Influence of Temperature on Symbiotic Bacterium Composition in Successive Generations of Egg Parasitoid, *Anagrus nilaparvatae*

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**Abstract:** *Anagrus nilaparvatae* is the dominant egg parasitoid of rice planthoppers and plays an important role in biological control. Symbiotic bacteria can significantly influence the development, survival, reproduction and population differentiation of their hosts. To study the influence of temperature on symbiotic bacterial composition in the successive generations of *A. nilaparvatae*, *A. nilaparvatae* were raised under different constant temperatures of 22 °C, 25 °C, 28 °C, 31 °C and 34 °C. Polymerase chain reaction-denaturing gradient gel electrophoresis was used to investigate the diversity of symbiotic bacteria. Our results revealed that the endophytic bacteria of *A. nilaparvatae* were Pantoea sp., Pseudomonas sp. and some uncultured bacteria. The bacterial community composition in *A. nilaparvatae* significantly varied among different temperatures and generations, which might be partially caused by temperature, feeding behavior and the physical changes of hosts. However, the analysis of *wsp* gene showed that the *Wolbachia* in *A. nilaparvatae* belonged to group A, sub-group Mors and sub-group Dro. Sub-group Mors was absolutely dominant, and this *Wolbachia* composition remained stable in different temperatures and generations, except for the 3rd generation under 34 °C during which sub-group Dro became the dominant *Wolbachia*. The above results suggest that the continuous high temperature of 34 °C can influence the *Wolbachia* community composition in *A. nilaparvatae*.

**Key words:** temperature; *Anagrus nilaparvatae*; *Wolbachia*; bacterial community; polymerase chain reaction-denaturing gradient gel electrophoresis; rice; brown planthopper

*Anagrus nilaparvatae* (Pang and Wang) (Hymenoptera: Mymaridae) is the dominant egg parasitoid of rice planthoppers and leafhoppers in Asian rice growing countries. It plays an important role in enhancing biological control of brown planthopper (BPH) *Nilaparvata lugens* (Stål) (Yu et al, 2001; Mao et al, 2002; Lou et al, 2005; Gurr et al, 2011). Temperature is a critical environmental factor significantly affecting insect development, survival, lifespan, fecundity and intrinsic increase rate (Zhu et al, 1991; Zhuo et al, 1992; Liu et al, 2012). The optimum growth temperature for *A. nilaparvatae* population is 27 °C (Chiappini and Lin, 1998). When cultured with the eggs of BPH at high temperature, both the fecundity and survival of *A. nilaparvatae* at the immature stages are greatly reduced, and their emergence rate is cumulatively declined (Chiappini and Lin, 1998; Liu et al, 2012). Therefore, we have to understand the influence of temperature on the natural enemies of planthoppers, which is important for maximizing biological control during global warming (Bottrell and Schoenly, 2012). Parasitic wasps harbor numerous bacterial endosymbionts which interact with their hosts during
the long-term co-evolution. These interactions range from parasitism to mutualism (Bandi et al, 1999; Kaltenpoth et al, 2005; Copeland et al, 2008). Wolbachia are one of the most important bacterial endosymbionts in arthropods and cytoplasmically inherited bacteria widely existing in the reproductive tissues of arthropods. Wolbachia can significantly influence the development, survival, reproduction and population differentiation of their hosts (de Almeida, 2004, 2010; Vasquez et al, 2011). Temperature can cause great variations in cytoplasmic incompatibility, parthenogenesis inducing, genetic male feminization and male killing (Cook and Butcher, 1999; Liu et al, 2011). Temperature can cause great variations in Wolbachia populations which will then influence the ecological and physiological characteristics of their hosts (Pintureau et al, 2003; Xiang et al, 2006). The optimum temperature for the development of Wolbachia-infected Trichogramma cacoeciae ranges from 24 °C to 28 °C. The growth of Wolbachia is inhibited under 30 °C while the expression of Wolbachia wsp gene turns negative after four generations (Xiang et al, 2006). However, little is known about the dynamics of bacterial community (including Wolbachia) in A. nilaparvatae, and the impact of temperature on the composition and state of bacterial community.

Challenges are faced in investigating the dynamics of bacterial endosymbionts because the majority of these bacteria have not been cultured outside their host cells. Fortunately, some new molecular methods can effectively analyze the bacterial community in diverse samples (Muyzer et al, 2004). The current study investigated the potential impacts of temperature on the endosymbionts in A. nilaparvatae through analyzing the bacterial community in the successive generations of A. nilaparvatae cultured under different temperatures.

**MATERIALS AND METHODS**

**Rearing of A. nilaparvatae at different temperatures**

BPH adults were collected from paddy fields at the China National Rice Research Institute, Hangzhou, China. BPH population was successively maintained on susceptible TN1 rice plants. The egg parasitoid A. nilaparvatae were trapped by exposing the potted 45-day-old TN1 rice plants carrying 24-hour-old BPH eggs to paddy fields for 3 d. The parasitized eggs (red color) were dissected under microscope, and the parasitoids were cultured in thimble tubes with wet filter paper. The newly emerged parasitoid adults (8-hour-old) were identified according to the method described by Yu et al (2001) and were continuously fed with BPH eggs by infesting with gravid BPH females for two generations at (26 ± 1) °C.

Five constant temperatures used in climate chambers were 22 °C, 25 °C, 28 °C, 31 °C and 34 °C. Forty-five-day old TN1 rice plants were trimmed and covered individually by mylar cages, and kept in climate chambers. Five gravid BPH females were infested into each cage for oviposition, and five pairs of 8-hour-old parasitoid A. nilaparvatae were also introduced into the same cage. When newly hatched BPH nymphs were observed, the mylar cages were replaced by clean ones with black jackets whose tapering top were attached with an inverted screw vials. The newly emerged A. nilaparvatae adults were attracted by light and then moved to glass vials. They were collected in 8 h intervals and sexually identified. Half of them were frozen at -72 °C for genomic DNA extraction, and the rest were continuously reared with BPH eggs under the previous temperature for four successive generations.

**Genomic DNA extraction**

The frozen adults of A. nilaparvatae were taken out from the refrigerator, and thawed on a clean bench at room temperature for 30 min. The parasitoid body surface were sterilized in 70% ethanol for 5 min and rinsed in sterile water for five times.

The genomic DNA of A. nilaparvatae was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Germany). The pretreated samples of A. nilaparvatae were transferred into new sterile 2 mL Eppendorf tubes, and then 180 µL DNeasy ATL buffer was added. The wasps were homogenized with sterile glass rod until their bodies and tissues were invisible by naked eye. The mixtures were treated with 2 mg/mL lysozyme (Sangon, China) at 37 °C for 2 h and then incubated with Proteinase K (Qiagen, Germany) at 56 °C overnight. All subsequent steps were performed according to the manufacturers’ instructions. The DNA extracts were quantified using A NanoDrop-2000 spectrophotometer (Thermo, USA) and stored at -20 °C.
Amplification of V3 region of 16S rDNA gene and wsp gene fragments

The total DNAs extracted from the successive generations of *A. nilaparvatae* cultured under different temperatures were used as templates for polymerase chain reaction (PCR). The V3 regions of bacterial 16S rDNA gene were amplified by PCR to analyze bacterial community change. wsp gene fragments were used for *Wolbachia* screening. For denatured gradient gel electrophoresis (DGGE), V3 regions were amplified with the primers 341F-GC (the complement of EUB341 with a 40-bp GC clamp, 5'-CGCCCGCCGCCGCGGGGCGGGGCGGGGGCACGGGGG
GCCTACGGGAGGCAGCAG-3') and 517R (the complement of EUB517, 5'-ATTACCGCGGCTGCTTGG-3') (Muyzer et al, 1993). A ‘touchdown PCR’ (Don et al, 1991) was performed according to the protocol described by Riemann et al (1999). The fragments of wsp gene were amplified with the primers 81F (5'-TGGTCCAATAAGTGTATGAAGAAC-3') (Braig et al, 1998) and 691R (5'-AAAGGGAGACTGATGATGT-3') (Zhou et al, 1998). A 40-bp GC-clamp was also attached to the 5'-end of the primer 81F. The PCR mixtures (25 μL) contained the genomic DNA template (10–20 ng), 200 nmol/L each primer, 200 nmol/L each dNTP (TransGen, China) and 1 U TransTaq-T DNA polymerase (TransGen, China) in 1 × TransTaq-T buffer (TransGen, China). The amplification conditions were 94 °C for 4 min; 31 cycles of 94 °C for 1 min, 51 °C for 45 s, and 72 °C for 50 s; and 72 °C for 10 min. PCR products were separated by 1% agarose gel electrophoresis and detected by ethidium bromide staining using molecular mass standards and the Quantity One-1-D software (Bio-Rad, USA).

Analysis of 16S rDNA and wsp gene fragments

Bio-Rad Dcode was used for the DGGE analysis on the PCR products of V3 region and wsp gene fragments. The 8% polyacrylamide gel with the denaturant gradients from 40% to 60% (100% is defined as 7 mol/L urea and 40% formamide) was used to analyze the V3 region PCR products. And the 6% polyacrylamide gel with the denaturant gradients from 20% to 40% was used to analyze the PCR products of wsp gene fragments. Equal amounts (600 ng per well) of PCR products were loaded on6% or 8% polyacrylamide gels. DGGE was performed in 1 × Tris acetic acid EDTA buffer (20 mmol/L Tris, 10 mmol/L acetate and 0.5 mmol/L EDTA, pH 8.0) at 60 °C, 120 V for 6 h. After electrophoresis, gels were stained with the 1 × Tris-acetic acid EDTA buffer containing ethidium bromide (0.5 mg/L) for 20 min, destained in distilled water for 15 min, and photographed with Tannon-2500 (Tannon, China). The PCR-DGGE replicates from the same DNA sample generated an identical pattern.

Recovering, cloning and sequencing of DGGE bands

The dominant bands were excised from DGGE gels, transferred to Eppendorf tubes and washed with sterilized water for five times. DNA was eluted in 30 μL Tris-EDTA buffer at 4 °C overnight. Then, 16S rDNA or wsp fragments were re-amplified from the excised bands and analyzed by a second DGGE to ensure that the aimed bands were resolved. PCR products were purified using the AxyPrep PCR purification kit (Axygen, USA) and ligated with PMD18-T vector (Takara, Japan). The ligation products were transformed into *Escherichia coli* DH5α competent cells, and the transformants containing DNA inserts were sent to Sangon (Shanghai, China) for sequencing. Sequences were analyzed with the NCBI BLASTn program (http://www.ncbi.nlm.nih.gov/), and a phylogenetic tree was constructed using the MEGA 4.1 software.

DGGE profile analysis

DGGE profiles were analyzed using the Quantity One-1-D software (Bio-Rad, USA) to measure the positions and intensities of individual bands. The background fluorescence of DGGE profiles were subtracted from each lane, and then band intensities were normalized to the total intensity of all bands in a given lane to calculate relative band intensities. Different lanes and their relevant band intensities composed a matrix, and principal component analysis was conducted in Matrix Laboratory, which could reveal the change of bacterial community in the successive generations of *A. nilaparvatae* under different temperatures.

RESULTS

Bacterial community structure in successive generations of *A. nilaparvatae*

The host *A. nilaparvatae* could not complete their life cycle at high temperature of 34 °C after the 3rd generation. Our results showed that the main bacterial
populations were similar, but the dominant strains were significantly different in various temperatures and generations (Fig. 1). After being analyzed and normalized, two principal components expressed 65.0% of the total variability in bacterial community structure. The bacterial communities changed in different generations under different temperatures, and these changes were much more significant under high temperatures, such as 31 °C and 34 °C. However, there was no significant regularity in the change of bacterial community structure in the successive generations of *A. nilaparvatae* under different temperatures (Fig. 2). Thirteen main bands were recovered and the DNA sequences were amplified by PCR, and were then cloned and sequenced (Fig. 1). The bacteria with bands 1, 10 and 11 were the main strains in all the generations of *A. nilaparvatae* under different temperatures, and were the dominant ones in some *A. nilaparvatae* samples.

The DNA sequences were aligned with the closely related 16S rDNA sequences from GenBank, and a phylogenetic tree was constructed (Fig. 3). The bacteria with bands 1, 10 and 11 belonged to uncultured bacterium, *Pantoea* sp. and *Pseudomonas* sp., respectively. The bacteria with other bands belonged to *Bacillus* sp., *Brevundimonas* sp., *Delftia* sp., *Xanthomonas* sp. and *Acinetobacter* sp.

**Changes of Wolbachia in successive generations of *A. nilaparvatae***

The *wsp* gene fragments were amplified with the primers 81F (with a GC-clamp attached to the 5’-end) and 691R, and then analyzed by PCR-DGGE. *Wolbachia* were detected in all the generations of *A. nilaparvatae* under different temperatures. The DGGE profiles showed an absolutely dominant band and a very weak band in all the samples except the 4th generation wasps under 34 °C which showed two distinct bands (Fig. 4). The bands were recovered, while the DNA fragments were cloned and sequenced. After the bands were aligned with closely related *wsp* gene sequences from GenBank, a phylogenetic tree was constructed using a neighbor-joining method (Fig. 5). The results showed that the *Wolbachia* in *A. nilaparvatae* belonged to group A, sub-group Mors and sub-group Dro. In addition, the *Wolbachia* of...
sub-group Mors was absolutely dominant under 34 °C until the 3rd generation after which the dominant Wolbachia changed to sub-group Dro.

**DISCUSSION**

The microbes harbored in insect guts have developed different interactions with their host during the long-term co-evolution (Dillon and Dillon, 2004). They play key roles in insect nutrition, colonization, resistance against exotic microbe invasion, multitrophic interactions between host and other biological factors and enhancement of host immune response (Oliver et al, 2005; Xiang and Huang, 2008). Some bacteria are indispensable for the development and reproduction of their host insects (Brummel et al, 2004). Microbial diversity and community structure are the foundation of their ecological functions. The environmental factors directly or indirectly influencing insect gut microbes also affect the ecological and physiological characteristics of host insects. Undoubtedly, understanding the mechanisms underlying the influences of environmental factors on insect gut microbes has important significance for insect biological control. The bacteria in insect gut are sensitive to environment changes, such as the change of diet. They can directly cause the variation of gut microbes. The physiological characteristics of insects can adjust to environmental variation which indirectly influences gut bacterial composition. A recent study discovers that adult oriental fruit flies (Bactrocera dorsalis) harbor diverse microorganisms, and a few species serve as the major components. However, different surroundings and diets can significantly influence bacterial composition (Wang et al, 2011). The ecological influence caused by global warming has increasingly drawn public attention (Walther et al, 2002). In the organisms engaged in mutualisms, high temperature may directly or indirectly influence their population performance through the mediated effects on their mutualists (Prado et al, 2010).

It is well known that temperature can influence the ecological and physiological characteristics of insects.

**Fig. 3.** Phylogenetic tree of bacteria associated with generations of A. nilaparvatae cultured under different temperatures.

Distances were calculated using the maximum-likelihood method, and the tree was constructed using neighbor-joining method.

**Fig. 4.** Denatured gradient gel electrophoresis (DGGE) profile of wsp gene fragments of Wolbachia in generations of A. nilaparvatae cultured under different temperatures.

Distances were calculated using the maximum-likelihood method, and the tree was constructed using neighbor-joining method.

**Fig. 5.** Phylogenetic tree of wsp gene of Wolbachia in generations of A. nilaparvatae cultured under different temperatures.
(Zhu et al, 1991; Cui et al, 2007; Anbutsu et al, 2008; Zhou et al, 2009; Prado et al, 2010). The change of physiological condition in host A. nilaparvatae may influence their symbiotic bacteria. In current study, A. nilaparvatae harbored several bacteria. The main bacterial species were similar under different constant temperatures. However, the structure of bacterial community differed significantly. The dominant bacterial species in different generations of A. nilaparvatae were stable under low temperatures (below 28 °C), but were significantly changed under high temperatures, 31 °C and 34 °C. It indicates that high temperature can influence the bacterial community in A. nilaparvatae.

Wolbachia may be the most abundant and widespread parasitic bacteria in the nature world. It has much higher infection efficiency than other insect species in parasitic wasps (Werren et al, 1995). Vertical transmission is the dominant pattern for transferring to host generations (Huigens et al, 2004; Serbus et al, 2008). A recent study reports that the Wolbachia density in host is influenced by temperature, host age and nutritional status (Uncleek et al, 2009). In current study, Wolbachia infections were almost the same in A. nilaparvatae under different temperatures, which suggests that the setting temperature has no significant influence on Wolbachia in A. nilaparvatae. Another previous study reports a similar result (Cui et al, 2007). Although high temperature (38 °C) has a negative influence on Wolbachia infected Trichogramma dendrolimi, the short time under high temperature cannot influence the action of Wolbachia (Cui et al, 2007).

Wolbachia remained stable in different temperatures and generations, except for the 4th generation during which the dominant Wolbachia changed to sub-group Dro. It has been reported that continuous high temperature can influence the Wolbachia density in insects. A study on Wolbachia-infected Encarsia formosa discovers that the continuous high temperature of 31 °C can influence Wolbachia’s control on host’s reproduction pattern (Zhou et al, 2009). Stouthamer et al (1990) discovered that the activity of Wolbachia is inhibited by the continuous temperature above 30 °C, which makes Trichogramma wasps become bisexual. Another study reported that the Wolbachia density in mosquito is significantly decreased by exposure to the high temperature of 37 °C (Wiwatanaratanabantr and Kittayapong, 2009). In our experiments, A. nilaparvatae population could not continue after three generations under the temperature of 34 °C, and the changes of Wolbachia in A. nilaparvatae was undiscovered. However, our results suggest that the temperature of 34 °C has continuous influence on the Wolbachia population in A. nilaparvatae. The density of each Wolbachia strain was changed in the 4th generation of A. nilaparvatae under the temperature of 34 °C. Moreover, the change of sub-group Maros to sub-group Dro in Wolbachia at high temperatures may be responsible for the death of A. nilaparvatae. Further works should be done to provide additional information on the ecological fitness of different Wolbachia species corresponding to different environmental factors, and the competitive relationship between Wolbachia and other endosymbionts in wasps, which are important for understanding the impacts of Wolbachia shift on the population dynamics of A. nilaparvatae and the parasitism of planthopper eggs.

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