Journal of Asia-Pacific Entomology 19 (2016) 473–476

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

The infection frequencies and dynamics of three secondary endosymbionts in the laboratory environments on *Sitobion avenae* (Fabricius) as determined by long PCR

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ARTICLE INFO

Article history:
Received 23 November 2015
Revised 6 April 2016
Accepted 12 April 2016
Available online 20 April 2016

Keywords:
Secondary endosymbionts
Sitobion avenae
Long-term reproduction
Long PCR

ABSTRACT

Stable infections of maternally transmitted secondary endosymbionts in aphids are frequently found in field populations; however, whether this phenomenon changes in artificial lab conditions has not been clear. To investigate, (1): we collected *Sitobion avenae* (Fabricius) from six different regions and detected the three common secondary endosymbionts (*Regiella insecticola*, *Hamiltonella defensa* and *Serratia symbiotica*); (2): we raised *Sitobion avenae* from Chuzhou, Anhui for 14 months in the laboratory, and analyzed these aphids' DNA using the long PCR protocol after the 1st month, 3rd month, 5th month, 7th month, 9th month, 11th month, 12th month, 13th month and 14th month.

The results showed that *S. symbiotica* and *R. insecticola* could be detected in all aphid populations, while *H. defense* were detected in three locations. After reared in the lab, the infection frequencies of *Serratia symbiotica* did not change across the 14 study months, but the infection frequencies of *Regiella insecticola* after the 9th month and of *Hamiltonella defensa* after the 11th month decreased significantly. Additionally, the results show that facultative endosymbiont infections may decline in artificial lab conditions, suggesting that temporal changes of endosymbionts may occur within the host under different environmental pressures. This result may partially explain why different geographic populations harbor a diverse variety of endosymbionts. The results of this study can be helpful in understanding the stability of secondary endosymbionts in *Sitobion avenae* under long-term reproduction in controlled laboratory conditions.

Introduction

Many insect species harbor endosymbionts that are indispensable for survival and common reproduction or that may help protect the hosts against ecological influences. Almost all *S. avenae* are infected by the primary endosymbiont *Buchnera aphidicola*, which can synthesize essential amino acids and other nutrients that the host aphids typically obtain in only small quantities from their restricted diets such as plant phloem (Douglas, 1989, 1998; Baumann et al., 1995). Meanwhile, aphids may also be infected with one or more nonessential secondary or facultative endosymbionts belonging to distinct bacterial lineages (Oliver et al., 2010).

Although such secondary endosymbionts do not present in every single individual and are not indispensable for aphid survival and reproduction, they can significantly influence the host aphids. Some effects of secondary symbionts include heat shock resistance (*S. symbiotica* (Montllor et al., 2002)), parasitoid resistance (*H. defensa* and *S. symbiotica* (Oliver et al., 2003)), natural enemies (*H. defensa* (Haine, 2008)), resistance to fungal pathogens (*R. insecticola*, *Spiroplasma* and *Rickettsiella* (Scarborough et al., 2005; Łukasik et al., 2013a; Parker et al., 2013)), reproductive manipulation (*Spiroplasma* (Simon et al., 2011)), host utilization (*R. insecticola* (Tsuchida et al., 2004)), and body color modification (*Rickettsiella* (Tsuchida et al., 2010)).

The English grain aphid *Sitobion avenae* (Fabricius) is one of the most widespread and economically damaging pests on cereal crops because it causes host damage directly through feeding and transmission of several diseases (Dedryver et al., 2010). Although we know a considerable amount about the secondary endosymbionts and their effects on the grain aphid (Łukasik et al., 2013b), we know little about the transmission fidelity of secondary endosymbionts in laboratory conditions, even though the majority of related experiments were from studies

Abbreviations: PASS, Serratic symbiotica; PAUS, Regiella insecticola; PABS, Hamiltonella defense; PCR, polymerase chain reaction.

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http://dx.doi.org/10.1016/j.aspen.2016.04.006
1226-8615/

performed in laboratory conditions. Here, we report a series of laboratory experiments that investigated whether long-term reproduction of *S. avenae* under laboratory conditions has any impact on the dynamics of secondary endosymbionts.

A previous study showed that the long PCR is approximately six to eight times more sensitive than standard PCR through amplification of the *wsp* and the 16S rDNA genes in diverse arthropod species (Jeyaprakash and Hoy, 2000). Long PCR uses a second DNA polymerase with proofreading activity and has yielded consistent results, with a sensitivity that is orders of magnitude greater than standard PCR (Guruprasad et al., 2012). Prolonged denaturation could cause breakage of the DNA template and depurination of bases, which could stop the extension by Taq (Heath et al., 1999; Huigens et al., 2000). Therefore, long PCR can be applied to amplify endosymbionts' DNA as well.

In the present study, we investigate (1) the diversity of secondary endosymbionts in 6 different geographic populations of *S. avenae* and (2) whether long-term reproduction of *S. avenae* in a laboratory setting affects endosymbiont host populations, using detection by long PCR protocols. The results of this study will be helpful in understanding the stability of secondary endosymbionts in this species under long-term reproduction in the laboratory setting.

Materials and methods

Aphids

All samples of *S. avenae* used in this study were collected from wheat (*Triticum aestivum* L.) in April 2013 from Yangling, Wudigguan, Shaanxi; Taigu, Shanxi; Chuzhou, Anhui; Zhengzhou, Henan; and Shihezi, Xinjiang, in China. At each locality, three quadrats (approximately 2×2 m) were identified that contained a thick growth of host plants for collecting *S. avenae*. Approximately 30 individuals from the wild populations in every quadrat were collected and preserved immediately in pure alcohol. These insects were subsequently maintained at −20 °C until DNA extraction for molecular analysis. Some individuals from the wild population in every quadrat were collected for long-term reproduction in the laboratory environment (climatic cabinets (20 °C, 75% humidity; BIC-300, Shuangxu)) using a 12 h light−12 h dark regime.

DNA extraction

Before DNA extraction, every aphid was washed with 70% ethanol and sterile water several times to remove surface contamination. More than 10 individual aphids were collected in each quadrat of each locality, and DNA was extracted from each of those 10 individual aphids separately using the Genomic DNA Extraction Kit (TransGen, Beijing) for the following analyses.

Specific PCR detection, cloning, and sequencing

For detection of certain endosymbiotic bacteria, diagnostic PCR analysis was conducted using specific primers (Table 1). Long PCR was performed in 20 μl containing 1 unit of Pwo and 5 units of Taq DNA polymerases (Barnes, 1994). Long PCR was carried out using three linked profiles over 35 cycles, as follows: (1) 1 cycle of denaturation at 94 °C for 2 min, (2) 10 cycles each consisting of denaturation at 94 °C for 10 s, annealing at 59 °C for 30 s, and extension at 68 °C for 1 min, and (3) 25 cycles each consisting of denaturation at 94 °C for 10 s, annealing at 59 °C for 30 s and extension at 68 °C for 1 min, plus an additional 20 s for every consecutive cycle between 11 and 36 for both the primers (Guruprasad et al., 2012). The PCR products were separated by electrophoresis in a 2% agarose gel and were visualized under ultraviolet light after staining with ethidium bromide. The PCR products corresponding to three samples from each quadrat were cloned and sequenced to minimize possible PCR errors. GenBank was used to search for the sequences of the 16S rDNA gene obtained from the wheat aphid. The sequences were then compared using BLAST and uploaded to GenBank.

Long-term reproduction of S. avenae (Chuzhou, Anhui)

The wild geographic population of aphids from Chuzhou, Anhui, was reared in the laboratory in climatic cabinets (BIC-300, Shuangxu) on seedlings of fresh wheat (variety '1376') at 20 °C using a 12 h light−12 h dark regime, while the geographic population of aphids from 3 quadrats (Chuzhou, Anhui) were reared separately in 3 different cube cages (side length = 25 cm). The geographic population of Chuzhou, Anhui, was the same population analyzed by PCR at the beginning of the study for detecting the endosymbionts of wild *S. avenae*. The wheat seedlings were renewed approximately every 10 days. Samples of *S. avenae* (Chuzhou, Anhui) were collected after the 1st, 3rd, 5th, 7th, 9th, 11th, 12th, 13th, and 14th months, preserved immediately in pure alcohol and subsequently maintained at −20 °C until DNA extraction for molecular analysis.

Results

Infection rates of endosymbionts in different wild geographic grain aphid populations

The infection rates (Table 2) for *S. symbiotica* and *R. insecticola* were 100% in the 6 locations and the infection frequencies for *H. defensa* were 30%, 66%, 17%, 0%, 0%, and 0% in the Yangling, Chuzhou, Shihezi, Zhengzhou, Wudingguan and Taigu regions, respectively. The 16S rDNA sequences (*S. symbiotica*, *R. insecticola*, and *H. defensa*) from the Yangling, Wudingguan, Taigu, Chuzhou, Zhengzhou, and Shihezi regions were uploaded to GenBank. For these six regions, the accession numbers for *S. symbiotica* are KM035992, KM035989, KM035993, KM035988, KM035990, and KM035991, respectively, while for *R. insecticola*, they are KM035998, KM035995, KM035999, KM035994, KM035996, and KM035997, respectively. The *H. defensa* sequences' accession numbers for Yangling, Shaaxi; Chuzhou, Anhui; and Shihezi, Xinjiang are KM036000, KM036001, and KM036002, respectively.

Changes of endosymbionts of the S. avenae (Chuzhou, Anhui)

The average infection frequencies for *H. defensa* and *R. insecticola* (Table 3) showed clear declines after the 11th month and the 9th month, respectively, based on the long PCR protocol analysis, and the

Table 1
Endosymbiotic bacteria examined in this study.

Target symbiont	Target gene	Primer name	Primer sequence(5′–3′)	Product size (kb)	Refs
R. insecticola	16SrDNA	U99F	ATCGGGGAGTAGCTTGCTAC	0.2	Sandström et al., 2001
		16SB4	CTAGAGATCGTCGCCTAGGTA		Fukatsu, 2001
	gryB	UF	TAATTCCTATAAAGTCTCCG	.32	This study
		UR	GGAAATACCCGAGTTAAGA		This study
H. defensa	16SrDNA	PABSF	AGCACAGTTTACTGAGTTCA	1.3	Darby et al., 2001
		16SB1	TACGGYTACCTTGTTACGACTT		Fukatsu and Shimada, 1999
S. symbiotica	16SrDNA	16SA1	AGAGTTTGATCMTGGCTCAG	0.48	Fukatsu and Shimada, 1999
		PASScmp	GCAATGTCTTATTAACACAT		Fukatsu et al., 2000

Table 2
Sample locations of the different geographic populations used for the study and the infection rates of *R. insecticola*, *H. defensa*, and *S. symbiotica*. The numbers in parentheses denote the infection frequency of endosymbionts; the notation n/10 indicates that n (number) individuals were infected among 10 individuals; a blank entry means the endosymbionts were not detected in that population.

No.	Locality	Longitude/ latitude	Infection rate		
			R. insecticola	*H. defensa*	*S. symbiotica*
1	Yangling, Shaanxi	34.36°N, 108.72°E	1	0.30 (3/10; 2/10; 4/10)	1
2	Wudingguan, Shaanxi	32.33°N, 118.31°E	1		1
3	Chuzhou, Anhui	32.83°N, 106.25°E	1	0.66 (7/10; 6/10; 7/10)	1
4	Tigu, Shanxi	34.36°N, 110.15°E	1		1
5	Zhengzhou, Henan	34.46°N, 113.40°E	1		1
6	Shihezi, Xinjiang	44.18°N, 86.00°E	1	0.17 (2/10; 1/10; 2/10)	1

infection frequency trends of the three respective repeats in every group were similar. Table 3 shows the detailed change of infection rates of endosymbionts of *S. avenae* (Chuzhou, Anhui) at different months when raised in the lab. The average infection frequencies of *H. defensa* and *R. insecticola* were unstable at different months. For *H. defensa*, there was no significant decrease in the first 9 months (from 70% to 60%). However, from the 11th month onward, there was a significant reduction. The infection rates from the 11th, 12th, 13th, and 14th months were 23%, 17%, 0%, and 0%, respectively. For *R. insecticola*, from the 9th month onward, there was a significant decrease, but this decrease was not sustained in the following months. The average infection rates for the first 7 months were all 100%, but those of the 9th, 11th, 12th, 13th, and 14th months were 80%, 80%, 83%, 87%, and 87%, respectively. Interestingly, the levels of *S. symbiotica* did not change over the 14-month study period.

Discussion

Facultative endosymbionts such as *S. symbiotica*, *R. insecticola*, and *H. defensa* have been widely reported and researched in many insects, particularly for the aphid (Unterman et al., 1989; Moran et al., 1994; Chen et al., 1996; Chen and Purcell, 1997; Chen et al., 2000; Fukatsu et al., 2000; Darby et al., 2001; Fukatsu, 2001; Sandström et al., 2001; Park et al., 2012; Peccoud et al., 2014). However, the impact of long-term reproduction of *S. avenae* in laboratories on endosymbionts are poorly understood—even though the majority of related experiments were conducted in laboratory conditions. This study is the first systematic survey of the effect of laboratory rearing on the endosymbionts of *S. avenae*; however, Oliver et al. (2014) researched the impact of long-term maintenance in laboratory conditions on *H. defensa* in pea aphid populations.

Table 3
Changes of endosymbionts (*R. insecticola*, *H. defensa*, and *S. symbiotica*) of the *S. avenae* (Chuzhou, Anhui) raised in artificial lab conditions in different months after collection. The numbers in parentheses denote the infection frequency of endosymbionts; the notation n/10 indicates that n (number) individuals were infected among 10 individuals.

Month	Infection rates		
	H. defensa	*R. insecticola*	*S. symbiotica*
1	0.60 ± 0.057 a (6/10; 5/10; 7/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
3	0.60 ± 0.000 a (6/10; 6/10; 6/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
5	0.70 ± 0.000 a (7/10; 7/10; 7/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
7	0.63 ± 0.033 a (6/10; 7/10; 6/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
9	0.67 ± 0.033 a (6/10; 7/10; 7/10)	0.80 ± 0.058 b (9/10; 8/10; 7/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
11	0.23 ± 0.033 b (2/10; 3/10;2/10)	0.80 ± 0.058 b (9/10; 7/10; 8/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
12	0.17 ± 0.067 b (1/10; 1/10; 3/10)	0.83 ± 0.033 b (8/10; 8/10; 9/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
13	0 ± 0.000 c (0/10; 0/10; 0/10)	0.87 ± 0.067 b (8/10; 8/10; 10/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)
14	0 ± 0.000 c (0/10; 0/10; 0/10)	0.87 ± 0.033 b (8/10; 9/10; 9/10)	1.00 ± 0.000 a (10/10; 10/10; 10/10)

Different letters (a, b, c) within a column indicate statistically significant differences ($P < 0.05$; one-way ANOVA with Tukey correction).

Of the 180 wild individuals of *S. avenae* collected from 6 localities in China, each harbored at least two of the three common secondary endosymbionts (*Regiella insecticola*, *Hamiltonella defensa*, and *Serratia symbiotica*). These results are consistent with Toju and Fukatsu (2011) and Ferrari et al. (2011) and demonstrate that multiple endosymbiont infections are commonplace in Chinese populations of *S. avenae*. *H. defensa* was not found in *A. pisum* populations in Japan (Tsuchida et al., 2002); however, in our study, *H. defensa* was detected in Chinese populations of *S. avenae* at rates similar to those in the UK populations (46%) (Łukasik et al., 2013a), suggesting that the secondary endosymbiont microbiota in *S. avenae* populations may differ significantly across distant geographical regions (Tsuchida et al., 2002). Further detailed studies of the secondary endosymbiont microbiota in natural populations of *S. avenae* in the UK, China, and other regions are needed.

The infection rates of *S. symbiotica* and *R. insecticola* in the 6 different regions were very high. Several studies have revealed a high frequency of secondary symbionts within aphid populations. For example, the *Yamatocallis* secondary mycetocyte symbiont (YSMS), a γ-proteobacterial symbiont, was found to infect 100% of surveyed individuals belonging to two species within the aphid genus *Yamatocallis* (Fukatsu, 2001). More than 99% of the mosquitoes harbored *Wolbachia* in *Aedes albopictus* (Park et al., 2015), and the infection rate of *Serratia* in *Curculio sikkimensis* was 82.3% (Toju and Fukatsu, 2011). In addition, a survey conducted in *A. pisum* populations (California, USA) reported *S. symbiotica* infections in 50 of 57 individuals (Chen and Purcell, 1997). Therefore, *S. symbiotica* and *R. insecticola* may commonly be present in populations of *S. avenae* in China.

In addition, rearing *S. avenae* in artificial labs may reduce the infection rates of *R. insecticola* and *H. defensa*. The infection rates of *R. insecticola* and *H. defensa* endosymbionts of *S. avenae* raised in artificial lab conditions decreased significantly from the 9th month and the 11th month onward, respectively. These results show that infection rates of the endosymbionts in *S. avenae* may change in labs and that the order of such changes is not synchronous, which are partly in agreement with previous results. The main reasons may be due to outside pressures. For example, ambient conditions may help certain endosymbionts reproduce while hindering the reproduction of others. Oliver et al. (2003) showed that the rate of *A. pisum* infections by *H. defensa* increased rapidly when facing pressure from parasitism and, conversely, observed a significant decline in infection frequency for both *H. defensa* and *S. symbiotica* when the population was exposed to parasitism. Similarly, the proportion of *Lolium arundinaceum* infected with the inherited endophytic fungal symbiont *Neotyphodium coenophialum* increased dramatically in the presence of herbivores (Clay et al., 2005). Apart from external pressures, balancing selection has been suggested as playing a large role in endosymbiont maintenance (Oliver et al., 2014), and endosymbiont-mediated protection is a potent mechanism that could maintain the prevalence of symbionts within insect populations (Brownlie and Johnson, 2009). Theoretical models show that if vertically transmitted symbionts can protect their hosts from pathogens, the transmission would act to maintain the symbiont within host populations (Lipsitch et al., 1996; Lively et al., 2005; Jones et al., 2007). In contrast, in the absence of these selection pressures (e.g., heat shock, parasitoid, host utilization, and fungal pathogens), facultative symbiont infections may decline in host populations. Because this study reared *S. avenae* under controlled laboratory conditions

(a stable environment—no heat shocks or parasitoids), the absence of such selection pressures may partly explain the case that several endosymbionts (*H. defensa* and *R. insecticola*) were lost over time, suggesting that temporal changes play a role in the costs and benefits of endosymbionts under different pressures (Oliver et al., 2014). In this study, we suggest that *H. defensa* and *R. insecticola* did not provide fitness advantages for the *S. avenae* raised in an artificial laboratory environment. Therefore, the host may restrict their reproduction. This hypothesis will be the focus of our next study. Here, we can only surmise that the types of endosymbionts in *S. avenae* may be related to the environment in which the aphids are living, but that may partly explain why different geographic population harbor diverse endosymbionts.

In this study, we did not investigate the changes of endosymbionts in wild *S. avenae* (Chuzhou, Anhui) over the corresponding 14 months because epigenetic factors and stress may also impact the presence and absence of endosymbionts dynamically (Oliver et al., 2003; Oliver et al., 2014). In addition, it is difficult to observe the dynamics of endosymbionts in wild *S. avenae* populations at the same time scale because the wild aphids overwinter. Recently, Zytynska and Weisser (2016) have begun a study of endosymbionts in aphids concerning eco-evolutionary dynamics in natural systems, which will be a good start for studying endosymbionts in wild aphids.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors are grateful to Dr. John Richard Schrock for his critical comments on earlier versions of this manuscript. This work was supported by the Fundamental Research Funds for the Central Universities in Northwest A&F University (grant no. QN2013011), the Programs for Science and Technology Development of the Shaanxi Province, China (grant no. 2013 K02–12), and project funding by the Postdoctoral Science Foundation of Shaanxi Province.

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