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研究报告

Bacteria associated with salivary glands of cicada *Hyalessa maculaticollis* (Motschulsky) (Hemiptera: Cicadidae)

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Abstract: [Objective] This paper aims to clarify the bacteria associated with the salivary glands of cicada *Hyalessa maculaticollis* (Motschulsky), and to address if the endosymbiont *Candidatus* Sulcia muelleri occurrs in the salivary glands. **[Methods]** We investigated the bacterial community in the salivary glands of cicada *H. maculaticollis* using 16S rRNA restriction fragment-length polymorphism (RFLP). **[Results]** We identified seven bacteria in the salivary glands, which belong to the Phyla Proteobacteria and Firmicutes. The bacterial community is dominated by *Pseudomonas aeruginosa* and *Enterobacter* sp., each of them accounted for 48.7% in the clone library. In total, the other five bacterial species (*Thermomonas brevis*, *Sphingomonas* sp., *Methyloversatilis* sp., *Anaerococcus* sp. and *Bacillus* sp.) together accounted for 2.05% in the clone library. **[Conclusion]** The results show that the bacterial species composition in the cicada salivary glands of *H. maculaticollis* was relatively simple, which were dominated by two bacterial species. The endosymbiont *Candidatus* Sulcia muelleri of Auchenorrhyncha, formerly detected from bacteriomes of related auchenorrhynchan hosts, were not detected in the salivary glands of *H. maculaticollis*. The bacteria harbored in salivary glands of cicadas probably play an important role in both food ingestion and interactions between the cicadas and their host plants. However, whether the related bacteria are commonly harbored in other cicadas and their certain function need to be further investigated.

Keywords: *Hyalessa maculaticollis*, Salivary glands, Microbiota, *Pseudomonas aeruginosa*, *Enterobacter*

斑透翅蝉唾液腺细菌群落组成研究

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摘 要:【目的】为明晰蝉类昆虫唾液腺中细菌的组成及其中是否存在内共生菌 *Candidatus* Sulcia muelleri。【方法】以斑透翅蝉 *Hyalessa maculaticollis* (Motschulsky)为材料，采用 16S rRNA 限制性内切酶片段长度多态性(RFLP)对其唾液腺细菌群落组成进行分析。【结果】斑透翅蝉唾液腺中

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共存在 7 种细菌，分别属于变形菌门和厚壁菌门；其中绿脓假单胞杆菌 *Pseudomonas aeruginosa* 和肠杆菌 *Enterobacter* sp.为优势细菌，分别占克隆总数的 48.7%；另外 5 种细菌(反硝化细菌热单胞菌 *Thermomonas brevis*、鞘胺醇单胞菌 *Sphingomonas* sp.、芽孢杆菌 *Bacillus* sp.、厌氧球菌 *Anaerococcus* sp.和 *Methyloversatilis* sp.)总共占克隆文库的 2.05%。【结论】首次采用分子生物学方法明晰斑透翅蝉唾液腺的细菌群落；其细菌群落组成相对简单，且两种细菌占主导地位；此外，头喙亚目昆虫体内特有的内共生菌 *Candidatus* Sulcia muelleri 未在斑透翅蝉唾液腺中检测出，表明该共细菌可能仅在腹部的贮菌体中分布；斑透翅蝉唾液腺中的细菌是否普遍存在于蝉科昆虫唾液腺中以及在取食韧皮部汁液过程中的功能有待进一步研究。

关键词: 斑透翅蝉，唾液腺，细菌群落，绿脓假单胞杆菌，肠杆菌属

1 Introduction

The salivary glands of insects are paired structures and immersed in haemolymph inside the head and thorax[1-2]. Researches on insect salivary glands have been involved in many aspects, including its structure, functions, saliva composition and properties, etc[3-6]. Saliva of insects contains a mixture of amino acid, protein, and many digestive enzymes, which plays an important role in both food ingestion and interactions between herbivores and their host plants[7-8]. Salivary glands of the Hemiptera are the most complex glands among insect groups, which are closely associated with the peculiar pierce-sucking feeding mode[9-10]. Regarding phytophagous hemipterans subsisting exclusively on plant xylem or phloem sap, the saliva plays an important physiochemical role during the mechanical penetration of plant tissues by the piercing-sucking mouthparts, and the saliva may vary in its chemical composition and physical consistency from one moment to the next to accomplish penetration[11-13].

Moreover, the salivary glands of some hemipteran insects are also one of the sites harboring microorganisms[14]. For example, the endosymbionts *Candidatus* Cardinium hertigii and *Candidatus* Phytoplasma vitis of the '*Bacteroidetes*' were found in the salivary glands of the leafhopper *Scaphoideus titanus*[15]. Acetic acid bacteria (AAB), especially members of the genera *Acetobacter* and *Gluconacetobacter*, and the versatile acetic acid bacterial symbiont *Asaia* sp., were reported in the salivary glands of *S. titanus* as well[16]. Coccoid or rod-shaped bacteria were consistently found in the salivary glands of planthopper *Pentastiridius leporinus* (L.)[17]. The endosymbionts *Candidatus* Liberibacter asiaticus has been densely aggregated within the cells of salivary glands of psyllid *Diaphorina citri* Kuwayama, which is the insect vector of citrus huanglongbing disease[18]. Facultative secondary symbionts *Rickettsia* were found in the salivary glands of whitefly *Bemisia tabaci* (Gennadius)[19]. Two groups of betaproteobacterial endosymbionts were reported in the salivary glands of aphid *Adelges tsugae* Annand[20]. Athough the microbial community in salivary glands of hemipteran insects may play potential importance for their hosts, few data on the complexity of this biota are available.

Like other sap-feeding hemipterans, cicadas habitat obligate vertically transmitted bacterial symbionts *Candidatus* Sulcia muelleri in their bacteriomes to supply the host with nutrients that are limiting in their diet[21-22], but it is unknown if the endosymbionts and/or other bacteria were distributed in their salivary glands. Zhong et al[23] and Zhong et al[24] studied the ultrastructure of salivary glands in cicadas *K. caelatata* Distant and *Platypleura kaempferi* Fabricius, respectively, and rod-shaped microorganisms were unexpectedly observed in *K. caelatata*, but no microorganisms were observed in that of *P. kaempferi*. However, the identity of the microorganisms residing in the salivary glands of *K. caelatata* remains unknown[23].

Considering information on the bacteria associated with cicada salivary glands was very limited, we investigated the bacterial communities in the salivary glands of a common cicada, *Hyalessa maculaticollis* (Motschulsky), in China using 16S rRNA-RFLP analysis, for the specific purpose of clarifying the occurrence of bacteria and/or special endosymbionts and their community in this special microenvironment. The results may be formative for further understanding the microbes in the salivary

glands of cicadas and their potential roles in cicadas' nutrition and feeding behavior.

2 Materials and Methods

2.1 Cicada collection and dissection

Three male and three female adults of the cicada *H. maculaticollis* were collected from the host plants *Salix babylonica* in Mts. Taibai (Meixian County, Shaanxi, China) in August of 2012. The cicadas were put into a voile-cage and transferred alive to the lab for dissection as soon as possible.

Before dissection, the cicadas were first narcotized by putting in 4 °C for a few minutes, and then the body were externally sterilized with 75% ethanol and rinsed with sterile water for several times. Cicadas were fixed on a wax-plate with sterilized 4# insect needles, and the cuticula were cut off along the back middle line from the anus to the head with sterilized 10 cm scissors under a Stereoscopic Zoom Microscope (Motic SMZ168, Xiamen, China). The salivary glands were carefully separated from other organs with sterilized fine 14 cm forceps, washed three times with sterilized water to reduce the contamination, and then put into a 1.5 mL microcentrifuge tube for DNA extraction. All works were done in the laminar flow cabinet (Taisite, Tianjin, China).

2.2 DNA extraction and PCR amplification

Genomic DNA was extracted from the pooled salivary glands tissue by using TIANamp Genomic DNA kit (TIANGEN Inc.) according to the manufacturer's directions. 16S rRNA gene was amplified from genomic DNA extracted from salivary glands of *H. maculaticollis* with paired primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TAGGGYTACCTT GTTACGACTT-3′). PCR was performed using a Peltier Thermal Cycler (Eppendorf, Germany). The amplification was performed in 25 μL PCR reactions system, containing: 1 μL Temple DNA, 2.5 μL of 10×PCR buffer, 1.5 μL of $MgCl_2$ (25 mmol/L), 2 μL of dNTP Mix (10 mmol/L), 1 μL of each primer (10 mmol/L), 0.25 μL of *Taq* DNA polymerase (5 U/μL, TaKaRa Bio. Inc.), and adding sterile ddH_2O to 25 μL. The PCR reaction condition was as follows: initial denaturation (2 min at 94 °C) followed by 25 cycles of denaturation (0.5 min at 94 °C), primer annealing (0.4 min at 55 °C), and primer extension (1 min at 72 °C) and a final extension step (7 min at 72 °C).

2.3 Cloning and restriction fragment-length polymorphism analysis

PCR products were purified using TaKaRa Agarose Gel DNA Purification Kit (Takara Bio. Inc.) following its instructions. In order to construct 16S rRNA gene clone library, PCR product was ligated into *Enterobacter coli* DH5α competent cell (TIANGEN Inc.) and then inserted into pMD19-T vector using heat-shock method according to the specification of the manufacturer (Takara Bio. Inc.). A dilution series of transformed *Enterobacter coli* cells ligated 16S rRNA inserts were plated on Luria-Bertani agar plates with 100 g/mL Ampicillin and 40 μL of 40 m/L X-gal and IPTG (Takara Bio. Inc.). The plates were incubated at 37 °C for 24 h, and then performed blue-white screening. Two hundred sixty of white clones were selected randomly and amplified using M13 vector primers to check if they were recombined plasmids. The PCR products amplified by M13 vector primers were digested independently with restriction endonucleases *Afa* I and *Hha* I for 4 h in 37 °C according to the specifications of manufacturer (TaKaRa Bio. Inc.). The restriction PCR fragments were separated by 2% agarose gel electrophoresis in 1×TAE buffer. The gel were stained with ethidium bromide and visualized under UV light. The restriction patterns of clones were compared and visually grouped. One to three representative clones for each unique RFLP pattern was selected for sequencing.

2.4 Nucleotide sequencing and phylogenetic analysis

All sequencing was done by Sangon Biotech Company (Shanghai, China). Sequences were manually corrected and assembled by SeqMan program (DNAStar, Inc.). Sequences were blasted both in GenBank and Ribosomal Database Project (http://rdp.cme.msu.edu/) to find their closest relatives. All sequences we obtained and their best matched sequences were aligned using Clustal 2.1[25] and then they were added to construct Maximum Likelihood tree in MEGA 5.0[26]. Sequences with above 97% identity were defined as same operational taxonomic unit (OTU), and their taxonomic status was determined based on the position of each sequence in the phylogenetic tree. Rarefaction curve was

conducted using Analytic Rarefaction 1.3 (http://strata.uga.edu/software/) to evaluate the efficiency of clones sampled. The diversity indices were calculated with the following equations[27].

Shannon-Wiener index:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i \ , \quad p_i = n_i / N$$

Where N is the number of total clones in the clone library; n_i is the clone number of ith bacteria; p_i is the proportion of clones in the ith bacteria; S is the number of bacteria species.

Simpson index:

$$D = 1 - \sum_{i=1}^{S} (p_i)^2 \ , \quad p_i = n_i / N$$

Where N is the number of total clones in the clone library; n_i is the clone number of ith bacteria; p_i is the proportion of clones in the ith bacteria; S is the number of bacteria species.

The 16S rRNA gene sequences of the bacteria isolated from salivary glands of *H. maculaticollis* are available under accession numbers KJ569762−KJ569768.

3 Results

We analyzed bacteria in the salivary glands of the cicada *H. maculaticollis* using PCR-RFLP analysis. Thirty-eight (38) different RFLP patterns were identified from 235 positive clones after digesting by two restriction endonucleases *Afa* I and *Hha* I (Figure 1). The representative clones of the 38 RFLP patterns were sequenced, and their sequence blasting results indicated that they were belonging to Proteobacteria and Firmicutes, representing seven bacterial species: *Pseudomonas aeruginosa*, *Thermomonas brevis*, *Enterobacter* sp., *Sphingomonas* sp., *Methyloversatilis* sp., *Anaerococcus* sp., and *Bacillus* sp. (Table 1).

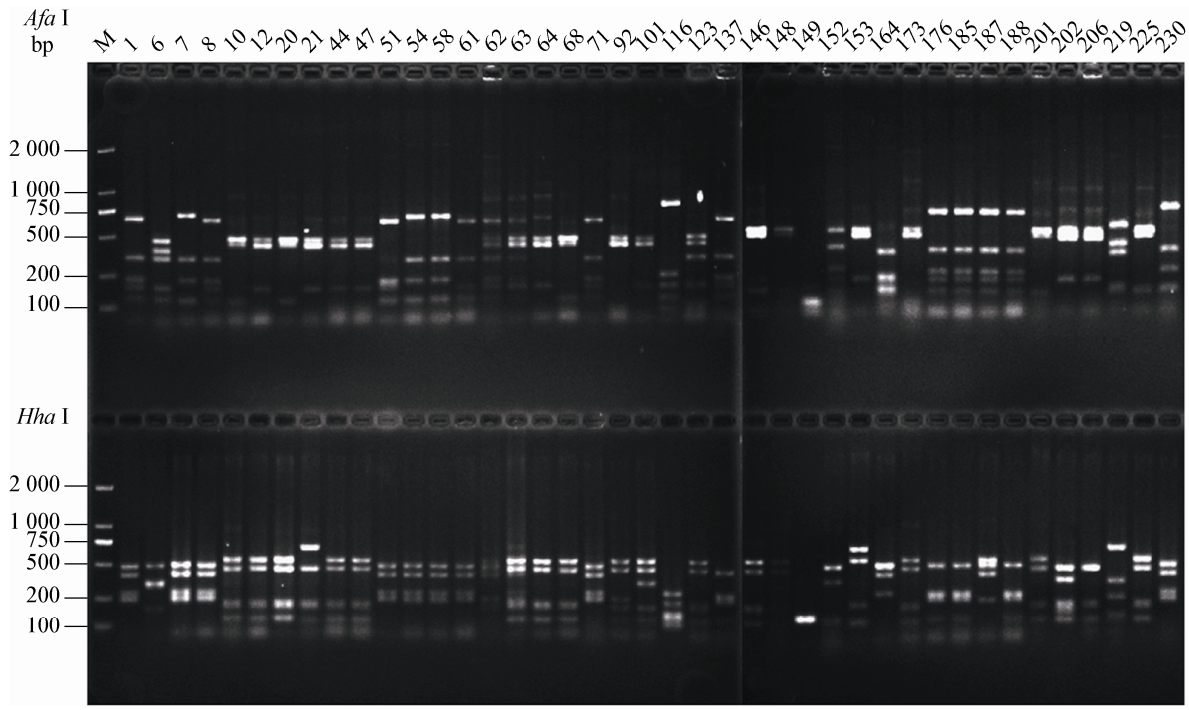

Figure 1 PCR-RFLP profiles of the 16S rRNA gene of the bacteria from the salivary glands of *H. maculaticollis*

图 1 斑透翅蝉唾液腺细菌克隆文库的 PCR-RFLP 图谱

Note: The restriction profiles were obtained after sample digestion with the restriction enzymes *Afa* I and *Hha* I, and *Afa* I restriction map is on the upper layer; *Hha* I restriction map is on the under layer; lanes 1 to 230 show representative clones of different restriction profiles.

注：该图谱由两种限制性内切酶 *Afa* I 和 *Hha* I 消化而成；*Afa* I 酶切图谱位于上层，*Hha* I 酶切图谱位于下层；条带标号代表了不同的限制性片段条带类型.

Table 1 NCBI BLAST results for the 16S rRNA-RFLP sequences of the representative clones isolated from salivary glands of *H. maculaticollis* (at 97% identity level)
表 1 斑透翅蝉唾液腺细菌克隆文库代表克隆子 16S rRNA 基因序列 NCBI 比对结果(97%相似度)

No. of representative clones	GenBank accession No.	The clone numbers (the % in clone library)	Closest match species in GenBank	Identity to closest match (%)
Clone-202	KJ569767	115 (48.7)	*Enterobacter* sp. (EU545406.1) (Gammaproteobacteria, Enterobacteriaceae)	99
Clone-54	KJ569763	115 (48.7)	*Pseudomonas aeruginosa* (JF513147.1) (Gammaproteobacteria, Pseudomonadaceae)	99
Clone-219	KJ569768	1 (0.43)	*Thermomonas brevis* (NR_025578.1) (Gammaproteobacteria, Xanthomonadaceae)	97
Clone-116	KJ569764	1(0.43)	*Sphingomonas* sp. (JQ396558.1) (Alphaproteobacteria, Sphingomonadaceae)	99
Clone-152	KJ569765	1 (0.43)	*Methyloversatilis* sp. (KC860260.1) (Betaproteobacteria, Rhodocyclaceae)	99
Clone-164	KJ569766	1 (0.43)	*Anaerococcus* sp. (HM587322.1) (Firmicutes, Clostridiales)	96
Clone-6	KJ569762	1 (0.43)	*Bacillus* sp. (EU784649.1) (Firmicutes, Planococcaceae)	99

Among the detected bacterial species, *P. aeruginosa* and *Enterobacter* sp. were the most frequently species obtained in the clone library of the salivary glands of *H. maculaticollis* (each of them accounting for 48.7% of the clone library). Whereas other five bacterial species were only discovered at very low levels with the same relative abundance which accounting totally for 2.05% of all clones. The bacterial diversity of the library was low (Shannon index: H'=0.82, 1-Simpson index: 1-D=0.52, which indicated that two individuals picked at random from this community would be different species in 52% of all cases). The endosymbiont *Candidatus* Sulcia muelleri were not detected in the salivary glands of *H. maculaticollis* which has been found in other cicadas[22].

Although the rarefaction curve has a rising trend at 3% difference between sequences (95% confidence intervals) (Figure 2), nearly 97.4% clones were found to belong to the two dominant bacterial species *Enterobacter* sp. and *Pseudomonas aeruginosa*. Hence, the number of clones sampled was sufficient to provide an accurate estimation of bacterial diversity in salivary glands of *H. maculaticollis*.

The Maximum Likelihood (ML) phylogenetic tree was constructed using the sequences of the seven representative clones isolated from the salivary glands of *H. maculaticollis* and their best matched sequences downloaded from GenBank (Figure 3). The results of

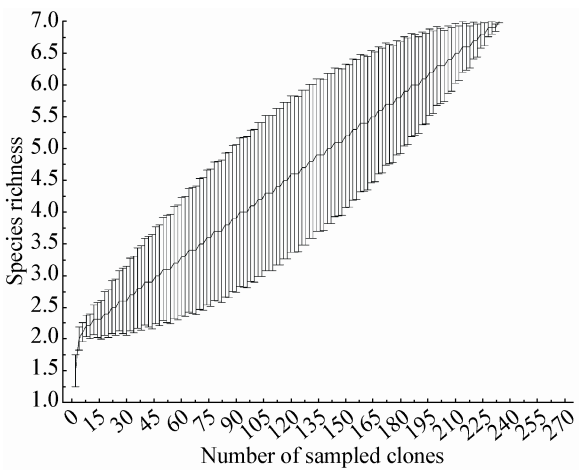

Figure 2 Rarefaction curve for 16S rRNA gene libraries constructed from salivary glands of *H. maculaticouis*
图 2 斑透翅蝉唾液腺细菌 16S rRNA 基因克隆文库稀有度曲线

Note: The rarefaction curve describe the equilibration of discovering bacteria species in the clone library of cicada *H. maculaticollis*; the error bars represent 95% confidence intervals which were automatically generated by the software Analytic Rarefaction for richness values.

注：稀有度曲线描述了斑透翅蝉唾液腺细菌克隆文库中所发现细菌种类的饱和度；误差线代表了稀有度分析软件自动生成的用于评价丰富度的 95%置信区间.

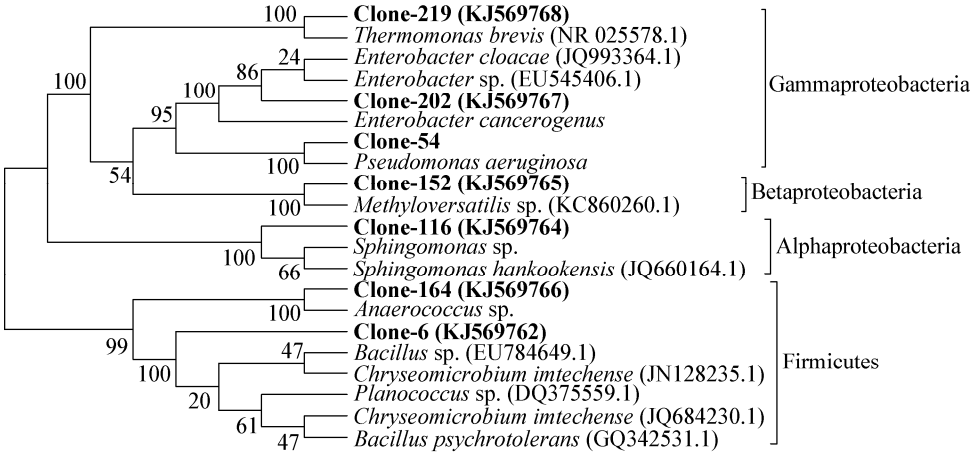

Figure 3 The Maximum likelihood tree based on bacteria 16S rRNA gene sequences of the salivary glands of *H. maculaticollis*, including selected database sequences

图 3 斑透翅蝉唾液腺细菌 16S rRNA 基因序列以及数据库中比对序列的最大似然发育树

Note: The tree was constructed through MEGA 5.0 with Tamura-Nei parameter model and complete deletion treatment for gaps and missing data; GenBank accession Numbers are provided in the parentheses; Numbers at each branch point indicate the percentage supported by bootstrap values based on 2 000 replications; The representative clones identified in this study are listed in boldface type followed by GenBank accession numbers.

注：发育树由 MEGA 5.0 软件根据 Tamura-Nei 参数模型自展 2 000 次构建，比对时对序列进行校正；括号内为该序列的序列号；分支节点处的数字为置信度；代表克隆子序号以及 GenBank 序列号用粗体表示.

phylogenetic analysis indicate that all these bacteria were belonging to four distinct groups, i.e., Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria and Firmicutes.

Clone-219 (KJ569768) was identified as bacterium *Thermomonas brevis* (NR_025578.1) with 97% support. Clone-54 (KJ569763) was identified as bacterium *Pseudomonas aeruginosa* (JF513147.1) with 99% support. Clone-202 (KJ569767) was clustered with bacteria *Enterobacter cloacae* (JQ993364.1), *Enterobacter* sp. (EU545406.1) and *Enterobacter cancerogenus* (JN644607.1) with 99% support, but its identity was unclear. All the above-mentioned clones formed a unique, well-supported branch, belonging to the class Gammaproteobacteria.

Clone-152 (KJ569765) was grouped with *Methyloversatilis* sp. (KC860260.1) with 99% support; while clone-116 (KJ569764) was grouped with *Sphingomonas* sp. (JQ396558.1) and *Sphingomonas hankookensis* (JQ660164.1) with 99% support. These two isolated bacteria were identified in the class Betaproteobacteria and Alphaproteobacteria, respectively.

Clone-164 (KJ569766) was identified as *Anaerococcus* sp. (HM587322.1) with 96% support. Clone-6 (KJ569762) was clustered with bacteria of *Bacillus*, *Planococcus* and *Chryseomicrobium* with 99% support, but its identity remains unknown. Clones-164 and clone-6 are members of the class Firmicutes, but belong to different families, viz. Clostridiales and Planococcaceae, respectively.

4 Discussion

The microbiota inhabiting in the salivary glands of the cicada *H. maculaticollis* were investigated and characterized using culture-independent PCR-RPLP techniques for the first time. Seven bacterial species were identified in the clone library, and bacteria in the genera *Pseudomonas* and *Enterobacter* were dominant. The salivary glands of insects are known as one of the most important barriers to the transmission of pathogens[18,28]. Many antimicrobial peptide and proteins have been found in the salivary glands and saliva of several herbivore insect species can help overcome defensive responses of host plants[5-6]. The occurrence of bacteria in the salivary glands of different insects (e.g., fruit flies, mosquitos and leafhoppers) has been suggested to serve as a route for

their horizontal transmission to plants or other intermediate hosts[29-30]. Herein, the seven bacteria found in the salivary glands of *H. maculaticollis* may imply that these bacteria can overcome the barrier and adapt the special microenvironment of salivary glands to live. However, more studies are required to clarify the bacterial community and their exact functions in the salivary glands of the Cicadidae.

Members of *Enterobacter* are common components of insect gut microbiota, which have been found in many insects[31-33]. For example, some bacteria of *Enterobacter* were found to be the most prominent microorganisms in the gut of mosquitoes *Aedes triseriatus* (Say), *Culex pipiens* L., and *Psorophora columbiae* (Dyar & Knab)[34]. Several species of this bacterial genus have been recognized as pathogens[35]. However, some species of *Enterobacter* residing in insects may be valuable to their hosts. For example, the dominant presence of the enterobacterial community in the gut of medfly *Ceratitis capitata* (Wiedemann) contributes to its longevity and fitness[36], and they are also capable of contributing to the nitrogen and carbon metabolism, copulatory success and development of their hosts[37-38]. Davidson et al[39] showed that *Enterobacter cloace* is mildly pathogenic and penetrates the insect gut cells on ingestion. *Enterobacter* also aids in the insecticidal activity of *Bacillus thuringenesis* in the gypsy moth[40]. In addition, according to studies on various insects such as aphids, whiteflies, parasitic wasps and tsetse flies, the Enterobacteriaceae has generated numerous facultative symbionts[41-42]. Facultative symbionts, especially those from Enterobacteriaceae, appear to be particularly common in hosts that possess bacteriome-associated obligate symbionts, including aphids, whiteflies, tsetse flies, and mealybugs[43]. Herein, we infer that the predominant bacterium *Enterobacter* sp. (EU545406.1) harbored in the salivary glands of *H. maculaticollis* might play a beneficial role to the cicada host, and it would be interesting in a future study to verify whether this bacterium has been evolved into a facultative symbiont in cicadas.

Pseudomonas aeruginosa is the other dominant bacterium isolated from salivary glands of *H. maculaticollis*. This bacterium is usually a common opportunistic pathogen that occupies a wide variety of environmental niches[44-45]. It has been reported in the guts of several insects, such as ants, beetles, mosquitoes, and termites, etc.[34,36-37,46-47]. He et al[48] detected high levels of *P. aeruginosa* in the crop, midgut and hindgut of the ant *Camponotus fragilis* (Pergande) from the lab-raised colonies, but it was absent in the field-collected colonies. They inferred that the infection of *P. aeruginosa* is a laboratory artifact and may be harmful to laboratory colonies[48]. However, *P. aeruginosa* can play a positive effect to its host insects, e.g., preventing parasite establishment in the midgut of mosquitoes[49], and having a detoxification function in *Paederus* beetles[50]. Indiragandhi et al[51] indicated that *Pseudomonas* sp. may have antagonistic activity towards entomopathogenic fungi in the diamondback moth. Though we revealed that *P. aeruginosa* was one of the most abundant species in the salivary glands of *H. maculaticollis*, further work is needed to clarify the effect of the infection of this bacterium in the salivary glands of *H. maculaticollis*, and to investigate if it is ubiquitous in cicadas.

Other five bacterial species occurred in very small amount. The endosymbiont *Candidatus* Sulcia muelleri were not detected in the salivary glands of cicada *H. maculaticollis*, which has been reported generally distributed in the bacteriomes of cicadas and can supply the host with nutrients limiting in their diet[22]. This implies that this endosymbiont probably only distributes in the bacteriomes of *H. maculaticollis*.

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征 稿 简 则

3.3 摘要写作注意事项

3.3.1 英文摘要：1) 建议使用第一人称，以此可区分研究结果是引用文献还是作者得出的；2) 建议用主动语态，被动语态表达拖拉模糊，尽量不用，这样可以避免长句，以求简单清晰；3) 建议使用过去时态，要求语法正确，句子通顺；4) 英文摘要的内容应与中文摘要一致，但可比中文摘要更详尽，写完后务必请英文较好且专业知识强的专家审阅定稿后再返回编辑部。5) 摘要中不要使用缩写语，除非是人人皆知的，如：DNA，ATP 等；6) 在英文摘要中，不要使用中文字体标点符号。

3.3.2 关键词：应明确、具体，一些模糊、笼统的词语最好不用，如基因、表达……

4 特别说明

4.1 关于测序类论文

凡涉及测定 DNA、RNA 或蛋白质序列的论文，请先通过国际基因库 EMBL (欧洲)或 GenBank (美国)或 DDBJ (日本)，申请得到国际基因库登录号 (Accession No.)后再投来。

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