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Phylogenetic analysis of bacterial community in the gut of American cockroach (*Periplaneta americana*)

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Abstract: [**Objective**] The present study was to fully evaluate the intestinal bacterial community of *Periplaneta americana*, an important model to study insects. [**Methods**] We investigated the bacterial community of *P. americana* gut by culture-independent methods, involving constructing the 16S rRNA gene library and microbial diversity analysis. [**Results**] The phylotypes were affiliated with *Proteobacteria* (66.4%), *Bacteroidetes* (17.8%), *Firmicutes* (14.5%), *Fusobacteria* (0.6%) and unclassified bacteria (0.6%). Phylogenetic analysis shows that 15% of the sequences clustered with that from a closely related omnivorous cockroach; and 59% clustered with that from more distantly related animals, including omnivorous, herbivorous, and carnivorous animals, which differ greatly in feeding habits. Moreover, 18% of the clones showed high sequence identity with potential pathogens closely related to human diseases, which also reinforces the concept of the cockroach as a carrier of pathogens. [**Conclusion**] Due to their habits of feeding on a variety of foodstuffs, omnivorous cockroaches harbor a large and diverse microbial community in the gut. The host phylogeny and dietary habits might be critical for the intestinal bacterial community composition of cockroaches.

Keywords: American cockroach, 16S rRNA gene, microbial community
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There are 3500 to 4000 species of cockroaches, and they are considered one of the most significant and objectionable pests [1]. Approximately 50 species of cockroaches have been reported as pests in human architecture and households worldwide, some of which may be potential health problems to their human neighbors. In China, the subtropical climate is favorable for the growth and development of *Periplaneta americana*, which is the dominant species in southern China, making up 70 percent of the total [2].

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American cockroaches are carriers, either on the cuticle or in the gut, of a variety of bacteria, including potential pathogens; some pathogens may carry resistance to antibiotics or drugs [3-5]. When the cockroaches fly, they have many opportunities to pick up, carry, and transmit various bacteria [3]. Although definitive evidence that cockroaches are vectors for human disease is still lacking, the prevalence of cockroaches near human and animal wastes, human food, and human environments creates sufficient concern about their role as vectors [3]. Therefore, omnivorous cockroach *Periplaneta americana*, along with *Blattella germanica*, *Blatta orientalis*, and *Supella longipalpa* have been classified into Group I of the "Dirty 22" by the Food and Drug Administration [6].

Because of its ubiquity, relatively large size, and omnivorous nature, *P. americana* is an important model for studies of insect physiology, biochemistry, and behavior [7]. By employing various culture-dependent techniques, including scanning and transmission electron microscopy and selective and non-selective medium [7-9], the association and transmission of living bacteria within the digestive tract of *P. americana* have been investigated. However, because of the high selectivity of culture-based approaches, they may not fully assess the complete picture of bacterial community composition of *P. americana*. Therefore, comprehensive cataloguing and comparative analysis of gut bacteria from *P. americana* are needed, which will broaden our knowledge of the cockroach-associated microbiota in American cockroach and further profile the contribution of intestinal bacteria to the omnivorous lifestyle of the cockroach.

To better understand microbial ecology in the American cockroach intestine, we surveyed the intestinal bacterial community of *P. americana* by constructing a 16S rRNA gene library and revealed that both host phylogeny and omnivorous diet shaped the bacterial community. The results also shed light on the possible role of *P. americana* as a reservoir for pathogenic vectors.

1 Materials and methods

1.1 Sampling

American cockroaches (*P. americana*) were provided as a gift by Hefei Centers for Disease Control. The insects were reared in cages at 25℃ and fed milled corn and bran cob, and water.

The cockroaches were anesthetized using diethyl ether and sterilized using 75% alcohol before dissection. The cockroach guts were dissected, and the fat body was removed. The intestinal microbes from the gut of the cockroaches were prepared with a few modifications according to the methods of Walter *et al*[10]. The guts were minced and pooled into 50 mL sterile phosphate-buffered saline (PBS, pH 7.4). After vigorous shaking under sterile conditions, the intestinal debris was centrifuged at 4℃ and 9 000 ×*g* for 3 min. Then, the precipitate was suspended in 40 mL of fresh PBS (pH 7.4) buffer and centrifuged at 150 ×g and 4℃ for 5 min to remove debris. The supernatant, which contained bacterial cells, was centrifuged again at 200 ×g for 5 min. After gradient centrifugation, the bacterial cells were recovered by centrifugation at 9 000 ×g and 4℃ for 10 min and resuspended in 2 mL STE buffer (0.2 mol/L NaCl, 100 mmol/L ethylenediamine tetraacetic acid, and 10 mmol/L Tris, pH 8.0).

1.2 DNA extraction and 16S rRNA gene amplification

Genomic DNA was extracted using a BacteriaGen DNA kit (CWBIO, Beijing, China) according to the manufacturer's protocol: (1) samples were incubated with proteinase K (20 μL, 20 mg/mL) at 56℃ until the cells dissociated and then transferred to a spin column to recover genomic DNA through centrifugation at 12000 ×g for 5 min. (2) Samples were incubated with 20 mg/mL of lysozyme for 30 min at 37℃. Then, the sample was heated at 56℃ for another 30 min after adding proteinase K (20 μL, 20 mg/mL). The genomic DNA was recovered with a spin column by centrifugation at 12000 ×g for 5 min.

The polymerase chain reaction (PCR) amplification of 16S rRNA genes was performed using the primer pair Bact-27F (AGAGTTTGATCMTGGC TCAG) and Univ-1492R (GGTTACCTTGTTA CGACTT) with the extracted genomic DNA as the template. The reaction was performed in a 50 μL solution containing 100 μmol/L deoxyribonucleotide triphosphates, 0.5 μmol/L of each primer, 2 mmol/L $MgCl_2$, 10 ng DNA template, and 2.5 U Taq DNA polymerase (Transgen, Beijing, China) with 1 × reaction buffer supplied by the manufacturer. PCR was performed with a Mastercycler Personal (Eppendorf, Germany) with the following protocol: 25 cycles of denaturation at 94℃ for 90 s, annealing at 52℃ for 30 s, and extension at 72℃ for 1 min, and a final extension at 72℃ for 10 min. The PCR products were analyzed by 1.0% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

1.3 16S rRNA gene library construction and sequencing

After purification, the PCR products of the 16S rRNA genes were ligated into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into chemically competent *E. coli* DH5α cells (Transgen, Beijing, China). The transformed cells were plated on Luria-Bertani agar containing isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. White positive clones were placed in 384-well plates to construct a 16S rRNA gene library. Twenty clones were randomly selected from the library for insert length inspection using M13F (GTAAAACGACGGCCAG) and M13R (CAGGAAACAGCTATGAC) primers specific to the pGEM-T vector. The PCR conditions were as follows: an initial denaturation at 94℃ for 5 min, followed by 30 cycles of denaturation at 94℃ for 45 s, annealing at 42℃ for 45 s, and extension at 72℃ for 90 s and a final elongation at 72℃ for 10 min.

A total of 170 sequences were randomly selected and sequenced (Beijing Genomics Institute, Shanghai, China) with an ABI 377 genetic analyzer (Applied Biosystems, USA).

1.4 Taxonomic and phylogenetic analysis

All of the near full-length sequences were edited to exclude the flanking vector sequences and tested for putative chimeras using the Mallard program [11]. Taxonomy analysis of the 16S rRNA sequences was performed using a classifier program of the Ribosomal Database Project [12] and megaBLAST programs against the EzTaxon database [13]. All of the sequences without chimeras were assigned as operational taxonomic units (OTUs) based on alignment using the Mothur program, with 97% sequence similarity as the designated cutoff [14]. The representative of each OTU was used as a query to search for similar 16S rRNA gene sequences in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence alignment was performed using ClustalX 2.0 to obtain a uniform length, and a phylogenetic tree was created using the neighbor-joining method with the MEGA4.0 program. A total of 1000 bootstrap replicates were generated.

The coverage of the 16S rRNA gene library was calculated using the formula $[1 - (n/N)]$, where n is the number of OTUs represented by one clone, and N is the total number of clones [15]. Bacterial diversity and richness were calculated using the Shannon-Weaver index and Simpson Index, respectively [16]. Rarefaction analysis was performed using the Mothur program at 97%, 95% and 90% sequence similarity thresholds [14].

1.5 Nucleotide sequence accession number

The 16S rRNA genes in this study were deposited in the GenBank database under accession numbers JX457153-JX457317.

2 Results

2.1 Bacterial community in the *P. americana* gut

To examine the total gut bacterial community, we performed 16S rRNA gene sequencing. A total of 170 clones were randomly selected and sequenced, of which 5 were identified as chimeras; 13 showed high sequence identity to the bacteria from cockroach fat

body and were thus excluded. Thus, 152 of the 16S rRNA gene sequences were classified into 54 different phylotypes with 97% sequence similarity as the designated cutoff [14]. Based on sequence analysis, 21 phylotypes showed < 97% sequence identity and may be novel.

Of the 152 clones, 66.4% were affiliated with the phylum *Proteobacteria*, followed by *Bacteroidetes* (17.8%), *Firmicutes* (14.5%), *Fusobacteria* (0.6%) and unclassified bacteria (0.6%). The Shannon-Weaver index, a measure of diversity, was 3.395, and the Simpson index was 0.06. The rarefaction curve was not saturated at a cutoff of 97%, indicating that the number of analyzed clones may not be sufficient to describe bacterial diversity within the cockroach gut at the species level (Figure 1). However, a reasonable coverage of 81% was obtained, indicating that the majority of the intestinal bacteria were included.

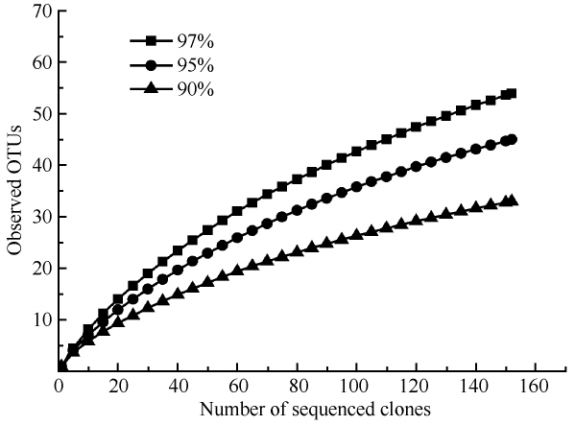

Figure 1. Rarefaction curves of 16S rRNA gene libraries recovered from the *P. americana*. The curves were generated based on analyses performed by the Mothur program. The expected number of clones was calculated from the number of clones analyzed at the species level with 97% sequence identity, genus level with 95% sequence identity, and family/class level with 90% sequence identity.

2.2 Phylotypes assigned to *Proteobacteria* and *Fusobacteria* phyla

A total of 101 clones belonging to 21 phylotypes were assigned to the phylum *Proteobacteria*, and most were related with the class *γ-proteobacteria* (Figure 2).

In the phylum *Proteobacteria*, 13 phylotypes (83 clones) clustered with bacteria from the guts or feces of distantly related animals, such as flies, mosquitoes, giant pandas, and humans. In addition, 6 clones assigned to two phylotypes clustered with uncultured bacteria from *Shelfordella lateralis* [17].

Only CR_38, a phylotype that was represented by only one clone, was assigned to the phylum *Fusobacteria* and clustered with uncultured *Fusobacterium* from *S. lateralis* [17] (Figure 2).

2.3 Phylotypes assigned to the *Bacteroidetes* phylum

A total of 27 clones were classified in the phylum *Bacteroidetes* (Figure 3). Seven phylotypes (13 clones) neighbored with gut bacteria from *S. lateralis*, which corresponded to 48% of the clones in the phylum. Five phylotypes clustered with the sequences from termites, with 93% -95% sequence identity.

2.4 Phylotypes assigned to the phylum *Firmicutes*

A total of 22 clones were classified in the phylum *Firmicutes*, and the majority belonged to the class *Bacilli*, order *Lactobacillales* (Figure 4). Under *Lactobacillales*, 8 phylotypes represented by 15 clones branched with *Enterococcus*, *Lactobacillus*, *Paralactobacillus*, and *Lactococcus* bacteria, which may be lactic acid bacteria that are involved in lactate and acetate production in the *P. americana* gut [7]. Two phylotypes branched with uncultured bacteria of *Enterococcaceae* and *Lactobacillaceae* families from the *S. lateralis* gut [17], with 94% and 99% sequence identity, respectively (Figure 5).

2.5 Clones with high sequence identity with potential pathogens

A list of the phylotypes with high sequence identity to potential pathogens along with their medical significance is shown in Table 1. In this study, 9 phylotypes represented by 28 clones shared high identity with known potential pathogens. Most of them were also identified in *P. americana*, *B. germanica*, and *B. orientalis* from houses and hospitals with culture-dependent methods, except for *Dysgonomonas* sp., *Myroides* sp., and *Providencia* sp. (Table 1).

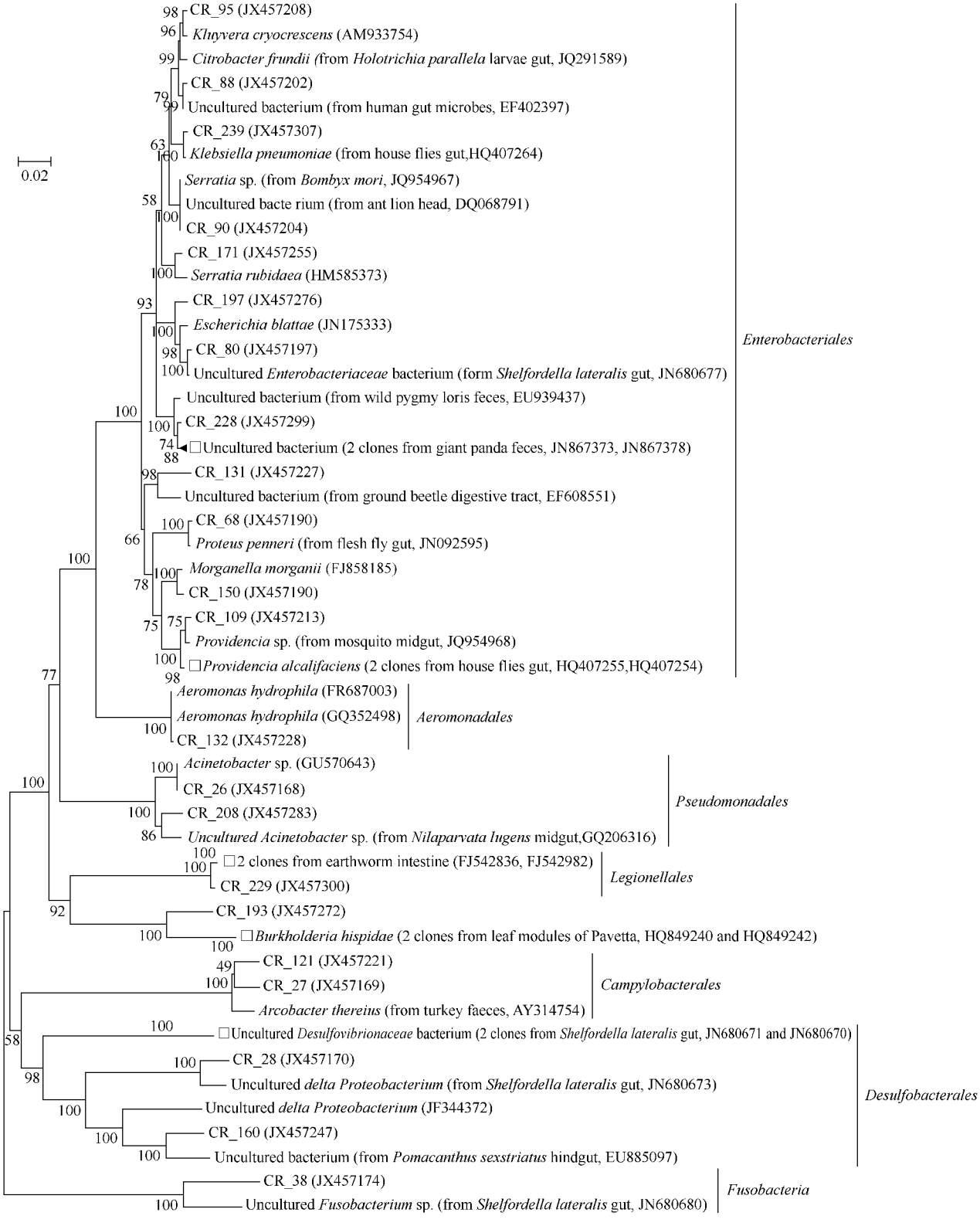

Figure 2. Phylogenetic tree of the intestinal bacteria affiliated with phylum *Proteobactria* and *Fusobacteria*. Numbers at each branch points indicate the percentage supported by bootstrap based on 1 000 replicates. Numbers in parentheses represent the sequence accession number in GenBank. The scale bar: 0.02 substitution per nucleotide.

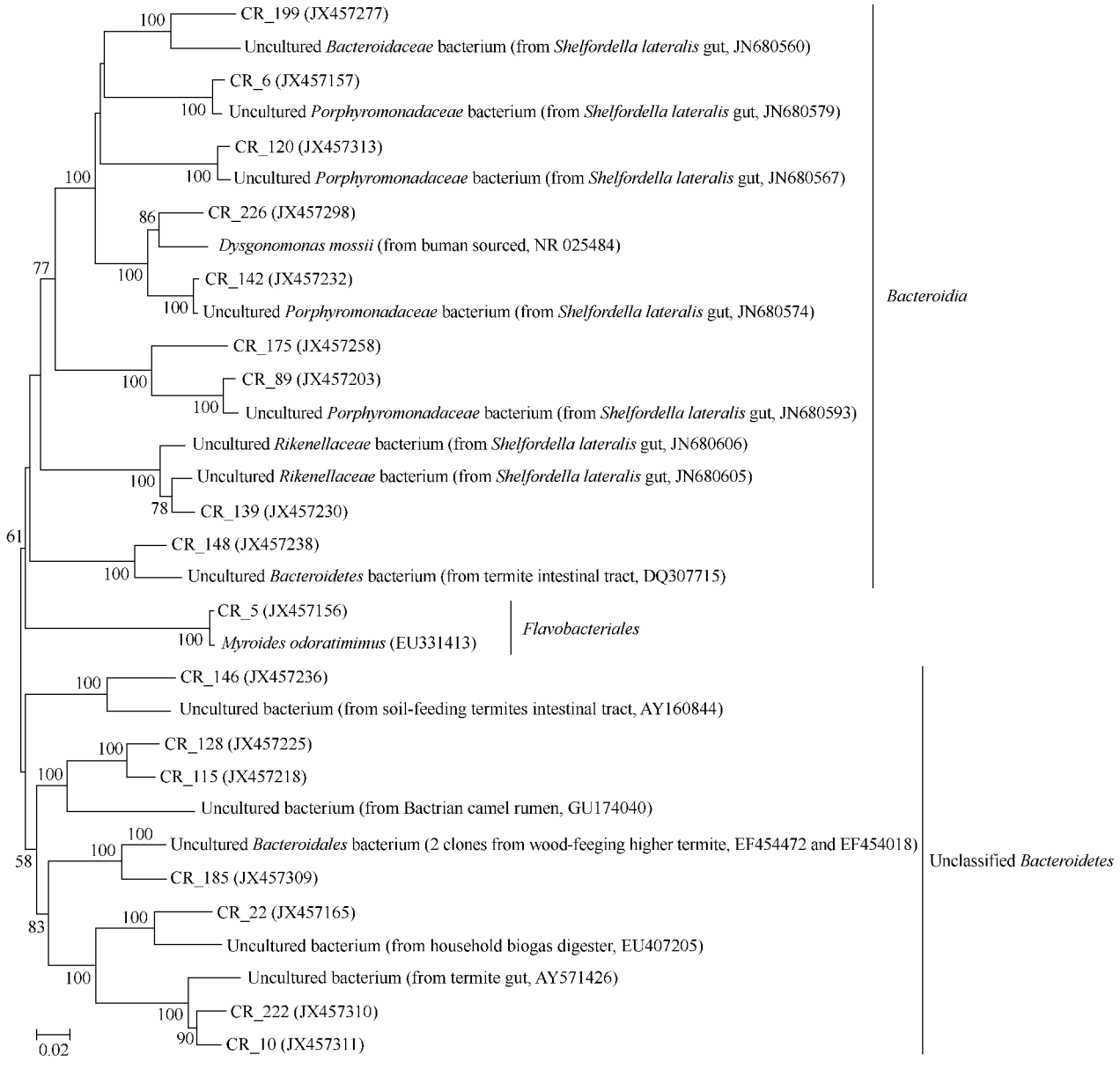

Figure 3. Phylogenetic tree of the intestinal bacteria affiliated with phylum *Bacteroidetes*. Numbers at each branch points indicate the percentage supported by bootstrap based on 1000 replicates. Numbers in parentheses represent the sequence accession number in GenBank. The scale bar: 0.02 substitution per nucleotide.

3 Discussion

To recover and analyze the 16S rRNA genes of gut microbial flora from the American cockroach, the gut was dissected to release the contents for DNA extraction. In our results, 13 clones showed 99% sequence identity to *Blattabacterium* sp. from the fat body of *P. americana*, which suggested that gut preparations of cockroaches were easily contaminated with fat body tissue. Nevertheless, the percentage of 16S rRNA gene sequences from fat body bacteria was only 7%, which indicated that contamination of the template from fat body bacterial symbionts was minor. Furthermore, 79% of the clones clustered with sequences derived from various gut environments, suggesting that the samples processed in this study were reliable.

In a study based on published 16S rRNA gene sequence data, Colman *et al.* performed comparative

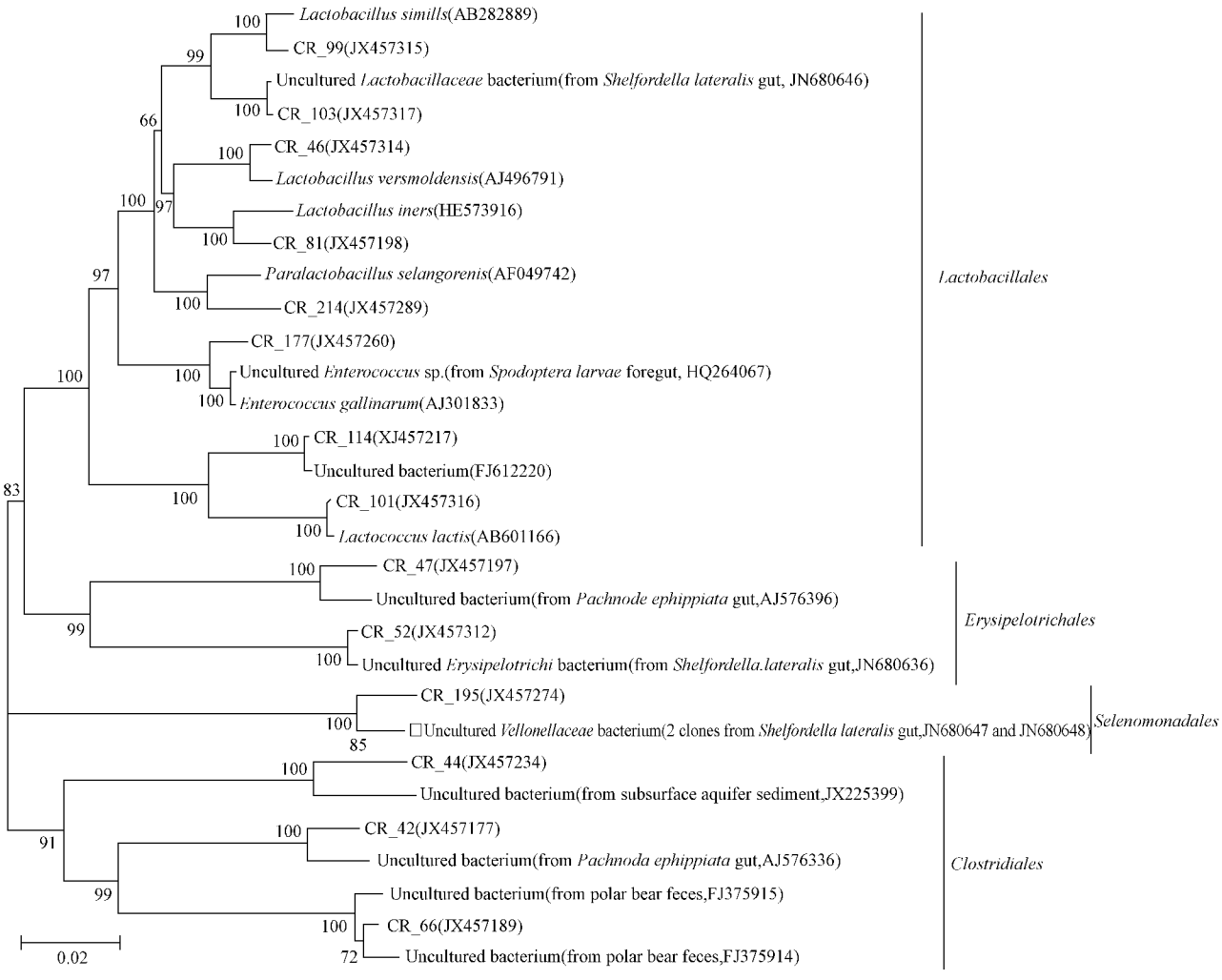

Figure 4. Phylogenetic tree of the intestinal bacteria affiliated with phylum *Firmicutes*. Numbers at each branch points indicate the percentage supported by bootstrap based on 1000 replicates. Numbers in parentheses represent the sequence accession number in GenBank. The scale bar: 0.02 substitution per nucleotide.

studies of intestinal bacterial communities from 62 insect species representing seven taxonomic orders and nine diet types [27]. However, no cockroach species were included. In the present study, some of our findings validated the observations in the 62 insects. In the *P. americana* gut, a predominance of *Proteobacteria* was found, which agreed with the findings of Colman and colleagues, who reported that *Proteobacteria* was the predominant bacterial phylum within the gut samples of non-termite insects [27]. However, this result was different from the results of *S. lateralis* colon, which closely resembled the hindgut microbiota of fungus-growing termites. Almost half of the clones from *S. lateralis* colon belonged to *Bacteroidetes*, followed by *Firmicutes* [17]. Moreover, analysis of the 16S rRNA gene sequences from the digestive system of *P. americana* revealed 54 species-level OTUs, which was almost as high as that of detritivores, the second richest of the diet guilds [27]. However, based on rarefaction analysis, the number of OTUs observed in *P. americana* was not fully saturated, which was similar to the rarefaction analysis of the *S. lateralis* colon, whose analysis indicated that the number of clones analyzed was not sufficient to describe bacterial diversity within the cockroach gut at either the genus or species level [17] (Figure 1). The low sampling coverage obtained in the cockroach intestinal bacterial community analysis was also observed in the

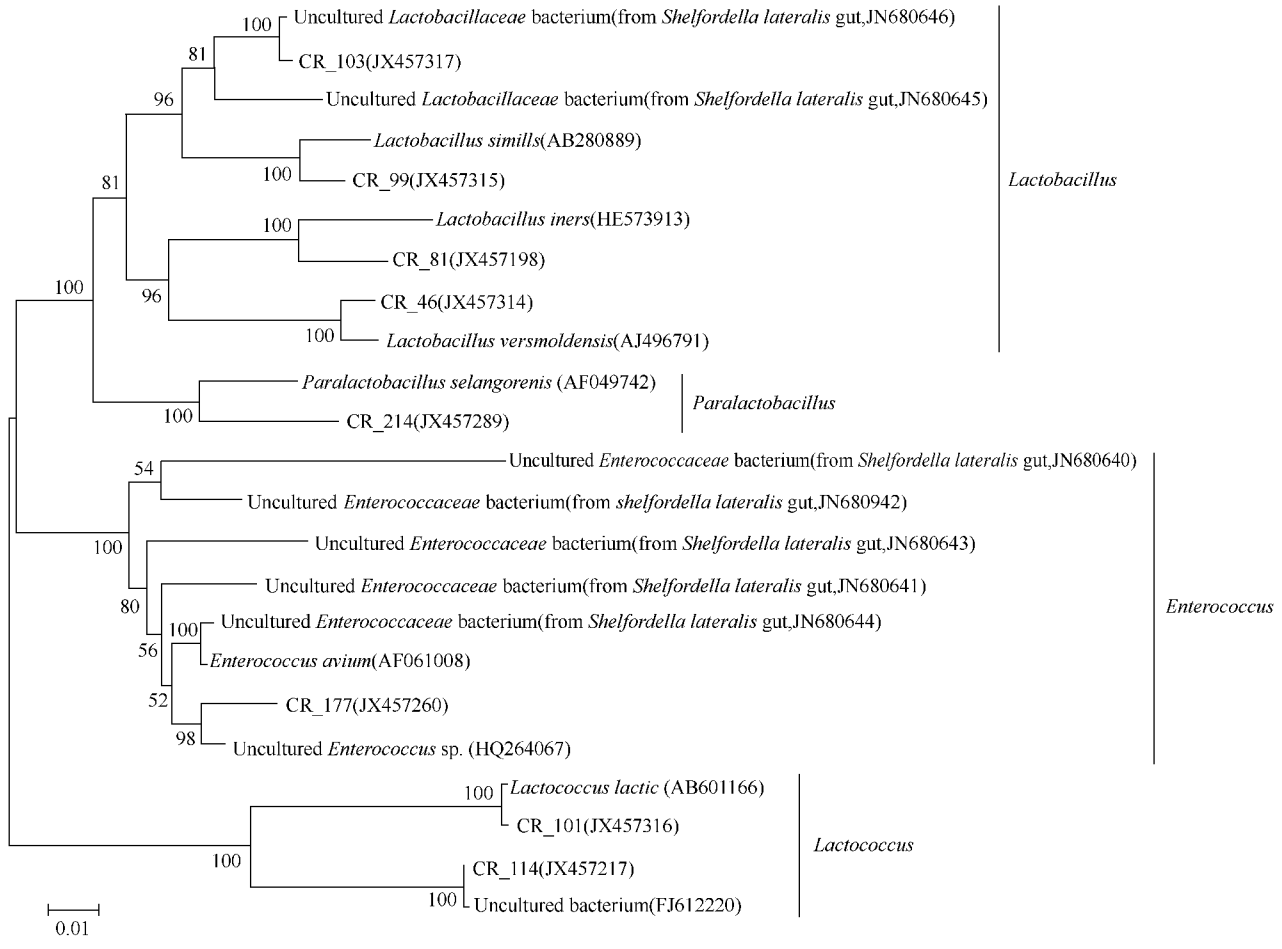

Figure 5. Phylogenetic tree of lactic acid bacteria from *P. americana* and *S. lateralis* intestines. Numbers at each branch points indicate the percentage supported by bootstrap based on 1000 replicates. Numbers in parentheses represent the sequences accession number in GenBank. The scale bar: 0.01 substitution per nucleotide.

most diverse samples, such as termites and omnivorous beetles [27]. Therefore, high intestinal microbial diversity may be a characteristic of cockroaches.

Host phylogeny is considered an important factor influencing the diversity of the gut communities of mammals as well as many insects [27-28]. As *Blattidae* cockroaches, *P. americana* and *S. lateralis* are neighboring omnivorous cockroaches that exhibit a less invasive and relative relationship [17]. This relationship was also reflected by the finding that 15% of the total clones in the *P. americana* library closely clustered with microbiota from the *S. lateralis* gut, and most were affiliated with the phylum *Bacteroidetes*. The findings suggested that host phylogeny influences the intestinal bacterial community of *P. americana*, which has been verified by observations that regardless of host diet, the gut samples from different taxonomic orders clustered exclusively together due to host taxonomy [27]. Furthermore, these bacterial lineages determined by host taxonomy may play an important role in metabolism [29]. For example, the potential lactic acid bacteria assigned to the order *Lactobacillales*, including *Lactobacillus*, *Lactococcus*, and *Enterococcus*, clustered with those from the *S. lateralis* intestine with 94% - 99% sequence identity (Figure 5). Interestingly, based on terminal restriction fragment length polymorphism analysis of *S. lateralis* gut bacteria, lactic acid bacteria were proved to play an important role. Similarly, lactic acid bacteria were also involved in lactate and acetate production from endogenous hexose to support the respiratory requirement of *P. americana* [6,10]. Therefore, some bacteria are

Table 1 List of potential pathogenic microbes isolated from *Periplaneta americana* based on taxonomic analysis

Phylotype (abundance)	Accession number	Nearest relatives	Similarity/%	Pathogenicity
CR_5 (1)	JX457156	*Myroides odoratimimus*	99.65	Nosocomial outbreak of urinary tract infection [18]
CR_132 (2)	JX457228	*Aeromonas hydrophila*	99.80	Producing a wide variety of virulence factors facilitates the organism in causing both intestinal and extra-intestinal infections [19]
CR_239 (1)	JX457307	*Klebsiella pneumoniae*	99.52	Opportunistic pathogens and can give rise to severe diseases such as septicemia, pneumonia, urinary tract infections, and soft tissue infections [20]
CR_109 (3)	JX457213	*Providencia rettgeri*	99.26	Opportunistic pathogenic involved in ocular infections [21]
CR_150 (2)	JX457239	*Morganella morganii*	99.19	Opportunistic pathogenic involved in urinary tract and wound infections [22]
CR_171 (1)	JX457255	*Serratia rubidaea*	99.89	Invasive infection [23]
CR_68 (2)	JX457190	*Proteus mirabilis*	99.13	Common pathogen responsible for complicated urinary tract infections and bacteremia [24]
CR_95 (9)	JX457208	*Citrobacter freundii*	99.26	Peritonitis and tunnel infection [25]
CR_88 (7)	JX457202	*Enterobacter aerogenes*	99.53	Important nosocomial pathogen [26]

speculated to play an important role in metabolism and thus originated from a common ancestor.

The dietary habit is another critical factor that affects the intestinal bacterial community composition of insects. Colman and colleagues found that microbial communities were highly similar and largely clustered together according to feeding habits, which has been verified in insects, including detritivorous species, carnivorous species, and xylophagous species [27]. However, no such investigation has been performed for omnivorous species. As omnivorous insects, houseflies have the ability to feed on a variety of organic substrates, of which many of the intestinal bacterial phylotypes exhibited high similarity with those associated with humans, domestic animals, and plants [16]. Similarly, the majority of the gut bacteria from *P. americana* and *S. lateralis* also clustered with bacteria from the gut environments of more distantly related animals, which suggest that the intestinal bacterial community may be influenced by dietary habit [17]. Because of the habitat preferences and unrestricted movement, the omnivorous cockroaches are able to include high- and low-fiber substrates in the diets. Previous studies on the cockroach *P. americana* have shown that gut-associated bacteria played the key role in digesting any food available [17]. Thus, to meet the requirement of digesting all types of food in different habitat, it may be necessary for the omnivorous cockroaches to possess a large and diverse gut microbial population, which is a typical feature of other omnivorous animals and may contribute to the omnivorous lifestyle.

Cockroaches are opportunistic and omnivorous insects that are in close contact with human beings and can thrive under poor sanitary conditions. Based on the 16S rRNA genes of the microbiota in the gut, potential pathogens have been identified in some insects, such as houseflies and *S. lateralis* [16-17]. Our survey revealed that 18.4% of the gut bacteria from the American cockroach exhibited high identity with potential pathogens, of which three genera, *Dysgonomonas*, *Myroides*, and *Providencia*, had not been previously been identified from cultured isolates (Table 1). Therefore, more effective control strategies aimed at reducing insect pest populations are important. To control termites, Shinzato *et al.* proposed defining termite-specific bacterial lineages in the intestine as targets to develop novel control strategies, e.g., disturb the metabolism of digestive symbionts or kill key microbes [29]. Similarly, common bacterial lineages in the intestine of various species of cockroaches may be a potential target for cockroach control. However, due to the limited information about the intestinal bacterial community of different species of cockroaches, cockroach-specific bacterial lineages remain unclear and are an interesting research object.

Along with the analysis of the bacterial community of *S. lateralis* colon, our findings from *P. americana* provide more data on the intestinal bacteria from cockroach species and provide evidence that host

taxonomy and omnivorous dietary habit determine the intestinal bacterial community composition of the insects. Furthermore, the findings presented here will most likely contribute to identifying potential common pathogenic bacterial lineages. Further studies are needed to evaluate the potential physiological functions of the common bacterial lineages, such as culture-based methods, which will contribute to identifying more effective methods of cockroach control.

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美洲大蠊(*Periplaneta americana*)肠道微生物多样性分析

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摘要:【目的】分析美洲大蠊(*Periplaneta americana*)肠道微生物群落的组成。【方法】以美洲大蠊肠道微生物基因组为模板,Bact-27F 和 Univ-1492R 为引物,PCR 扩增 16S rRNA 基因,连接 pGEM-T 载体,构建肠道微生物 16S rRNA 基因文库,并对肠道微生物的组成及多样性进行分析。【结果】美洲大蠊肠道微生物主要包括变形杆菌门(*Proteobacteria*, 66.4%),拟杆菌门(*Bacteroidetes*, 17.8%),厚壁菌门(*Firmicutes*, 14.5%),梭杆菌门(*Fusobacteria*, 0.6%),以及未分类微生物(unclassified bacteria, 0.6%)。系统发育分析显示,15% 的美洲大蠊肠道微生物 16S rRNA 基因序列与亲缘关系较近的杂食蟑螂肠道微生物的 16S rRNA 基因序列聚于一支;59% 的美洲大蠊肠道微生物 16S rRNA 基因序列与不同食性动物肠道微生物的 16S rRNA 基因序列聚于一支。另一方面,18% 的美洲大蠊肠道微生物 16S rRNA 基因序列与潜在的微生物致病菌一致性高于 99%,说明美洲大蠊是一类潜在的致病菌携带者。【结论】美洲大蠊肠道微生物群落组成多样,宿主系统进化以及杂食性生活方式对其肠道微生物的组成有较大影响。

关键词:美洲大蠊, 16S rRNA 基因, 微生物群落

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