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Analysis of Inter-Individual Bacterial Variation in Gut of Cicada *Meimuna mongolica* (Hemiptera: Cicadidae)

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ABSTRACT. Intestinal bacterial community plays a crucial role in the nutrition, development, survival, and reproduction of insects. When compared with other insects with piercing-sucking mouthparts, the habitats of cicada nymphs and adults are totally different. However, little is known about the differences in the gut bacterial communities in the nymphs and adults within any cicada species. The diversity of bacteria in the gut of nymphs and adults of both genders of *Meimuna mongolica* (Distant) was studied using the denaturing gradient gel electrophoresis (DGGE) method. Few inter-individual variations among gut microbiota were observed, suggesting that *M. mongolica* typically harbors a limited and consistent suite of bacterial species. Bacteria in the genera *Pseudomonas* and *Enterobacter* were the predominant components of the gut microflora of *M. mongolica* at all life stages. Bacteria of *Pantoea*, *Streptococcus*, and *Uruburuella* were also widespread in the cicada samples but at relatively lower concentrations. The relative stability and similarity of the PCR-DGGE patterns indicate that all individuals of this cicada species harbor a characteristic bacterial community which is independent from developmental stages and genders. Related endosymbionts that could be harbored in bacteromes of cicadas were not detected in any gut samples, which could be related to the cicada species and the distribution of these endosymbionts in the cicada cavity, or due to some of the possible limitations of PCR-DGGE community profiling. It is worthwhile to further address if related cicada endosymbiont clades distribute in the alimentary canals and other internal organs through diagnostic PCR using group-specific primer sets.

Key Words: *Meimuna mongolica*, PCR-DGGE, gut bacteria, inter-individual variation, characteristic bacterial community

Cicadas are involved in important ecological functions including nutrient cycling in the woodland ecosystem. Their adults feed exclusively on the xylem fluid from branches of their host plants (Lloyd and White 1987). Further injuries caused by the feeding of cicada usually go undetected since their nymphs are long-lived underground and feed exclusively on the xylem sap from roots of their host plants. Cicadas cause great harm including twig dieback in host plants when large numbers of certain cicada species insert eggs into the stems of trees and shrubs (Lloyd and White 1987).

Xylem sap has been reported to contain many organic compounds including carbohydrates (Satoh et al. 1992; Lopez-Millan et al. 2000; Escher et al. 2004), amino acids (Dickson 1979), and proteins (Biles and Abeles 1991; Rep et al. 2002; Buhtz et al. 2004; Kehr et al. 2005; Djordjevic et al. 2007; Aki et al. 2008). However, the proteins are present in xylem sap at very low concentrations (10–300 μg/ml) (Biles and Abeles 1991; Satoh et al. 1992; Buhtz et al. 2004; Alvarez et al. 2006), which raises the question: how can cicadas live by feeding on a difficult-to-access and a poor unbalanced diet from xylem fluid?

The answer to this question may be related to the micro-organisms hosted in the cicada gut, which could supply their host with essential nutrients lacking in their diets. Many insects contain a rich, diverse and complex community of microorganisms that participate in the insect life, ranging in function from pathogenesis to mutualism (Dillon and Dillon 2004; McCutcheon 2009). As for cicadas, they can harbor endosymbiont *Candidatus* Sulcia muelleri (hereafter *S. muelleri*) as well as *Candidatus* Hodgkinia cicadicola (hereafter *H. cicadicola*) in their specialized bacteromes, which appear to provide different resources for the host (Gosalbes et al. 2010). McCutcheon et al. (2009) detailed the metabolic contributions of *S. muelleri* and *H. cicadicola*, co-resident symbionts in the cicada *Diceroprocta semicincta*, and their results showed that *Sulcia* and *Hodgkinia* exhibit a striking level of metabolic interdependence.

As sap-suckers, the gut of cicadas is long and thin, and different regions of the gut perform different tasks under different conditions of pH and enzyme activity; this makes it more efficient to absorb nutrients (Dow et al. 1987). When compared with other insects with piercing-sucking mouthparts, the habitats of cicada nymphs and adults are totally different, i.e., nymphal cicadas live a long time underground, but the adults live on crowns of plants just for several weeks (Gourley and Kuang 2009). However, little is known about the similarities and differences in the gut bacterial communities in the nymphs and adults within any cicada species, which could be informative on the effect of habitat change on the gut bacterial communities within cicada species.

We investigate the gut microbial communities in nymphs and adults of the cicada *Meimuna mongolica* (Distant) (Hemiptera: Cicadidae) which is widely distributed in China, using the PCR-DGGE method, for the specific purposes of identifying the predominant bacterial species, detecting possible trends in microbial succession during cicada development, and establishing their potential contributions to the insect's carbon and nitrogen nutrition.

Materials and Methods

Cicada Collection. All nymphs and adults of *M. mongolica* were collected in the same wild poplar woods in Yangling, Shaanxi Province of China in the August of 2011. The adults were collected using a light trap in the poplar woods at night. Last instar nymphs were captured on trunks of poplars at night before eclosion. All captured cicadas were transferred live to a voile-cage and brought to the lab immediately for dissection.

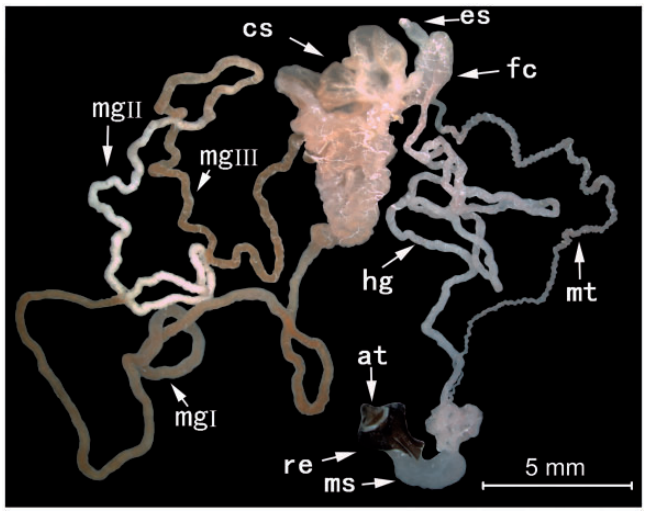

Fig. 1. Gross morphology of the gut of *M. mongolica*. at, annal tube; cs, conical segment; es, esophagus; fc, filter chamber; hg, hind gut; mgI, pro-midgut; mgII, mid-midgut; mgIII, hind-midgut; ms, membranous sac; mt, Malpighian tubule; re, rectum.

Gut Dissection. Before the dissection, each cicada was first narcotized in the refrigerator (4°C) for a few minutes, and then externally sterilized with 75% ethanol for 10 s and 1% mercury bichloride for 2 min, respectively, and then rinsed three times with sterilized water. Then the cicada was dissected along the dorsal middle line from anus to head with a pair of sterilized scissors. The whole gut (including esophagus, midgut, hindgut, filter chamber, conical segment, and rectum, see Fig. 1) was carefully separated from other organs with sterilized fine-tip forceps and washed twice with 0.9% NaCl solution as soon as exposed, and the hemolymph and NaCl solution around the gut were absorbed with sterilized tissue. Each sample contained one gut from each individual cicada. In total, 12 samples, including 6 nymphs, and 6 adults (Table 1), were prepared for the following experiments. All work was done in a laminar flow cabinet.

Genomic DNA Extraction. The genomic DNA was extracted from the aforementioned gut samples using TIANamp Genomic DNA kit (Tiangen Inc., Beijing, China) according to the manufacturer's directions. DNA was eluted with 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and stored at −20°C.

PCR Amplification and DGGE Analysis. The DNA samples were amplified using a forward primer 357F (5′-CCTACGGG AGGCAGCAG-3′) and a reverse primer 518R (5′-CGG TGTG TACAAGG CCC-3′), which were designed for amplifying the V3 region of bacterial 16S rRNA gene fragments for DGGE analysis (Liu et al. 2008). The forward primer was modified at the 5′ end with a GC-clamp sequence (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGG) which can terminate gel migration of products within a concentration gradient of urea/formamide. The reaction mixture (25 µl) contained 12.5 µl 2×Es Taq Master Mix (Beijing CoWin Bioscience Co., Ltd), 1 µl of each primer (10 mM), 9.5 µl RNase-free water, and 1 µl Template DNA. An initial denaturation step of 4 min at 95°C was followed by 29 cycles of amplification (30 s at 94°C, 30 s at 55°C, and 45 s at 72°C), and a final elongation step of 5 min at 72°C.

PCR products of samples (25 µl of each) were loaded on an 8% polyacrylamide gel containing a linear denaturing gradient of 35–60% (100% denaturing acrylamide was defined as containing 7-M urea and 40% formamide) using the DGGE system (Chishun Science & Technology Co., Ltd., Nanjing, China). The gel was run for 1 h at 80 V and then for 16 h at 60 V in 1×TAE buffer at a constant temperature of 60°C. The gel was stained with EB (ethidium bromide) and photographed under UV light.

Table 1. Richness (*S*), Evenness (E_H) and Shannon-Wiener index (H') of gut bacterial identified in samples of *M. mongolica* individuals

Index	Nymph						Adult					
	Female			Male			Female			Male		
	A	B	C	D	E	F	G	H	I	J	K	L
S	9	12	11	12	13	12	11	12	11	9	12	6
E_H	0.736	0.758	0.739	0.810	0.758	0.901	0.820	0.818	0.851	0.811	0.804	0.791
H'	1.618	1.885	1.773	2.014	1.944	2.239	1.966	2.033	2.041	1.782	1.998	1.538

Dominant and bright bands were excised from polyacrylamide gel, and DNA was eluted overnight at 4°C in 30 µl RNase-free distilled water, and 2 µl supernatant was used as template for the re-amplification with primer pair 357F and 518R without a GC clamp at the 5′ end. Subsequently, the PCR products were purified with a PCR purification kit (TaKaRa Bio. Inc., Japan). The purified DNA was cloned into PMD 19-T Vector and then transformed into *Escherichia coli* competent cells (strain DH5α) to identify positive clones based on the blue-white screening. Five white clones were selected randomly from each transformation to further verify if they are the positive clones, then the positive clones were sequenced by the Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. in China. The cloning method is as described previously by He et al. (2011).

Phylogenetic Analysis. 16S rRNA gene sequences were manually corrected using Chromas Lite 2.01 (Technelysium Pty. Ltd., Helensvale, Australia) if necessary and sequence assembly using ChromasPro Version 1.5 (Technelysium Pty. Ltd., Helensvale, Australia). All sequences were blasted in NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) to infer their taxonomic affiliation, and the closest hits in BLAST searches were downloaded. Multiple sequence alignments including 30 sequences retrieved from NCBI databases were performed with Clustal X2.0 (Larkin et al. 2007). Aligned sequences were loaded to construct a Maximum Likelihood tree with the 2,000 bootstrap method and Kimura 2-parameter model in MEGA 5 (Tamura et al. 2011). The DGGE sequences obtained in this study are available in GenBank KC900953–KC900971.

Bacterial Community Analysis. Quantity One software (Version 4.6.2, Bio-RAD) was used to analyze the DGGE band profile. Each DGGE band was digitized via auto detection of peak density and transferred into corresponding data, and then the diversity indices were calculated to investigate the dominant bacterial communities and to determine the variation among A–L individuals. Biodiversity indices, such as the Shannon-Wiener index (H'), Richnessw (S), and Evenness (E_H), were calculated from the DGGE patterns according to the following equations:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i = -\sum_{i=1}^{s} (N_i/N)\ln(N_i/N)$$

$$E_H = H/H_{\max} = H/\ln S$$

Where S is the number of bands in a lane, N_i is the peak density of the ith band and N is the total peak density of all bands in a lane. The significant differences between developmental stage (nymph and adult) or gender (femal and male) were analyzed by t-test in SPSS Version 19.0 (SPSS Inc. Chicago, IL). In order to further compare the similarity among 12 individuals, '1' and '0' matrix was formed according to the presence and absence of DGGE band in A–L, and the dendrogram was construct based on the similarity index with Pearson Correlation method using SPSS Version 19.0.

Results

Analyses of DGGE Profile. To compare the diversity and similarity of gut microbes between nymphs and adults in *M. mongolica*, a DGGE

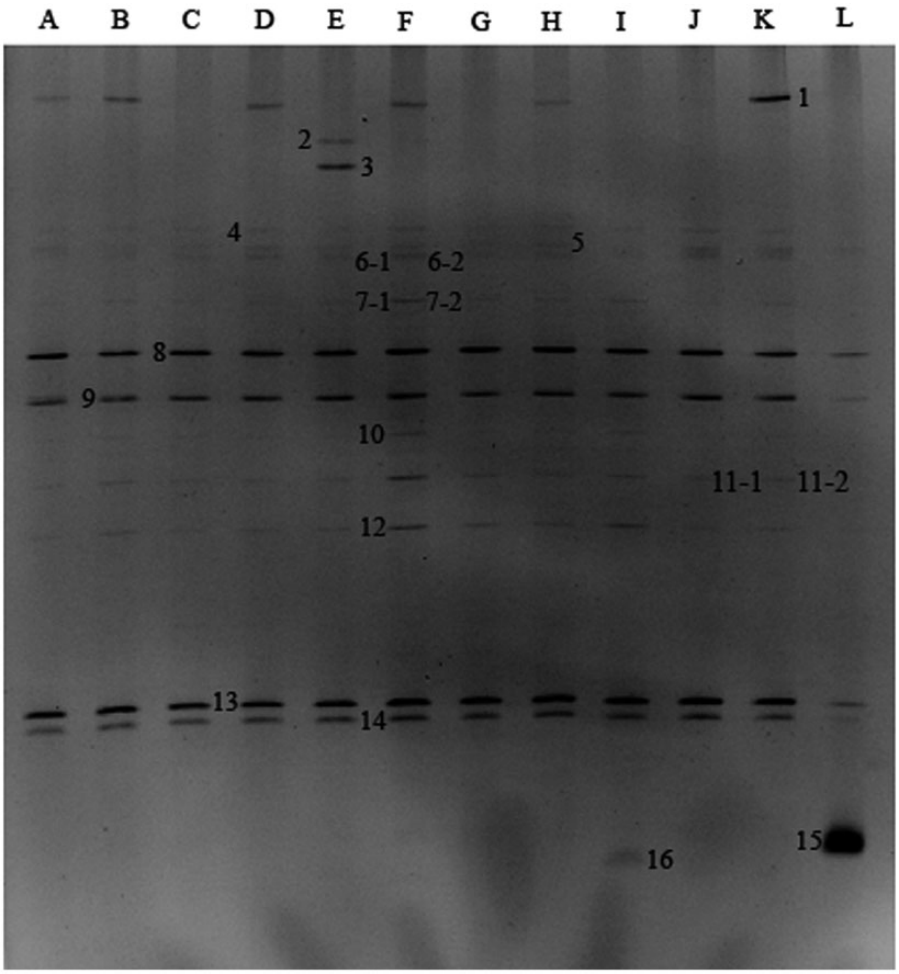

Fig. 2. DGGE profiles of bacterial community from the gut of tested *M. mongolica* individuals. Letters (A–L) represent the cicada individuals caught in the field (Table 1). Numbers in the lanes relate to bands excised from the gel for sequencing (Table 2).

profiles obtained from the guts of 12 individuals was created and a total of 16 different DGGE bands were checked in all samples (Fig. 2).

There is little variation found between different individuals from the DGGE profiles although one individual cicada (L) had fewer bands than the others (Fig. 2). Although some bands were restricted to one or a few individuals, most bands were widespread, occurring in almost every profile. For example, DGGE Band-2, Band-3, Band-15, and Band-16 were only detected once among all the samples; Band-4, Band-5, Band-8, Band-9, Band-13, and Band-14 were found in the gut of all tested individuals. The results indicate that *M. mongolica* harbors a great number of the same bacterial genotypes, and their gut bacterial community was relatively stable despite the developmental stage and gender differences.

Bacterial Diverisity and Similarity Analyses. The band brightness can throw light on the proportion of each bacterium in the gut of *M.mongolica*. The relatively brighter bands in the DGGE profiles include Band-1, Band-3, Band-8, Band-9, Band-13, Band-14, and Band-15; other bands are relatively weaker. The peak density of bands in the DGGE profile were obtained using Quantity One software, and the bacterial diversity indices were computed (Table 1). The richness (S) and Shannon-Wiener index (H') of bacteria in the individuals are between 6–12 and 1.538–2.239, respectively. There are no significant difference between the bacterial diversity of the nymphs and adults, or that of the males and females ($P > 0.05$). The Dendrogram (Fig. 3) based on DGGE-band similarity among individuals also showed that most individuals (H, K, B, D, F, C, G, J, I, and E) clustered together with high similarity (similarity index > 0.5370), and only A and L were clustered with low similarity (similarity index 0.441 and 0.153, respectively), while no obvious characteristics associated with gender or developmental stage are found in the Dendrogram.

DGGE Band Identification and Phylogenetic Analyses. Nucleotide sequences of partial 16S rRNA gene were determined from the 16 distinct bands on the DGGE gel. In total, 19 sequences were obtained, and the results of sequence alignments are shown in Table 2.

According to the NCBI blasting results, three DGGE bands (Band-6, Band-7, and Band-11) were found to contain two different nucleotide sequences, and we designated them as 6-1, 6-2, 7-1, 7-2, 11-1, and 11-2 to distinguish them. Some sequences obtained from different DGGE bands were assigned to the same bacterium. For example, sequences of Band-2 and Band-3 both showed high similarity to the uncultured *Spiroplasma* sp. (JF266585.1); sequences of the Band-5 and Band-9 were closely related to the uncultured *Pseudomonas* sp. (HQ144204.1); sequences of Band-10 and Band-11-2 were both assigned to the uncultured *Streptococcus* sp. (KC020726.1).

A ML phylogenetic tree was constructed using the sequences of bacteria obtained from the DGGE bands from the gut of *M. mongolica* and their closest related sequences in GenBank (Fig. 4). Nineteen sequences derived from 16 bands clustered into six major bacterial phyla: α-proteobacteria (one sequence, accounting for 5.3%), β-proteobacteria (two sequences, accounting for 10.5%), γ-proteobacteria (9 sequences, accounting for 47.4%), Actinobacteria (one sequence, accounting for 5.3%), Tenericutes (three sequences, accounting for 25%), and Firmicutes (three sequences, accounting for 25%).

Band-8, Band-9, Band-13, and Band-14 closely matched members of *Pseudomonas* and *Enterobacter*, respectively and were detected as

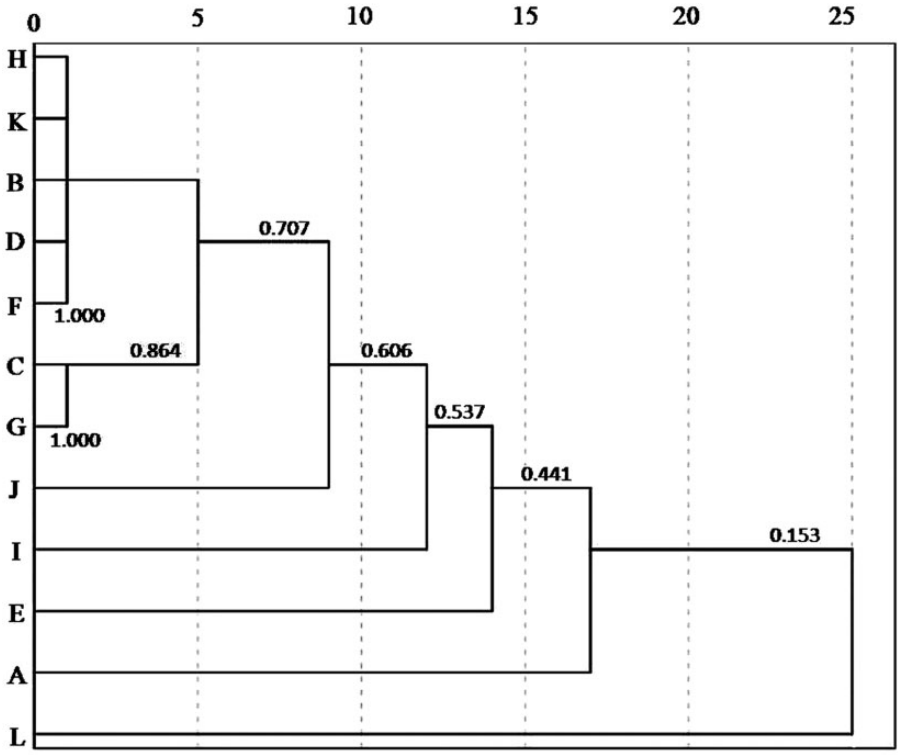

Fig. 3. Dendrogram of similarities among *M. mongolica* individuals using average linkage (between groups). The dendrogram was generated using SPSS software based on Pearson Correlation method. Similarity indices are presented in each cluster.

Table 2. Results of sequence analysis of DGGE bands isolated from guts of *M. mongolica* individuals

Band no.	GenBank	Closest match (accession no.)	% Identity to closest match	Individuals with corresponding bands	Number of samples
1	KC900953	*Spiroplasma* sp. (DQ288984.1)	95%	A, B, D, F, H, K	6
2	KC900954	Uncultured *Spiroplasma* sp. (JF266585.1)	99%	E	1
3	KC900955	Uncultured *Spiroplasma* sp. (JF266585.1)	99%	E	1
4	KC900956	Uncultured *Pseudomonas* sp. (JN030443.1)	99%	A, B, C, D, E, F, G, H, I, J, K	11
5	KC900957	Uncultured *Pseudomonas* sp. (HQ144204.1)	100%	A, B, C, D, E, F, G, H, J, K, L	11
6-1	KC900958	*Enterobacter asburiae* (KC136820.1)	100%	B, C, D, E, F, G, H, I, J, K	10
6-2	KC900959	*Pantoea* sp. (KC150862.1)	100%	B, C, D, E, F, G, H, I, J, K	10
7-1	KC900960	*Pseudomonas aeruginosa* (KC119335.1)	100%	B, C, D, E, F, G, H, I, K	9
7-2	KC900961	*Streptococcus* sp. (HM776056.1)	99%	B, C, D, E, F, G, H, I, K	9
8	KC900962	*Pseudomonas* sp. (JN208919.1)	100%	A, B, C, D, E, F, G, H, I, J, K, L	12
9	KC900963	Uncultured *Pseudomonas* sp. (HQ144204.1)	100%	A, B, C, D, E, F, G, H, I, J, K, L	12
10	KC900964	Uncultured *Streptococcus* sp. (KC020726.1)	100%	B, C, D, E, F, G, H, I, K	9
11-1	KC900965	*Uruburuella* sp. (JQ595501.1)	100%	A, B, C, D, E, F, G, H, I, J, K	11
11-2	KC900966	Uncultured *Streptococcus* sp. (KC020726.1)	99%	A, B, C, D, E, F, G, H, I, J, K	11
12	KC900967	*Uruburuella suis* (NR_042211.1)	100%	A, B, C, D, E, F, G, H, I, J, K	11
13	KC900968	Uncultured *Enterobacter* sp. (EF434254.1)	98%	A, B, C, D, E, F, G, H, I, J, K, L	12
14	KC900969	*Enterobacter* sp. (HM365935.1)	99%	A, B, C, D, E, F, G, H, I, J, K, L	12
15	KC900970	*R. salmoninarum* (NR_041773.1)	96%	L	1
16	KC900971	*L. crescens* (JX430025.1)	99%	I	1

strong bands in most samples (Fig. 2). Some weak bands representing bacterial species in the genera *Pseudomonas*, *Enterobacter*, *Pantoea*, *Streptococcus*, and *Uruburuella* were also present in most individuals. Band-2, Band-3, Band-15, and Band-16 only appeared once in the DGGE profiles and were identified as close to the uncultured *Spiroplasma* sp. (JF266585.1) (Band-2 and Band-3), *Renibacterium salmoninarum* (NR_041773.1) (Band-15) and *Liberibacter crescens* (JX430025.1) (Band-16), respectively.

Discussion

In this study, we identified bacterial species that presented in the gut of different *M. mongolica* individuals by PCR-DGGE targeting the V3 region of 16S rRNA genes. The result is expected to reflect the unique bacterial communities among *M. mongolica* individuals of different gender and developmental stages, although there could be some inevitable biases caused by the preparation of total community DNA and the PCR amplification process (Wintzingerode et al. 1997; Polz and Cavanaugh 1998; Frostegard et al. 1999). The results reveal that individuals of both nymphal and adult stages showed similar gut bacterial profiles, except one sample contained fewer band profiles (Fig. 2). Cicada nymphs usually develop slowly underground and feed exclusively on the xylem sap from root system of their host plants, whereas their adults feed only on the xylem fluid from branches (Smits et al. 2010), Our result suggests that there is a characteristic gut bacterial community associated with this cicada species, and there are no significant difference between the bacterial diversity of the nymphs and adults, or that of the males and females ($P > 0.05$).

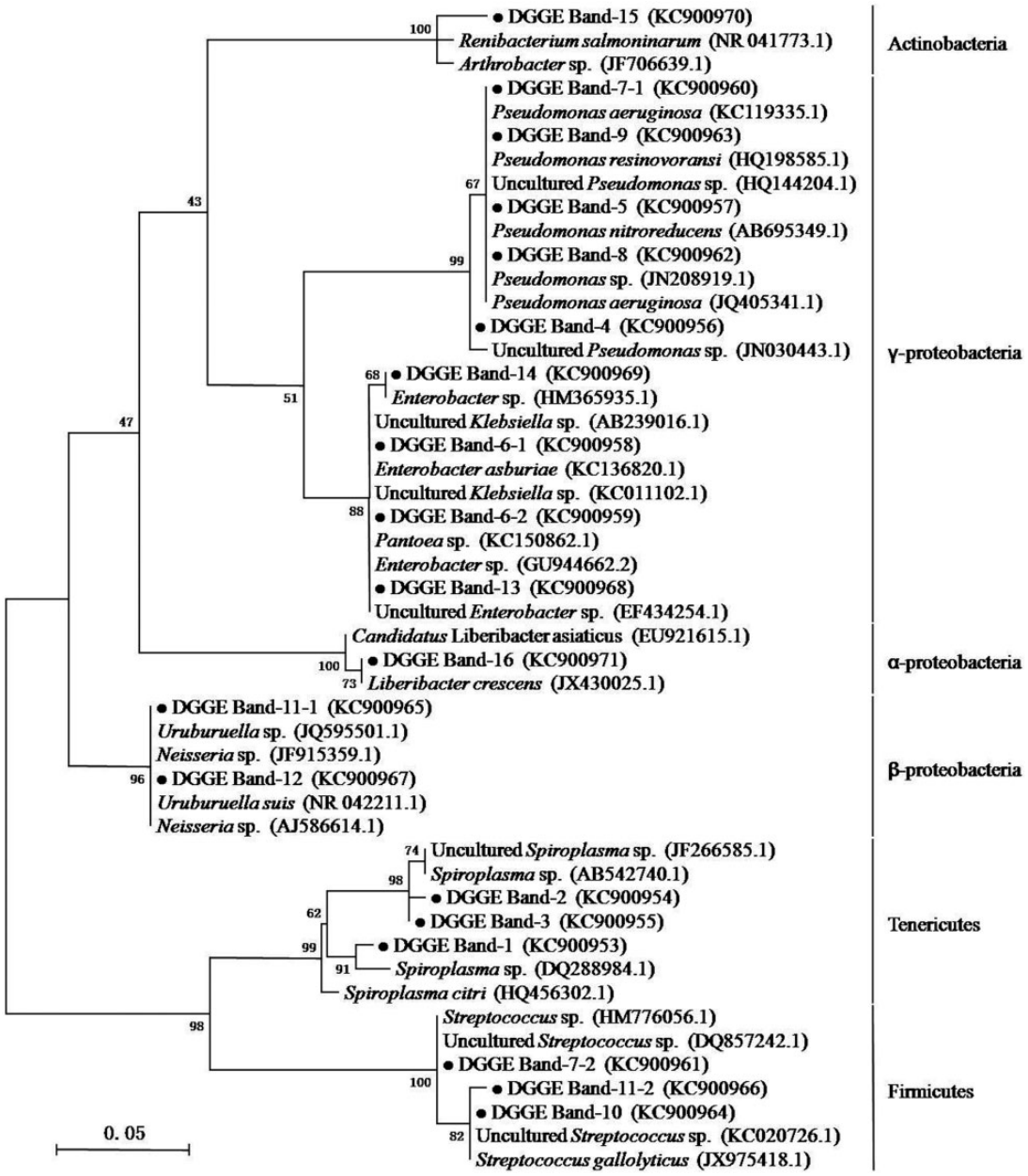

Fig. 4. A ML tree based on DGGE sequences from the gut of *M. mongolica* and their closest related sequences in GenBank. The tree was generated using Kimura 2-parameter model with 2,000 bootstrap method in MEGA5 software. DGGE band sequences in this article are indicated by dark spots, and their GenBank accession numbers are listed in parentheses.

Our observations reveal that most bacterial species predominant in the gut of *M. mongolica* belong to the genera *Pseudomonas* and *Enterobacter* in the class γ-proteobacteria (Fig. 4). *Pseudomonas* and *Enterobacter* were also the most prominent microorganisms in the gut of mosquitoes (Demaio et al. 1996). The predominance of them in related insect groups with vastly different diets indicates this is unlikely to be diet driven. Some *Pseudomonas* bacteria may play a beneficial role to their hosts, e.g., antagonistic activity towards entomopathogenic fungi in the diamondback moth (Indiragandhi et al. 2007), prevention of parasite establishment in the midgut of mosquitoes (Azambuja et al. 2005), and detoxification function in *Paederus* beetles (Piel 2002). Similarly, many species of the genus *Enterobacter* have a demonstrated role in insect nutrition, e.g., nitrogen fixation in the fruit-fly *Bactrocera tryoni* (Murphy et al. 1994), some termites (Ohkuma et al. 1999), and the apple maggot fly *Rhagoletis pomonella* (Lauzon et al. 2003). The predominant *Pseudomonas* and *Enterobacter* harbored in the gut of *M. mongolica* possibly also play a beneficial role to the cicada hosts.

The bacterium (KC900953) with a sequence showing 95% similarity to the *Spiroplasma* sp. (DQ288984.1) appeared to be exclusive to *M. mongolica*. Bacteria in the genus *Spiroplasma* can infect a wide range of arthropod hosts (Gasparich et al. 2004; Regassa and Gasparich 2006). They have diverse effects within different hosts, being mutualistic, pathogenic, or gender ratio distorters (Haselkorn et al. 2009). More *M. mongolica* nymphs than adults were found containing the bacterium (KC900953) in our study, which might be closely associated with the development, feeding habits and/or habitats of the insects, e.g., nymphs and adults feeding exclusively on the xylem fluid from branches and roots of their host plants, respectively. However, infection frequency by this bacterium in nymphs and adults of *M. mongolica* needs to be investigated further with more individuals in the future to clarify if it is more closely associated with the environment of the cicada nymphs, where they undergo a long-term subterranean life.

Bacteria in the genera *Pantoea*, *Streptococcus,* and *Uruburuella* were all present at a low concentration in the gut of *M. mongolica*.

However, they were widely distributed in the samples of both nymphal and adult stages and both genders. The functions of these bacteria in the gut of *M. mongolica* remain unknown. The bacterium *Pantoea agglomerans* can help produce antifungal phenols which are selectively bacteriocidal, and their toxins may have a wide role in host defense against pathogenic microorganisms in the desert locust *Schistocerca gregaria* (Dillon and Charnley 1995). Interestingly, the bacterium *Streptococcus faecalis* that was found to be predominant in the gut of the larval honeycomb moth *Galleria mellonella* also showed bacteriolytic activity (Jarosz 1975). Whether the bacteria of *Pantoea*, *Streptococcus*, and *Uruburuella* detected in the gut of *M. mongolica* has a role in host defense merits further investigation.

The endosymbionts *Candidatus* S. muelleri and *Candidatus* H. cicadicola that have been found from the bacteriomes positioned adjacent to the lateral margins of the anterior abdomen in some cicadas were not detected in any tested samples of *M. mongolica* in our study. Although bacteriome-specific bacteria of *M. mongolica* is not included in this study, the absence of these in the alimentary canal of this cicada species indicates that the above cicada endosymbionts are bacteriome-specific.

In conclusion, we revealed that the gut bacterial community of *M. mongolica* comprises a few bacterial species, and this bacterial community was relatively stable despite the gender and developmental stage/niche differences that existed between the sampled individuals. Additional method, such as high-throughput sequencing technique, will be necessary to obtain a more detailed overview of the gut microbiota of *M. mongolica* and other cicadas.

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