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The Interaction of Microbial Symbionts and the Host, *Geocoris pallidipennis*

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Research Article

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ABSTRACT

The interaction of microbial symbionts and *Geocoris pallidipennis* (Costa) was investigated. The study identified the microbial symbionts in the host insect, *G.pallidipennis* by 16S rDNA sequence analyses. The results showed that major symbiotic bacteria in *G. pallidipennis* were *Wolbachia*, *Lactobacillus* and *Uncultured bacterium*. The microbial symbionts in *G. pallidipennis* had significant effects on physiology and development of the host. The developmental times for 1st instar, 5th instar and preoviposition of *G. pallidipennis* in rifampin treatment (removal of microbial symbionts) were significantly longer compared with those of the control. In addition, female weight, average egg production per female each day and egg hatching rate in the treatment (removal of microbial symbionts) were significantly lower than those of the control. The results from current study indicate that the microbial symbionts in *G. pallidipennis* have significantly favorable effects on host physiology and promote the growth and fecundity of *G. pallidipennis*.

INTRODUCTION

Microbial symbionts were special microorganisms that long-term live together with the host. The hosts provide suitable environment and nutrients for symbiotic bacteria [1-4]. The symbiotic bacteria may provide host with essential amino acids and other nutrients by biosynthesis [5,6], the symbiotic bacteria can also help the host to degrade hazardous substances [7,8]. Insect symbiotic bacteria play important roles in the growth, development, food digestion, nutrient absorption and environmental adaptation of host insects [9-12]. In addition, the symbiotic bacteria spread through the cytoplasm of eggs to regulate the growth and ovarian development of the insect host was also reported [13,14].

Wolbachia was one of the most known symbiotic bacteria found in insect hosts [15].The infection rate of *Wolbachia* in insects, mites, crustaceans and nematodes [16,17] is common [18-20] Weinert et al. [21] reported the diversity and incidence of bacterial symbionts in ladybird beetles, and conclude that Rickettsia, *Wolbachia* and *Spiroplasma* were all can be found among ladybird beetles. Kikuchi and Fukatsu (2003) explored the presence of *Wolbachia* in Heteroptera bug and revealed several novel lineages of *Wolbachia*. Pike and Kingcombe (2009) revealed that *Wolbachia* was responsible for a huge variety of reproductive peculiarities. *Wolbachia* in *Drosophila* species also caused cytoplasmic incompatility [22], and in *Aedes albopictus* accelerated cytoplasmic drive [23]. In addition, *Wolbachia* in *D. melanogaster* had significantly favorable effects on survival rate and fecundity [24]. Besides, the reproduction [25] and population conformity [26], the egg and larval survival rate [27, 28] were also enhanced by *Wolbachia*. Because of *Wolbachia*'s ability to alter host reproduction, *Wolbachia* may have potential application in biological control.

Geocoris pallidipennis (Costa) is one of important predaceous insects in Hemiptera, Lygaeidae. It is one of important natural enemies of many insect pests and it can prey on woolly aphids, leafhopper, cotton bollworm eggs and small larvae of Lepidoptera. Zheng and Dong (1996)[29] found out that *G. pallidipennis* is one of the main natural enemies for controlling insect pests in cotton fields. A few characteristics of morphology, biology, ecology and predaceous function of *G. pallidipennis* were reported [30,31].

However, no interaction of *Wolbachia* and *G. pallidipennis* is reported. The goal of the study was to characterize the microbial symbiont community of *G. pallidipennis*, and to assess whether the symbionts might be important for host development and reproduction.

METHODS

16S rDNA sequence analyses: The genome DNA was isolated from *G. pallidipennis* by TIANamp Genomic DNA Kit (Tiangen®, Beijing, China) and used as template. The high fidelity Taq polymerase (TransGen Biotech, Beijing, China) was employed for the following PCR. The primers were 27 F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 R (5'-TACGGTTACCTTGTTACGACTT-3') [32] (synthetic by Biological engineering of Beijing, China). Each reaction was run for 30 cycles. Reaction in each cycle was denaturation at 94°C for 30 s, annealing at 51.3°C for 45 s and extension at 72°C for 2 min (10 min extension for the last cycle). The products of PCR were detected by 1% gel electrophoresis and cloned into PMD19-T vector (Takala, Dalian, China) to construct recombinant vector. The positive clones identified by PCR verification were sequenced into DH5a (TransGen Biotech, Beijing, China) competent cells after electrotransformation. The plasmid of positive clones was extracted by TIANprep Mini Plasmid Kit (Tiangen®, Beijing, China), and analyzed by restriction enzymes MspI (Tiangen®, Beijing, China). The enzyme system includes the following: plasmid 4 µl, 10× T Buffer 2 µl, BSA 2 µl, MspI 1 µl, ddH2O 11 µl. The enzyme reaction was carried out at 37°C for 16 h. The digest products were separated by electrophoresis on a 2% gel.

Host insects, the treatment and sample preparation: The host insect colony of *G. pallidipennis* was established in the laboratory in 2010 with individuals originally collected near Xiaoyue River, in Beijing. *G. pallidipennis* was reared on artificial diet [34]. The new hatched *G. pallidipennis* nymphs on the same day were used for the study reared on artificial diet at (25 ± 1)°C with a 16:8 (L: D) photoperiod under (70 ± 5)% RH in a climatic incubator. The treatment was 0.3mg/ml rifampin [35] (Jiangchen, Beijing, China) added to artificial diet and mixed well to remove microbial symbionts in *G. pallidipennis*, and artificial diet without antibiotics was used as a control. The *G. pallidipennis* were continuous feed by the treatment and the control over two generations. And then, five of 4th instar nymphs were collected and extracted genome DNA. The genome DNA was used as a template, the primers were *WSP* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and 691R (5'-AAAAATTAAACGCTA CTCCA-3') [36,37] for PCR amplification, each reaction was run for 30 cycles. Reaction in each cycle was denaturation at 94°C for 30 s, annealing at 44.6°C for 45 s and extension at 72°C for 2 min (7 min extension for the last cycle). The products of PCR were detected by 1% agarose gel electrophoresis.

Effects of microbial symbionts on host growth: For detection of the effects of microbial symbionts on the growth of *G. pallidipennis*, the host insects from the treatment or control described above over two generations were allowed to produce offspring, the newly hatched nymphs were placed individually in cylindrical Plexiglas tube (2 cm diameter, 4 cm height). One end of the tube was sealed with cotton and the other end was filled with artificial diet. The newly hatched nymphs in the same time was collected and checked at 6 pm every day for the development time (days) until adults. The development time for each instar was recorded, and the preoviposition time for adults was also recorded. All experiment insects was reared at (25 ± 1)°C with a 16:8 (L:D) photoperiod under (70 ± 5)% RH in a climatic incubator. The experiment designed was completely randomized with 30 replicates for the treatment and the control, respectively. The following data were assessed: developmental time from egg to adult; preoviposition time; weights of newly emerged adults.

Effects of microbial symbionts on host reproduction: For investigation of the effects of microbial symbionts on egg production of *G. pallidipennis*, the host insects from the treatment or control described above over two generations were allowed to produce offspring, the newly emerged adults were weighed using a Mettler microbalance (Saiduolisi, Germany) (0.1 mg sensitivity), one male and one female adult were then paired and reared together in a plastic dishes (9 cm diameter, 2 cm height) on the artificial diet. The eggs per female laid each day during the first two weeks were recorded. The experiment to examine the effects on the reproduction consisted of 24 replications for the treatment and control. For investigation of the effects of microbial symbionts on egg hatching rate of *G. pallidipennis*, the twenty eggs of the treatment and control were collected separately and the hatching rate was recorded, the experiment was completely randomized design with three replications.

Statistical analysis: Differences in development time of eggs and nymphs, adult weights, preoviposition time, egg production and hatching rate were analyzed using t-test with significance level set at p=0.05. And the means were separated using Duncan's Multiple Range Test with SAS (2004) [38].

RESULTS

16S rDNA sequence analyses to identify microbial symbionts: The 16S rDNA sequence analyses were conducted and the target fragment of 1500 bp as anticipated was obtained **(Figure 1)**. One hundred positive clones were identified and sequenced. The results of those positive clones plasmid digested with MspI showed that there were three kinds of symbiotic bacteria in *G. pallidipennis*. The three symbionts identified in *G. pallidipennis* were *Wolbachia*, *Lactobacillus* and *Uncultured bacterium*. The basic local alignment search (Blast) demonstrated that there were 49 cloned sequences which were 98% homologous with *Wolbachia*; 22 cloned sequences those were 99% homologous with *uncultured bacterium*, and 29 cloned sequences those were 100% homologous with *Lactobacillus* **(Table 1)**.

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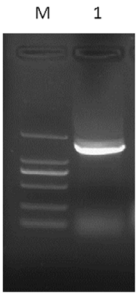

Figure 1. The PCR products of microbial symbiotes amplified. M: Molecular Markers DL 2000; 1: genome DNA of microbial symbiotes amplified.

Table 1. Identification and sequence analysis of the positive cloning.

Operating units	Reference bacterium	Register	Homologous	Percentage
M-1	*Uncultured bacterium*	JF429312.1	99%	22%
M-2	*Wolbachia*	HE583204.1	98%	49%
M-3	*Lactobacillus*	NR_075042.1	100%	29%

Removal of *Wolbachia* in *G. pallidipennis*: The existence of *Wolbachia* in *G. pallidipennis* over two generations after antibiotic treatment was determined by the *wsp* gene amplified method. The target fragment was found in both antibiotic treatment and control for the first-generation of *G. pallidipennis* **(Figure 2A)**, but at second generation, no *Wolbachia* could be detected in the antibiotic treatment **(Figures 2B and 2C)**. The elimination of *Wolbachia* from *G. pallidepennis* was successfully achieved through continuous feed rifampin over two generations.

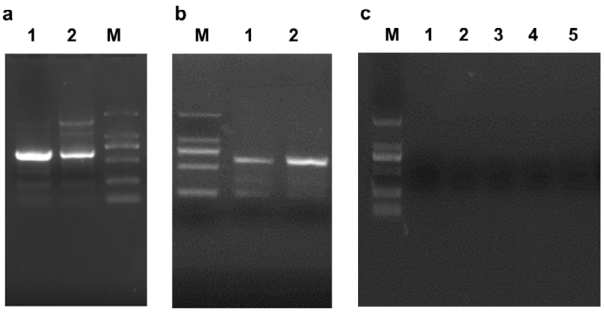

Figure 2. The PCR products of *Geocoris pallidipennis* genome DNA samples amplified by *wsp* gene (1: control (symbiosis); 2: treatment (aposymbiosis); M: Molecular Markers DL 2000). a: The PCR products of the first-generation, 1: control (symbiosis); 2: treatment (aposymbiosis). b: The PCR products of the second generation; 1-2: control (symbiosis); c: The PCR products of the second generation; 1-5: treatment (aposymbiosis).

Effects of microbial symbionts on host development: The effects of microbial symbionts on the development of *G. pallidepennis* were determined. The growth of *G. pallidepennis* was retarded after removing of microbial symbionts. The development times (days) for the treated insects were significantly longer (eggs: F=96.44; df=1, 58; P<0.0001; first instar: F=6.28; df=1, 54; P=0.0152; and fifth instar: F=8.12; df=1, 49; P=0.0064) compared with those of the control. Similarly, the preoviposition period (days) for the treated insects was also significantly longer than that of the control (F=6.49; df=1, 23; P=0.0180) **(Table 2)**.

Table 2. Developmental times (days) of *Geocoris pallidipennis* in treatment and control.

Developmental stages	Control	Treatment
Egg	10.1d ± 0.14a	13.93d ± 0.36b
1st instar	8.09d ± 0.14a	8.97d ± 0.28b
2st instar	7.16d ± 0.21a	7.18d ± 0.21a
3st instar	7.09d ± 0.19a	7.2d ± 0.21a
4st instar	6.76d ± 0.19a	7.38d ± 0.32a
5st instar	8.52d ± 0.16a	9.25d ± 0.20b
Preoviposition	6.92d ± 0.35a	8.54d ± 0.51b
Data are means ± standard error. Lower-case letters indicated significant difference at P<0.05.		

Effects of microbial symbionts on host reproduction: The effects of microbial symbionts on the egg production per female were determined. Average egg production per day for a *G. pallidepennis* female showed two peaks on both treated and control artificial diet. The peaks of egg production were on the fifth and tenth days, the days of lowest egg production were first and seventh. The trend of the first two week in average egg production was basically identical. The average egg production for treated insects in the first weeks was not significantly different from that of the control (F=0.81; df=1,12; P=0.6809). However, the average egg production per female for the treated insects was significantly lower than that of the control in the second week

(*F*=6.41, *df*=1,12; *P*=0.0264) **(Figure 3)**. Furthermore, the hatching rate of eggs of treated insects was significantly lower than that of control insects (*F*=64, *df*=1,4; *P*=0.0013) **(Figure 4)**.

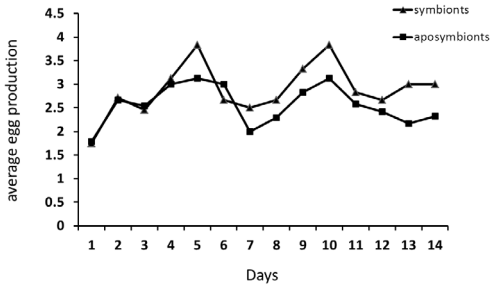

Figure 3. The average egg production per female of *Geocoris pallidipennis* each day (treatment (aposymbiosis) vs control (symbiosis)).

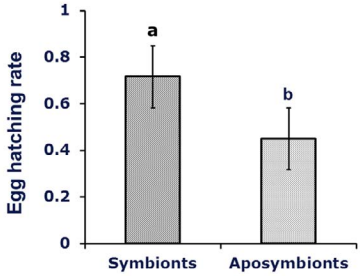

Figure 4. The egg hatching rate of *Geocoris pallidipennis* with treatment (aposymbiosis) and control (symbiosis). Error bars represent SE. Bars with the different letter are significantly different (*P*<0.05).

Effects of microbial symbionts on host weight: Male adult weight (2.14 mg) of *G. pallidipennis* treated with antibiotic was not significantly different from that (2.64 mg) of control (*F*=3.9; *df*=1,17; *P*=0.0648). However, the average female adult weight (2.33 mg) of *G. pallidipennis* treated with antibiotic was significantly different from that (3.07 mg) of control (*F*=23.5; *df*=1,24; *P*<0.0001) **(Figure 5)**.

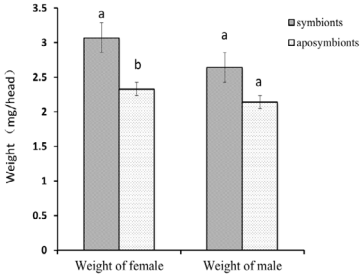

Figure 5. Weights of females and males of *Geocoris pallidipennis* in treatment (aposymbiosis) vs those in control (symbiosis). Error bars represent SE. Bars with the different letter are significantly different (*P*<0.05), according to Duncan's Multiple Range Test.

DISCUSSION

In generally, the removal of the *Wolbachia* and other microbial symbionts in insects can be achieved through antibiotic diet [34] and heat treatment over 30°C [39,40]. Pike et al. (2009) [41] had studied antibiotic treatment to eliminate *Wolbachia* and lead to sterility in the *Folsomia* candida. Gibson et al. (2005) [42] studied the role of yeasts associated with green lacewings using the methods of fungicides and heat treatment but unsuccessful. In this study, *G. pallidipennis* was treated by rifampin at concentration 0.3 mg/ml in order to remove *Wolbachia* and other microbial symbionts in *G. pallidipennis*. The results showed that this concentration did effectively remove *Wolbachia* infection without producing toxin to *G. pallidipennis*.

The *Wolbachia* has favorable effects on host development therefore inhibition or removal of the *Wolbachia* from host can have adverse effects on the host. Dong [26] found *Wolbachia* infections in *Liposcelis tricolor* decreased development periods and increase survivorship in some immature life stages. Effects of *Wolbachia* have been studied in some predatory bugs, such as *Wolbachia* in *Macrolophus pygmaeus* and *Orius strigicollis* [43,44]. The current study also showed that the *G. pallidipennis* without *Wolbachia* attained longer development time than the control. Similar results were reported by Hu et al. [41] and Lu et al., [43] they found that the development time of nymphs and life span of *Sitobion avenae* and *Nilaparvata ugens* stal were longer in rifampin treated group than those in control group. Furthermore, the weight of female of *G. pallidipennis* was dropped significantly after removal of *Wolbachia* and other microbial symbionts, and the average egg production per female per day also declined significantly on the second week in the treated insects. The results were similar to those symbionts found in parasitoid *wasp* [47] and *Wolbachia* in *D. melanogaster* [24]. The data from current study demonstrated that the growth and egg production were

affected by the removal of *Wolbachia* and other microbial symbionts in *G. pallidipennis*. The development and egg production of *G. pallidipennis* need a great deal of nutrients, the symbiotic bacteria may provide necessary nutrients to speed the host growth and production. However, it is necessary to conduct further research to determine the specific nutritious those *Wolbachia* or other microbial symbionts can provide for *G. pallidipennis*.

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