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-Millán 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). For as 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.
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′-ATTACCGG CGGCTGCTGG-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 (GGGCGCGCCGCCGC GGCGAGCCCGGGGCGGGCGGGGCGGGG) which can terminate gel migration of products within a concentration gradient of urea/formamide. The reaction mixture (25 μl) contained 12.5 μl 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.

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 \( H' \), Richness \( S \), 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 \( i \)th 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
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 Diversity 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
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 ($P > 0.05$).

**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, I, 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, 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. (JN08919.1)   | 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     | *Urburella* 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     | *Urburella 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                |
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 (Okhuma 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*.

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**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.
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