & Koga, 2000; Fukatsu, Tsuchida, Nikoh, & Koga, 2001). The infection frequencies between R. maidis and R. padi from 19 common collecting sites (Table 3) showed significant difference for five symbionts except for S. symbiotica and Wolbachia which were rarely detected in both aphids. This may result primarily from the fidelity of maternal transmission, whereas horizontal transmission happened occasionally with a low rate (Russell & Moran, 2005). H. defensa showed higher prevalence in R. maidis than in R. padi, as described by Fakhour et al. (2018) that different host species could exhibit different symbiont combinations. Furthermore, significant difference of infection frequencies can be found even from different genotypes of the same aphid species (Zepeda-Paulo, Villegas, & Lavandero, 2017). A higher proportion of European R. padi harbored three, four and five symbionts simultaneously compared with Chinese samples indicating that the infection frequency of facultative symbionts may differ significantly between distant geographical regions. The abiotic factors such as temperature, humidity, day-length, and rainfall intensity are different between the European and Chinese sampling sites which could affect the infection situation (Tsuchida et al., 2002). For example, the frequency of S. symbiotica in A. pism increased in two-thirds with increasing seasonal temperature in California (Montllor et al., 2002). Moreover, frequencies of symbionts with protective functions may also shift according to the changing of biotic factors such as parasitoid pressures (Smith et al., 2015).

4.2 Geographic distribution of facultative symbionts

Wolbachia has been detected in R. maidis and R. padi with low frequencies of 2.8% and 3.3%, respectively. Also, this bacterium was distributed in northern China (above Henan province) and absent in R. padi collected from Europe, however, it has been found in other aphids from southern Europe (Greece, Portugal, Spain) (Gómez-Valero et al., 2004), Iran and Israel (Augustinos et al., 2011), China (Wang, Su, Wen, Jiang, & Qiao, 2014), USA (Russell et al., 2013), and Africa countries (De Clerck et al., 2014). The linear correlation analysis demonstrated that the frequencies of Wolbachia in R. maidis, H. defensa in R. padi, and Arsenophonus in both R. maidis and R. padi were correlated with the latitude of collecting locations to some degree. A recent study found that high altitudes act negatively on bacterial communities abundance (Fakhour et al., 2018) and China exhibits diverse ambient conditions from south to north of the latitude ranging from 4°N to 53°N which may affect the symbiont frequency but need further study to verify.

4.3 Frequency and phylogenetic analysis of H. defensa

Among the tested aphids, 10.8% of R. padi and 16.0% R. maidis were infected with H. defensa, the presence of this symbiotic bacterium could be related with its potential effect on parasitoid wasp defense in aphid host (Cayetano & Vorburger, 2015; Leybourne et al., 2018; Oliver et al., 2005). What’s more, host–parasitoid coevolution could be modified by the presence of H. defensa (Vorburger, 2014). The infection frequency of H. defensa in aphids is affected by transmission efficiency, cost of infection as well as protection against parasitoids (Oliver et al., 2014; Vorburger, 2014). For instance, a longevity cost of harboring H. defensa was demonstrated in A. fabae (Vorburger & Gouskov, 2011). In our study, only one haplotype was obtained from R. padi and two haplotypes from R. maidis indicating the high conservation of this genotype (Telesnicki, Ghera, Martinez-Ghera, & Arneodo, 2012). As the phylogenetic tree illustrated, haplotype 1 of H. defensa in R. maidis could have diverged earlier than haplotype 2 and that R. maidis and R. padi may acquire H. defensa independently on different occasions (Russell et al., 2013). Little transfer of H. defensa between R. maidis and R. padi has occurred yet although a shared feeding niche (West, Cook, Werren, & Godfray, 1998). Our results demonstrated that the two Rhopalosiphum species were infected by different H. defensa strains which may be determined by host × symbiont genotype interactions (Vorburger & Gouskov, 2011). Furthermore, genotype × genotype interactions exhibited among aphid, symbiont, and parasitoid which could play important role in their coevolution (Vorburger, 2014; Vorburger & Gouskov, 2011; Vorburger, Sandrock, Gouskov, Castañeda, & Ferrari, 2009).

5 Conclusion

To conclude, both R. maidis and R. padi presented wide symbiotic relationship with the detected symbionts especially R. insecticola and Rickettsia, whereas these two Rhopalosiphum species were rarely infected with S. symbiotica. We hypothesize that the low infection frequency of S. symbiotica may be related to the environmental temperature of the collecting regions since S. symbiotica has been demonstrated to confer heat tolerance in aphid (Chen, Montllor, & Purcell, 2000; Montllor et al., 2002; Russell & Moran, 2006) which could be tested in the future. Multiple infections were common in these two aphid species, however, single or double infection occupy the highest frequencies. Linear correlation analysis showed the infection frequency of H. defensa, Wolbachia, and Arsenophonus were correlated with the latitude of the collection sites to some extent. The proportions of H. defensa, Rickettsia, and Arsenophonus in European samples were significantly higher than from Chinese ones, which need further investigation to figure out whether it is caused by the environmental factors. In our study, two Rhopalosiphum aphid species were collected from the same host plant and over the same period of time which allowed us to compare and contrast their symbiont communities between different geographical locations. Additionally, further work is required to detect the phylogenetic relationship of other symbionts except for H. defensa and figure out the symbiont-mediated adaptation for these aphid species to local conditions which can facilitate insect pest control programs.
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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTION

J.G. mainly carried out experiment, analyzed the data and was the primary composer of the manuscript; X.L. helped with a part of experiment; N.P. helped with optimizing PCR reaction conditions; K.H. assisted in experiment design; F.F. and Z.W. involved with experiment design and provided supervision. All authors contributed and agreed on the content of the final version.

ETHICS STATEMENT

None required.

DATA ACCESSIBILITY

All data are available in the results section of this paper apart from the three fragments of the 16S rDNA sequence of H. defensa which were deposited at www.ncbi.nlm.nih.gov/genbank/ with accession numbers of KJ550361, KJ550362, and KJ550363.

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APPENDIX

| Corn region | Province | Index | Population | Locality       | Geo-coordinates     | Date            | Number |
|-------------|----------|-------|------------|----------------|--------------------|-----------------|--------|
| China       | Heilongjiang | 1     | HEB        | Harbin         | 45°49′N, 126°48′E  | August 14, 2014 | 14     |
|             |          | 2     | HBP        | Harbin         | 45°38′N, 126°38′E  | July 24, 2016   | 20     |
|             |          | 3     | HBD        | Harbin         | 45°50′N, 126°50′E  | July 24, 2016   | 16     |
|             |          | 4     | HG         | Hegang         | 47°8′N, 130°17′E   | August 5, 2014  | 17     |
|             |          | 5     | SYS        | Shuangyashan   | 46°46′N, 131°06′E  | August 7, 2014  | 3      |
| Jilin       |          | 6     | TH         | Tonghua        | 42°22′N, 125°25′E  | August 1, 2014  | 24     |
| Liaoning    |          | 7     | SY         | Shenyang       | 41°49′N, 123°33′E  | July 28, 2014   | 24     |
| Inner       |          | 8     | TL         | Tongliao       | 43°40′N, 122°21′E  | August 11, 2014 | 24     |
| Mongolia    |          | 9     | TMT        | Tumd Right Banner | 40°36′N, 110°34′E | September 4, 2016 | 24    |
| Ningxia     |          | 10    | QTX        | Qingtongxia    | 38°1′N, 106°4′E   | April 4, 2016   | 24     |
| Gansu       |          | 11    | ZY         | Zhangye        | 38°56′N, 100°27′E  | August 30, 2016 | 20     |
| Hebei       |          | 12    | ZJK        | Zhangjiakou    | 40°44′N, 114°52′E  | August 21, 2014 | 24     |
| Shanxi      |          | 13    | XZ         | Xinzhou        | 38°25′N, 112°43′E  | August 26, 2014 | 24     |
| Shaanxi     |          | 14    | YuL        | Yulin          | 38°20′N, 109°46′E  | August 28, 2014 | 24     |
| Hebei       |          | 15    | YaL        | Yangling       | 34°16′N, 108°3′E   | September 23, 2014 | 11    |
| Shandong    |          | 16    | HD         | Handan         | 36°56′N, 114°52′E  | August 27, 2014 | 24     |
|             |          | 17    | LF         | Langfang       | 39°28′N, 116°38′E  | August 31, 2014 | 24     |
| Henan       |          | 18    | JN         | Jining         | 35°5′N, 116°34′E   | September 4, 2014 | 12    |
|             |          | 19    | WF         | Weifang        | 36°54′N, 119°10′E  | September 3, 2014 | 11    |
| Beijing     |          | 20    | XX         | Xinxiang       | 35°18′N, 113°53′E  | September 15, 2014 | 8     |
| Anhui       |          | 21    | BJ         | Beijing        | 40°1′N, 116°16′E   | August 19, 2014 | 23     |
|             |          | 22    | SZ          | Suzhou         | 33°38′N, 117°4′E   | September 19, 2014 | 24    |
| Yunnan      |          | 23    | MS          | Mangshi        | 24°26′N, 98°35′E   | August 24, 2014 | 21     |
| Sichuan     |          | 24    | MZ          | Mianzhu        | 31°24′N, 104°18′E  | July 5, 2016    | 14     |
| Guizhou     |          | 25    | GY          | Guiyang        | 26°30′N, 106°39′E  | August 8, 2016  | 20     |
| Xinjiang    |          | 26    | YN          | Yining         | 43°59′N, 81°32′E   | August 14, 2014 | 18     |
|             |          | 27    | KS          | Kashi          | 39°28′N, 75°59′E   | August 14, 2014 | 6      |
|             |          | 28    | QT          | Qitai          | 44°4′N, 89°44′E    | August 26, 2016 | 18     |
| Belgium     | Namur    | 29    | DN          | Dinant         | 50°34′N, 4°41′E    | September 26, 2015 | 14    |
| Luxembourg  | Hesperingen | 30    | LSB         | Atzingen       | 49°34′N, 6°9′E    | September 28, 2015 | 4     |
| France      | Bas-Rhin | 31    | FR          | Strasbourg     | 48°38′N, 7°37′E    | October 2, 2015 | 27     |
| Germany     | Bayern   | 32    | GM          | Ingolstadt     | 48°44′N, 11°25′E   | October 4, 2015 | 24     |

TABLE A2 Collecting information of *Rhopalosiphum maidis* samples investigated

| Corn region | Province | Index | Population | Locality       | Geo-coordinates     | Date            | Number |
|-------------|----------|-------|------------|----------------|--------------------|-----------------|--------|
| China       | Heilongjiang | 1     | HEB        | Harbin         | 45°49′N, 126°48′E  | August 16, 2014 | 24     |
| Jilin       |          | 2     | GZL        | Gongzhuling    | 43°31′N, 124°48′E  | September 4, 2014 | 26    |
| Liaoning    |          | 3     | SY         | Shenyang       | 41°49′N, 123°33′E  | August 28, 2014 | 24     |
| Inner Mongolia |        | 4     | TL         | Tongliao       | 43°40′N, 122°21′E  | August 11, 2014 | 19     |
|             |          | 5     | TMT        | Tumd Right Banner | 40°36′N, 110°34′E | September 4, 2016 | 11    |
| Hebei       |          | 6     | ZJK        | Zhangjiakou    | 40°45′N, 114°53′E  | August 21, 2014 | 24     |
| Shanxi      |          | 7     | LP          | Luanping       | 40°56′N, 117°19′E  | September 4, 2015 | 22    |
|             |          | 8     | XZ          | Xinzhou        | 38°25′N, 112°44′E  | August 26, 2014 | 24     |
### Table A3

| Symbionts          | Primer name | Primer sequence (5′-3′)        | Product size (bp) | References |
|--------------------|-------------|--------------------------------|-------------------|------------|
| *Serratia symbiotica* | 16SA1       | AGAGTTTGATCMTGGGCTCAG          | 480               | (1)        |
|                    | PASScmp     | GCAATGTCTTATTAACACAT           |                   | (2)        |
| *Hamiltonella defensa* | PABSF      | AGCACAGTTTACTGAGTTCA           | 1,660             | (3)        |
|                    | 16SB1       | TACGGYTACCTTGTTACGACTT         |                   | (1)        |
| *Regiella insecticola* | U99F       | ATCCGGGAGGTACGGTTGCTAC         | 200               | (4)        |
|                    | 16SB4       | CTAGAGATCGTGCTCGCTAGGTA        |                   | (5)        |
| *Rickettsia*       | 16SA1       | AGAGTTTGATCMTGGGCTCAG          | 200               | (1)        |
|                    | Rick16SR    | CATCCATACCGGATAATCTTTCC       |                   | (6)        |
| *Spiroplasma*      | 16SA1       | AGAGTTTGATCMTGGGCTCAG          | 510               | (1)        |
|                    | TKSSspR     | TAGCCGCTGTCTTCTGGA             |                   | (2)        |
| *Wolbachia*        | 81F         | TGGTCCATAATGTAGTAGGAAACAGAAGGC | 610               | (7)        |
|                    | 691R        | AAAATTAACAGCTACTCCA            |                   | (7)        |
| *Arsenophonus*     | 16SA1       | AGAGTTTGATCMTGGGCTCAG          | 960               | (1)        |
|                    | Ars16SR     | TTAGCTCCGGAGGCACAGT            |                   | (5)        |
| Gene               | GenBank accession | Host species                  |
|--------------------|-------------------|-------------------------------|
| *Hamiltonella defensa* | AB780465          | Acyrthosiphon pisum           |
| *H. defensa*       | AY136136          | Aphis craccivora              |
| *H. defensa*       | KM375938          | Aphis fabae                   |
| *H. defensa*       | KF835615          | Aphis mendocina               |
| *H. defensa*       | AY264675          | Bemisia argentifolii         |
| *H. defensa*       | AF400475          | Bemisia tabaci                |
| *H. defensa*       | KT336571          | Brevicoryne brassicae         |
| *H. defensa*       | EU348313          | Cinara pinmaritimae           |
| *H. defensa*       | JQ293090          | Metopolophium dirhodum        |
| *H. defensa*       | JX533645          | Sitobion avenae               |
| *H. defensa*       | KM375935          | Sitobion fragariae            |
| *H. defensa*       | HM156641          | Sitobion miscanthi            |
| *H. defensa*       | AF293622          | Uroleucon ambrosiae           |
| *H. defensa*       | AY136162          | Uroleucon nigrotuberculatum   |
| *H. defensa*       | AY136163          | Uroleucon pieloui             |
| *H. defensa*       | AY136164          | Uroleucon reynoldense         |
| *H. defensa*       | AY136166          | Uroleucon rudbeckiae          |