Dominant Egg Surface Bacteria of Holotrichia Oblita (Coleoptera: Scarabaeidae) Inhibit The Multiplication of Bacillus Thuringiensis and Beauveria Bassiana

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Research Article

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Abstract

Many species of scarabs (Coleoptera: Scarabaeidae) are important pests. *Bacillus thuringiensis* (Bt) and *Beauveria bassiana* (Bb) are entomopathogens that have been used as biocontrol agents of various pests. However, neither provides adequate control of scarab pests in the field. In this study, we used *Holotrichia oblita*, an important scarab pest in China, as a model to explore the reason of difficult to control. Compared to other scarabs, *H. oblita* could form a unique egg cocoon structure, in which the young larvae complete the initial development process. We analyzed the microbiome of eggs surface, egg cocoon and bulk soil respectively, and investigated the role of these microorganisms in pathogen infection. 16S rRNA gene analysis revealed low bacterial richness in egg surface with *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Fusobacteria* dominating in terms of OTUs number, and indicated that the microbial community structure varied among egg and soil samples. Some dominant species of the egg surface have been reported to exhibit antimicrobial activity, which may contribute to the low susceptibility of young larvae to entomopathogens. The further confrontation culture analysis verified this hypothesis. We found that 11 of 28 cultivable isolates possessed antimicrobial activity against both Bt and Bb, and all isolates from egg surface samples showed antagonistic ability. Four isolates belonging to *Pseudomonas* showed strong Bt- and weak Bb-antagonistic ability, and this genus significantly increased in egg surface samples. This study demonstrated that *H. oblita* egg has a unique microbial community structure, containing microorganisms with antimicrobial activity against entomopathogens. These microorganisms protect the newly hatched larvae from pathogen infection.

Introduction

Many species of Scarabaeidae (Coleoptera) are plant pests that cause significant economic losses in agriculture, horticulture, and forestry [1,2]. The adults typically feed on aerial plant structures, while the larvae, known as white grubs, feed on plant roots. Some root-feeding species are major pests.

Application of chemical insecticides is the main method used to control grubs. However, the extensive use of chemicals poses a threat to the environment and public health. Environmentally friendly microbial agents that could control scarabs would be a safer alternative. *Bacillus thuringiensis* (Bt) is a gram-positive bacterium that produces one or more insecticidal crystal proteins (Cry, Cyt) or vegetative insecticidal proteins (Vip), which exhibit specific toxicity against many insects, especially Lepidoptera and Coleoptera [3,4]. Following the ingestion of the endotoxin crystals by susceptible insects, the crystals are solubilized by the alkaline conditions in the midgut and are subsequently proteolytically converted into a toxic core fragment. The activated toxin binds to midgut receptors and forms ionic pores that kill midgut cells, or induces a cell death signaling pathway, finally leading to insect death [5,6]. Different Bt strains, such as HBF-1, HBF-18, Bt185, and B-JJX, have insecticidal activity against scarab pests [7-10]. Fungi are also used as biological insecticides for the control of insect pests. Compared to bacteria and viruses, fungi can directly penetrate through the insect cuticle and multiply in the hemocoel. This makes them useful biological control agents of white grubs [11,12]. *Beauveria bassiana* (Bb) is the most widely

distributed entomopathogenic fungus in the world. It infects a variety of insect species and causes white muscardine disease. Several Bb-based products are commercially available for the control of the coffee borer beetle (*Hypothenemus hampei*) and various species of Curculionidae [13-15].

Both Bt and Bb are effective against scarab larvae in lab experiments, but, under field conditions, they are often not as effective. In China, *Holotrichia oblita*, *H. parallela*, and *Anomala corpulenta* are the most important grub pest species. They damage many plants, such as sweet potatoes, soybeans, peanuts, and the Siberian elm [16]. Exposed to the same Bt or Bb agents, *H. oblita* was less susceptible than *H. parallela* or *A. corpulenta*[3,9,10,17]. *H. oblita* was able to survive using different defense mechanisms. Our previous study indicated that the larval gut bacteria may potentially affect the susceptibility of scarabs to Bt strains. The proportion of gut isolates with antibacterial activity against Bt strains was 90% in *H. oblita*, 75% in *H. parallela* and 80% in *A. corpulenta*[18].

Scarab adults usually oviposit in the soil near their preferred host plants. Compared to *H. parallela* and *A. corpulenta*, *H. oblita* forms a unique egg cocoon structure by mixing its eggs with the surrounding soil. There is a variety of microbes in the soil, some of which can produce compounds adversely affecting the growth of pathogens [19-21]. Biocontrol agents generally target young larvae; such as 3 to 5-day-old emerging larvae in lab experiments [9,10], however, under natural conditions, the grubs can often resist pathogen invasion and hatch normally. This unique egg cocoon structure may contribute to the relatively low susceptibility of young larvae to entomopathogens.

To study the role of egg bacteria in pathogen infection, we collected 20 samples from the *H. oblita* egg surface (E), egg cocoon (C), and bulk soil (B), and analyzed the microbial community structure using 16S rRNA gene sequencing. We tested the antimicrobial potential of 28 cultivable isolates against scarab-specific Bt and Bb strains using confrontation culture analysis. The results suggest that dominant egg surface bacteria can inhibit the multiplication of Bt and Bb strains.

Methods

Sampling and DNA extraction. H. oblita was collected from a field in Cangzhou, Hebei Province, China. The adults were reared in plastic boxes (66 cm by 41 cm by 18 cm) filled with soil containing willow leaves at a temperature of 25℃ until they laid eggs.

Egg cocoons were collected and peeled off, then the eggs were transferred to a sterile 2 ml plastic centrifuge tube containing 1 ml sterile water and sonicated for 5 min in an ultrasonic cleaning bath (Bransonic 32) to dislodge bacteria. After centrifugation at 10,000×g for 5 min, the microorganisms in the wash buffer were collected and defined as the egg surface (E) sample, and resuspended with 1 ml sterile water. The egg cocoon soils (1 g) and soils (1 g) 10 cm away from the egg cocoons were suspended in 5 ml sterile water and centrifuged at 10,000×g for 5 min, and the pellets were defined as the egg cocoon (C) sample and the bulk soil (B) sample (Figure 7) and resuspended with 1 ml sterile water. A total of 20 samples were collected, including 6 E samples, 7 C samples, and 7 B samples.

For egg surface (E) samples, 1 µl of the microorganism suspension was directly used as a template for PCR. For soil samples (B and C), a 900 µl suspension was used to extract genomic DNA, using a PowerSoil® DNA Isolation kit (MoBio Laboratories, Carlsbad, CA, USA), and 1 µl of DNA was used as a template for PCR amplification. The remaining 100 µl suspension of 20 samples was kept for conventional culture using solid Luria Bertani (LB) agar medium, and single colonies were picked from the plates and repeatedly grown on solid agar plates until pure cultures were obtained. A total of 28 cultivable isolates were collected, including 3 isolates from E samples, 7 isolates from C samples, and 18 isolates from B samples. Genomic DNA of each isolate was extracted as previously described [49].

16S rRNA gene sequencing and bioinformatic analysis. The V3-V4 region of microbial 16S rRNA genes of 20 samples were amplified by PCR using the specific primers, 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-'3). PCR products were purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The TruSeq ® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) was used for 16S rRNA gene amplicon library construction. The Qubit @ 2.0 Fluorometer (Thermo Scientific, Rockford, IL, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA, USA) were used for library quality assessment. Finally, the library was sequenced on the Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA), and 250 bp paired-end reads were generated. Raw Data were trimmed using Trimmomatic (version 0.36) [50] with default parameters. Then the clean paired-end reads were assembled into raw tags using Usearch (version 9.2.64) [51]. The primer sequences in the raw tags were trimmed, and effective tags were obtained. The 16S rRNA sequence data of 20 samples were deposited in Sequence Read Archive (SRA) database under BioProject ID PRJNA637400, with accession number SRR11931252–SRR11931271.

The operational taxonomic units (OTUs) were clustered at 97% identity cutoff with a Usearch UPARSE algorithm [52]. Then the chimera sequences were removed on the basis of the UPARSE pipeline analysis. The OTU annotation was performed using the Usearch SINTAX algorithm [53], against RDP training set (version 16) 16S rRNA Database with a confidence threshold of 0.8. OTUs annotated as chloroplast or mitochondria or OTUs not annotated to the kingdom level were abandoned. QIIME (version 1.7.0) pyNAST algorithm [54] was used for species annotation against the GreenGene Database[55]. Usearch (version 9.2.64) [51] was used to calculate Alpha diversity metrics, including the indexes (shannon, chao1) reflecting the sample community richness, and indexes (simpson, dominance, and equitability) reflecting the sample community evenness. QIIME (version 1.7.0) [56] was used to calculate Beta diversity to estimate variation between samples. Principal Component Analysis (PCA) and Non-Metric Multi-Dimensional Scaling (NMDS) analysis were performed using R package (https://www.r-project.org/) to visualize complex relationships between samples. Lda Effective Size (LEfSe) test for variability of microbiota was calculated using lefse (Version 1.0.7) (http://huttenhower.sph.harvard.edu/galaxy/).

For 28 cultivable isolates, the 16S rRNA genes of each isolate were amplified using the specific primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-'3). Then the 16S rRNA

sequences were identified through aligning against NCBI 16S rRNA sequence (Bacteria and Archaea) database with BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Confrontation culture analysis. Four entomopathogen strains were used: 3 scarab-specific Bt strains (HBF-1, HBF-18, Bt185)[7-9], and one Bb strain BBNS-J9-16 (preservation number: CGMCC No.5288).

The dual culture tests for antagonistic ability of 28 cultivable isolates against Bt strains were processed using the cup-plate confrontation culture method, as previously described [18]. Sterile water was used as a negative control. The observable inhibition zones were used as indicators of the antibacterial activity of the 28 isolates against Bt strains.

For the antagonistic ability analysis of the 28 isolates against Bb strain, the isolates were cultured at 30℃ with shaking at 220 rpm. The Bb strain was cultured on PDA for 2–4 d. Subsequently, fungal culture plugs were placed in the middle of LB agar plates. Five dishes of sterile blotter paper (6 mm diam.) were placed on the surface of the plate and inoculated with 10 µl of the cultured bacterial suspension. The amphotericin-B and sterile water were used as positive and negative controls, respectively. The plates were incubated at 28℃ for 5 d, and inhibition zones induced by 28 isolates against Bb strain were recorded.

Genome sequencing and secondary metabolite analysis. Four cultivable isolates with strong antagonistic ability against Bt strains and weak antagonistic ability against Bb strain were selected for draft genome sequencing, using the Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA, USA). The produced reads were cleaned by removing reads with Ns or more than 20% low-quality bases, and 1 Gb 2×100 bp pair-end clean reads for each isolate were obtained. The SPAdes [57] was used for genome assembly, and GeneMark [58] was used for gene prediction. The antiSMASH 2.0 pipeline [59] was used for secondary metabolite analysis of these four isolates.

Phylogenetic analysis. All the 16S rRNA sequences of 28 cultivable isolates were analyzed using MEGA (version 7) [60] and an online tool iTOL: Interactive Tree of Life (http://itol.embl.de/) [61]. The analysis included bootstrapping values with 1000 replications.

For the phylogenetic analysis of four genome sequenced isolates, we constructed the whole-genome-based tree using CVTree [26] with *k-string* = 6, and PHYLIP [62]. The iTOL was used to annotate the tree [61].

Results

Microbial diversity comparisons between egg and soil samples. The bacterial composition of different samples from H. oblita egg surface (E), egg cocoon (C), and bulk soil (B) was determined by sequencing analysis of the 16S rRNA gene. A total of 2,748,824 raw reads were generated from 20 samples, including 667,427 raw reads from six E samples, 924,724 raw reads from seven C samples, and 1,156,673 raw reads from seven B samples. After removing the short reads and trimming the low-quality regions, a total of 1,894,784 effective tags were identified, with an average length of 415 bp. All the effective sequences

were clustered into 49,503 OTUs at 97% DNA sequence similarity, including 1,863 OTUs from E samples, 22,827 OTUs from C samples, and 24,813 OTUs from B samples (Tables S1 and S2).

Alpha diversity analysis was then performed to assess the diversity and evenness of the microbial population from different samples. The alpha diversity patterns were variable across the soil samples (B and C) and egg surface samples (E) (Figure 1). The number of observed OTUs and alpha diversity analysis based on Shannon and Chao1 indexes indicated that soil samples had more microbial diversity than the egg surface samples (Figure 1A and Table S2). A previous study in our laboratory showed that the microorganism collection method could affect the community structure, where the phyllosphere community diversity was lower for samples subjected to DNA extraction than for those subjected to direct PCR [22]. In the present study, we performed direct PCR for E samples and added a DNA extraction process before PCR for B and C samples. The results confirmed that the community diversity of soil samples was much higher than the egg surface samples. The results of Simpson, dominance, and equitability indexes indicated that, compared to the B and C samples, the evenness of sample E decreased (Figure 1B and Table S2). The rarefaction curve based on the Shannon index showed that all samples reached a plateau, suggesting that our sampling effort was sufficient to obtain a full estimate of OTU richness (Figure S1).

Among all samples, 26 phyla, 143 families, and 300 genera were identified. *Proteobacteria* was the dominant phylum and comprised the majority of all detected microorganisms (approximately 44.63%) (Figure 2A). This is typically observed in other soil libraries [23-25]. *Actinobacteria*, *Acidobacteria*, and *Bacteroidetes* were also abundant in egg cocoon (C) samples and bulk soil (B) samples. In E samples, *Firmicutes*, *Bacteroidetes*, and *Fusobacteria* were the most abundant phyla (Figure 2A). The community structure varied markedly among different samples, outlined by the Lefse LDA results (Figure 3A). Compared with bulk soil samples, the composition of *Firmicutes*, *Bacteroidetes*, and *Fusobacteria* and the composition of *Actinobacteria* and *Acidobacteria* significantly increased in egg surface samples and egg cocoon samples, respectively (Figure 3A). Bray-Curtis tree and PCA analysis also indicated that microbiota in different samples were clearly separated at the phylum level (Figure 2A and Figure 3B). PC1 and PC2 explained 73.8% and 14.8% of the global variation, respectively (Figure 3B). Similar results were observed in the NMDS analysis based on Weighted UniFrac distances (Figure S2).

At the family level, Sphingomonadaceae and Xanthomonadaceae in phylum *Proteobacteria* and Chitinophagaceae in phylum *Bacteroidetes* were enriched in B samples. Rhodospirillaceae in phylum *Proteobacteria* and Micrococcaceae in phylum *Actinobacteria* were enriched in C samples. The families Enterobacteriaceae, Moraxellaceae, and Desulfovibrionaceae in phylum *Proteobacteria*, Porphyromonadaceae in phylum *Bacteroidetes*, Leptotrichiaceae in phylum *Fusobacteria*, and Ruminococcaceae and Lachnospiraceae in phylum *Firmicutes* were enriched in E samples (Figure 2B). Differences were also observed at the class, order, and genus level (Figure S3).

From 20 samples, we isolated 28 strains with different colony morphology and found the number of cultivable isolates from bulk soil samples (18 strains from B) was much higher than egg cocoon samples (seven strains from C) and egg surface samples (3 strains from E). Then we performed 16S rRNA gene sequencing to identify these 28 isolated strains. All the sequences were aligned against the NCBI database using BLAST, and the results showed that these 28 isolates belonged to two phyla, *Proteobacteria* and *Firmicutes*. Phylogenetic analysis on the basis of the 16S rRNA sequences revealed that these 28 isolates clustered into four major groups at the family level, i.e., Alcaligenaceae, Pseudomonadaceae, Enterobacteriaceae, and Bacillaceae (Figure 4). Alcaligenaceae, Enterobacteriaceae, and Pseudomonadaceae belonged to the *Proteobacteria* phylum, which constituted the largest group (23 isolates). The other five Bacillaceae strains belonged to the *Firmicutes* phylum (Table S3).

The 18 isolates from bulk soil samples were composed of 7 different genera, *Alcaligenes*, *Citrobacter*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Enterobacte*, and *Serratia*. The seven isolates from egg cocoon samples were composed of four genera, *Alcaligenes*, *Bacillus*, *Citrobacter*, and *Klebsiella*. The three isolates from egg surface samples were composed of two genera, *Alcaligenes* and *Pseudomonas* (Table S3).

The effects of cultivable isolates against pathogens. We assessed the antimicrobial activity of the 28 cultivable isolates against scarab-specific Bt and Bb strains. The confrontation culture analysis showed that strains (LD01, LD9) from H. oblita egg surface (E) samples and strains (T03, T162) from bulk soil (B) samples had strong antagonistic ability against all three scarab-specific Bt strains and weak antagonistic ability against the Bb strain. All of these four strains were Pseudomonas. Strain LD02 from E samples, strain L05 from C samples, and strains (T10, T16, T101, T161, T164) from B samples showed weak antagonistic ability against all three Bt strains but showed strong antagonistic ability against the Bb strain. These seven strains belonged to Alcaligenes. The remaining 17 strains showed no antagonistic ability against the Bt and Bb strains, including 12 Proteobacteria strains and 5 Firmicutes strains (Figure 5 and Table S3).

All the three isolates from E samples showed antagonistic ability (100%, N=3) against pathogens, where the proportions of antimicrobial isolates in B and C samples were 38.89% (N=18) and 14.29% (N=7), respectively.

Genome sequencing and secondary metabolite analysis of strains with antimicrobial activity. The four strains (LD01, LD9, T03, and T162) with strong Bt-antagonistic ability and weak Bb-antagonistic ability were genome sequenced using the Illumina platform. The 16S rRNA gene sequence identification showed that these four strains belonged to the genus Pseudomonas and had the highest similarity with P. aeruginosa strain DSM50071 (99.51%–99.79%). Therefore, we collected 20 additional Pseudomonas strain genomes from the NCBI GeneBank database (http://www.ncbi.nlm.nih.gov/), including 11 P. aeruginosa strains, 7 P. mendocina strains, 1 P. denitrificans strain, and 1 P. reidholzensis strain (Figure 6 and Table S4). The whole-genome-based phylogenetic tree was constructed using CVTree and PHYLIP, with Bt kurstaki strain HD73 as an outgroup. The CVTree is an alignment-free method where each

organism is represented by a Composition Vector (CV) derived from all proteins present in its genome. CVTree has been effectively used in several phylogenetic studies of microorganisms including archaea, prokaryotes, and fungi [26-28]. The results showed that these four strains were clustered with P. aeruginosa strains, indicating they belonged to P. aeruginosa. The blue-green coloration produced during culture verified this result. Phylogenetic analysis also showed high genome similarity among these four P. aeruginosa strains, suggesting that they might be the same strain. As an opportunistic human pathogen, P. aeruginosa can be isolated from various sources, including humans, animals, hospitals, swimming pools, soil, rhizosphere, and plants [29]. P. aeruginosa is also a promising biocontrol agent for plant pathogens and pests such as Pythium sp. and the root-knot nematode (Meloidogyne incognita) [30,31]. Nga et al (2013) found that P. aeruginosa isolated from the rhizosphere of a watermelon plant showed high antagonistic ability against both bacterial and fungal pathogens on rice, watermelon, and cabbage [32]. Our study showed that P. aeruginosa also had antagonistic ability against entomopathogenic Bt and Bb strains.

Then we used antiSMASH 2.0 pipeline to identify and annotate the putative secondary metabolite biosynthesis gene clusters in the four strains. A total of 62 gene clusters were identified, including 18 NRPS (non-ribosomal peptide synthetase cluster), 9 NRPS-like fragments, 8 hserlactone (homoserine lactone cluster), 7 bacteriocin, 8 phenazine, 4 CDPS (tRNA-dependent cyclodipeptide synthases), 4 NAGGN (N-acetyl-glutaminyl-glutamine-amide), and 4 thiopeptides (Table S5), some of which belonged to antimicrobial compounds. For example, phenazines, redox-active small molecules, have antibiotic properties toward many bacteria and fungi and can damage mammalian cells [33-35]. Thiopeptide antibiotics are a prominent class of antimicrobials with potent activity against gram-positive bacteria and many drug-resistant pathogens [36].

In this study, we also found 7 *Alcaligenes* strains showed strong Bb-antagonistic ability and weak Bt-antagonistic ability. *Alcaligenes* is known among bacteria having antibacterial and antifungal activity, for example, active on *Microcrocystis* spp. and *Fusarium oxysporum* [37,38]. These 7 *Alcaligenes* strains in the present study may also possess some antimicrobial compounds.

Discussion

H. oblita is one of the main soil-dwelling scarab pests in China. Biocontrol agents Bt and Bb usually target young larvae, such as 3 to 5-day-old emerging larvae in lab experiments [9,10]. However, the control effect by these agents is not as efficient as expected under field conditions. Compared to other scarab pests, *H. oblita* could form a unique egg cocoon structure, and the young larva completes the initial development process inside this structure.

Microorganisms can confer various benefits on hosts, such as supplying nutrients, or protect against pathogen invasion [39]. The microbiota of different scarab insects exhibits some similarities, meanwhile can be affected by factors such as insect species, host developmental stage, habitats, pH, temperature,

even different tissues of the host [40,41]. For example, significant difference of community structure was observed in the midgut and hindgut of one scarab larvae, *Protaetia brevitarsis* [42]. We previously demonstrated that *H. oblita* larvae gut isolates contributed to the relatively low susceptibility of young larvae to entomopathogens. [18]. However, as an important part of *H. oblita* developmental stage, the microbiota of egg and egg cocoon has not been investigated before, and the role of the unique egg cocoon structure in pathogen infection remains poorly understood. To this end, we analyzed the microbiome of egg surface, egg cocoon and bulk soil in this work respectively. The average numbers of OTUs in egg surface (310.50 ± 46.97) significantly decreased than egg cocoon (3261.00 ± 310.52) and bulk soil (3544.71 ± 124.14), indicating the microbial diversity of egg surface was less than soil. And the average Simpson indexes of egg surface, egg cocoon and bulk soil were 0.105 (± 0.024), 0.008 (± 0.002), and 0.008 (± 0.001) respectively, indicating the community unevenness of egg surface increased. The further microbial composition analysis demonstrated the community of egg surface was selective. At the genus level, the composition of *Clostridium* from *Firmicutes*, *Pseudomonas, Acinetobacter, Desulfovibrio, Enhydrobacter, Delftia, Sphingobium* from *Proteobacteria*, *Dysgonomonas, Emticicia* from *Bacteroidetes*, *Sebaldella* from *Fusobacteria*, and *Deinococcus* from *Deinococcus-Thermus* significantly increased in egg surface samples (Figure S4), some of which have been reported to exhibit antimicrobial activity. For example, the genus *Clostridium* mainly occurs in soil, and *C. butyricum* of this genus has been shown to have antagonistic interaction against *Candida albicans*, *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp., *Vibrio* spp. and *Helicobacter pylori*, as well as some fish pathogens [43,44]. *Acinetobacter* is an emerging nosocomial pathogen, with a growing prevalence worldwide [45]. We previously reported that two *Acinetobacter* strains isolated from the grub gut exhibited strong antibacterial activity against Bt strains [18]. Many species of genus *Pseudomonas* have been proved synthesize a variety of compounds with antagonistic activity [32,46]. Species of *Sphingobium* and *Delftia* are also producing antimicrobial compounds, such as delftibactin A, which inhibit the growth of some common pathogenic microbes [47,48]. These species may play an important role in the defense process of *H. oblita* larvae against biocontrol agents. To further verify this hypothesis, we isolated 28 strains from egg and soil samples, and assessed the antimicrobial potential against Bt and Bb. All the four *Pseudomonas* isolates and seven *Alcaligenes* isolates showed antimicrobial activity against both Bt and Bb.

Scarab larvae gut isolates from *H. oblita*, *H. parallela*, and *A. corpulenta* belonged to four phlya, *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. Most of these gut isolates (81.25%, N=32) exhibited antibacterial activity against Bt strains. And *Proteobacteria* constituted the largest group (62.07%, N=29) [18], which was also the dominant group among all samples in the current study (approximately 44.63%). *Firmicute* and *Bacteroidetes* were the most dominant phyla in egg surface, and *Actinobacteria* was the dominant phylum in egg cocoon. Under natural conditions, the newly hatched larvae will eat the egg shells and feed on the soil around the eggs. During this process, the larvae may feed on these specific microorganisms, which could help them build a stable intestinal microbiota, and protect them from pathogen infection.

Declarations

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Author Contributions

C.S., G.W. and J.Z. designed the study, C.L. and L.G. provided the insect and collected samples, Q.L. carried out the experimental work, K.W. did the data analysis, K.W. and C.S. wrote the manuscript. All authors read and approved the final version of the manuscript.

Additional information

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

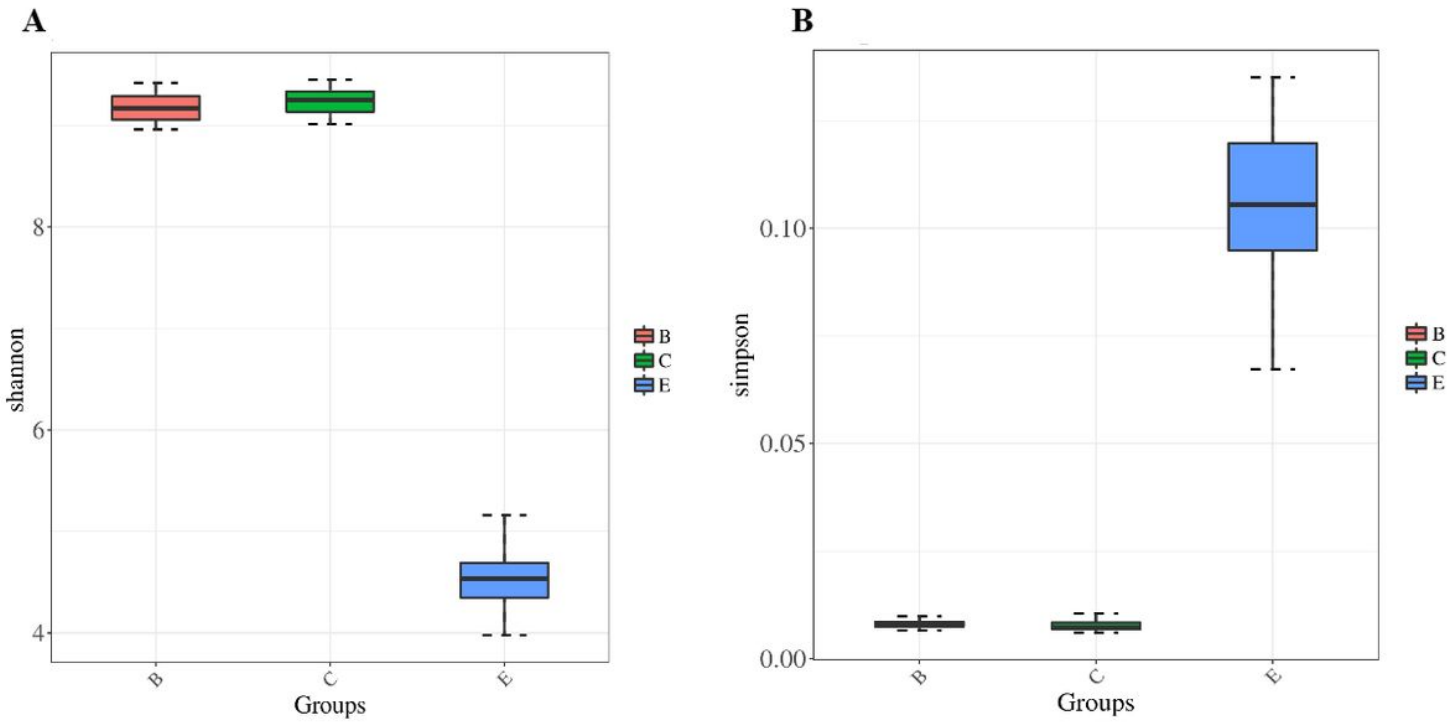

Figure 1

The distribution of alpha diversity indexes of OTUs from bulk soil (B), egg cocoon (C) and egg surface (E) samples. Shannon index (A) reflects the richness of the sample community, and Simpson index (B) reflects the evenness of the sample community.

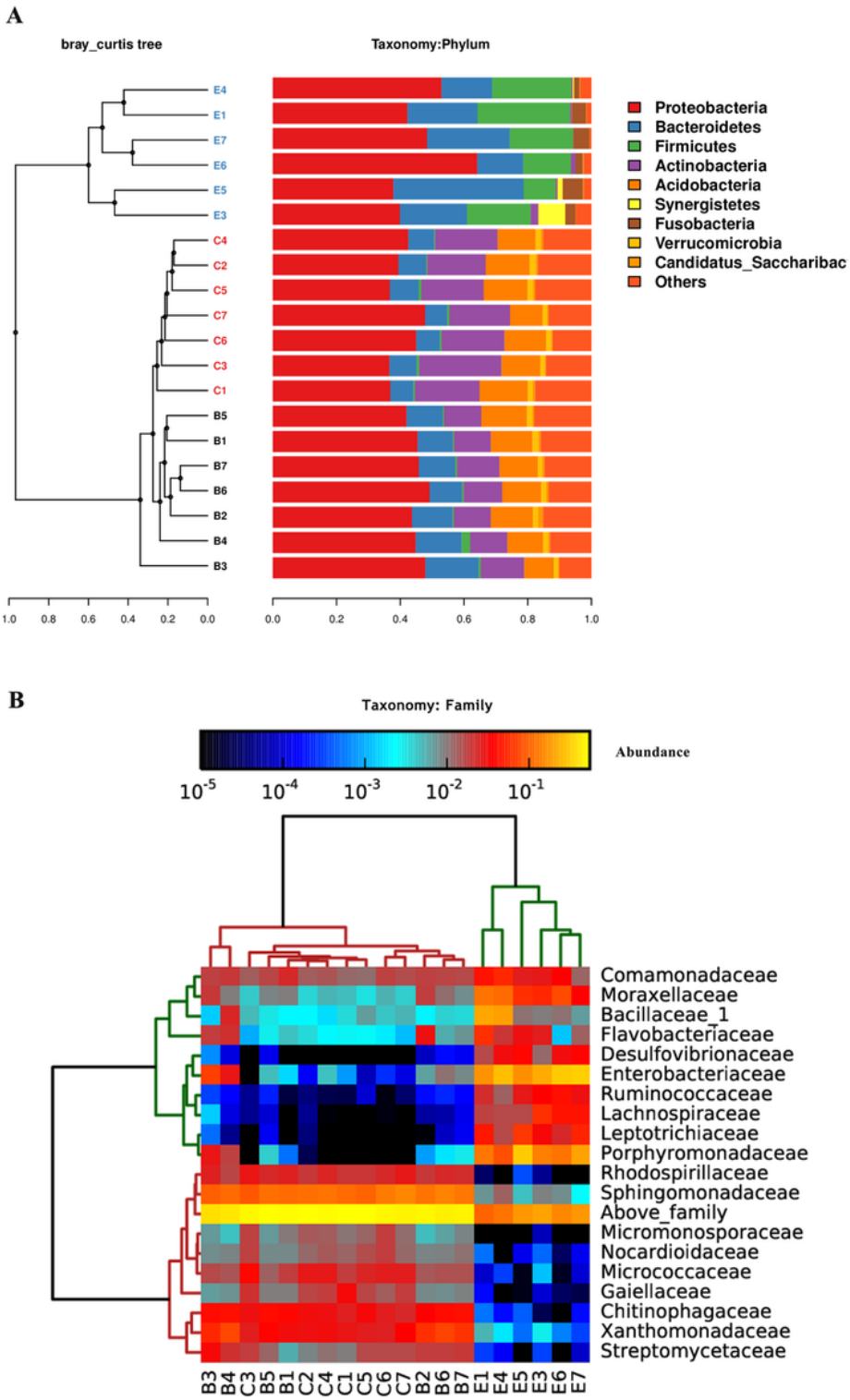

Figure 2

Taxonomic composition of microbial communities in bulk soil (B), egg cocoon (C) and egg surface (E) samples. Taxonomic resolution is at (A) phylum level and (B) family level respectively. The sample variation in the community structure is also highlighted in the Bray-Curtis tree (A) and cluster tree (B).

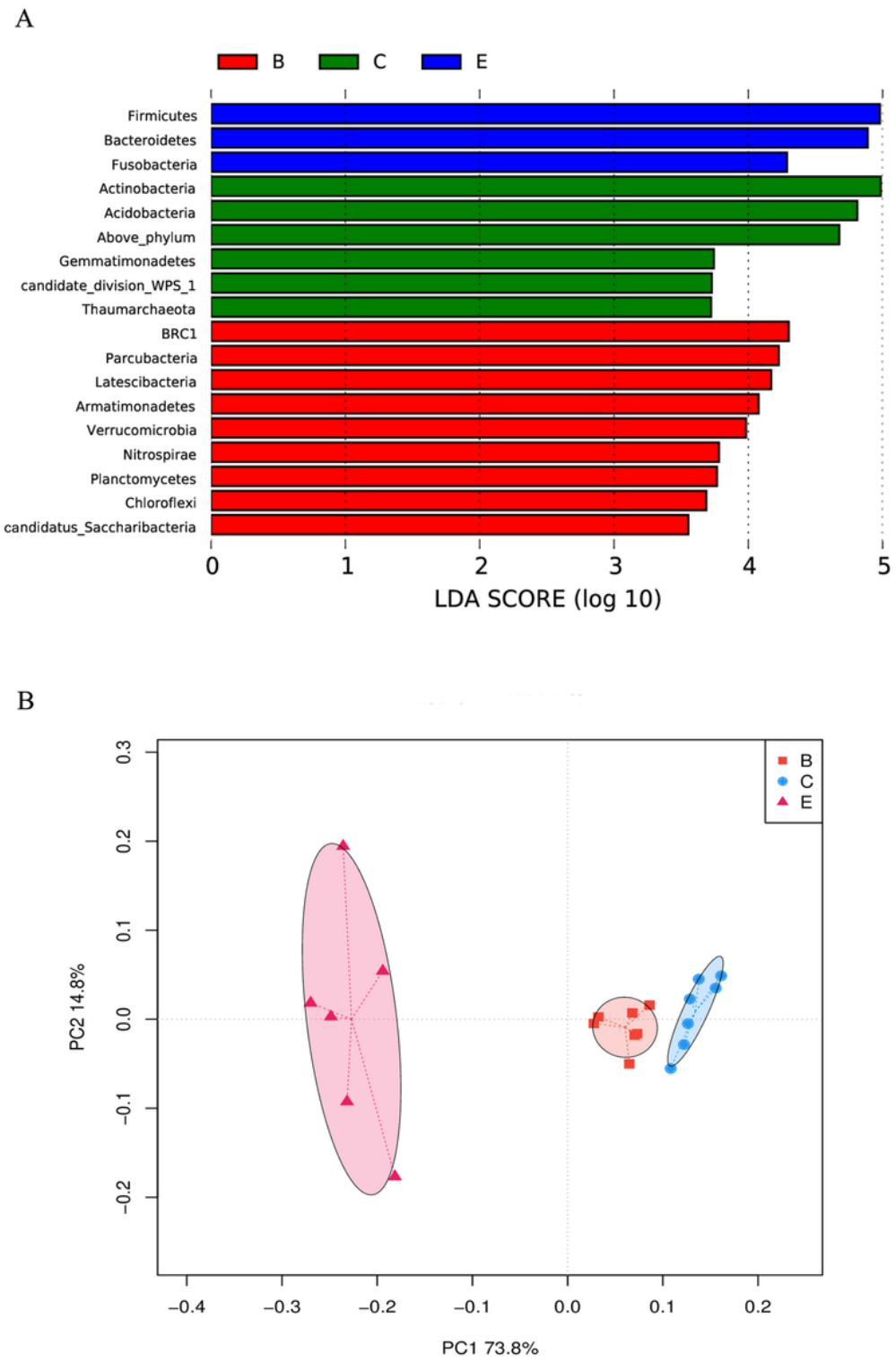

Figure 3

Comparison of microbiota using Lefse (LDA) test at the phylum level (A). LDA scores represent the significant microbial difference among different samples. PCA analysis for microbiota variability at the phylum level (B). Each symbol represents a sample. The variance explained by the PCs is indicated on the axes.

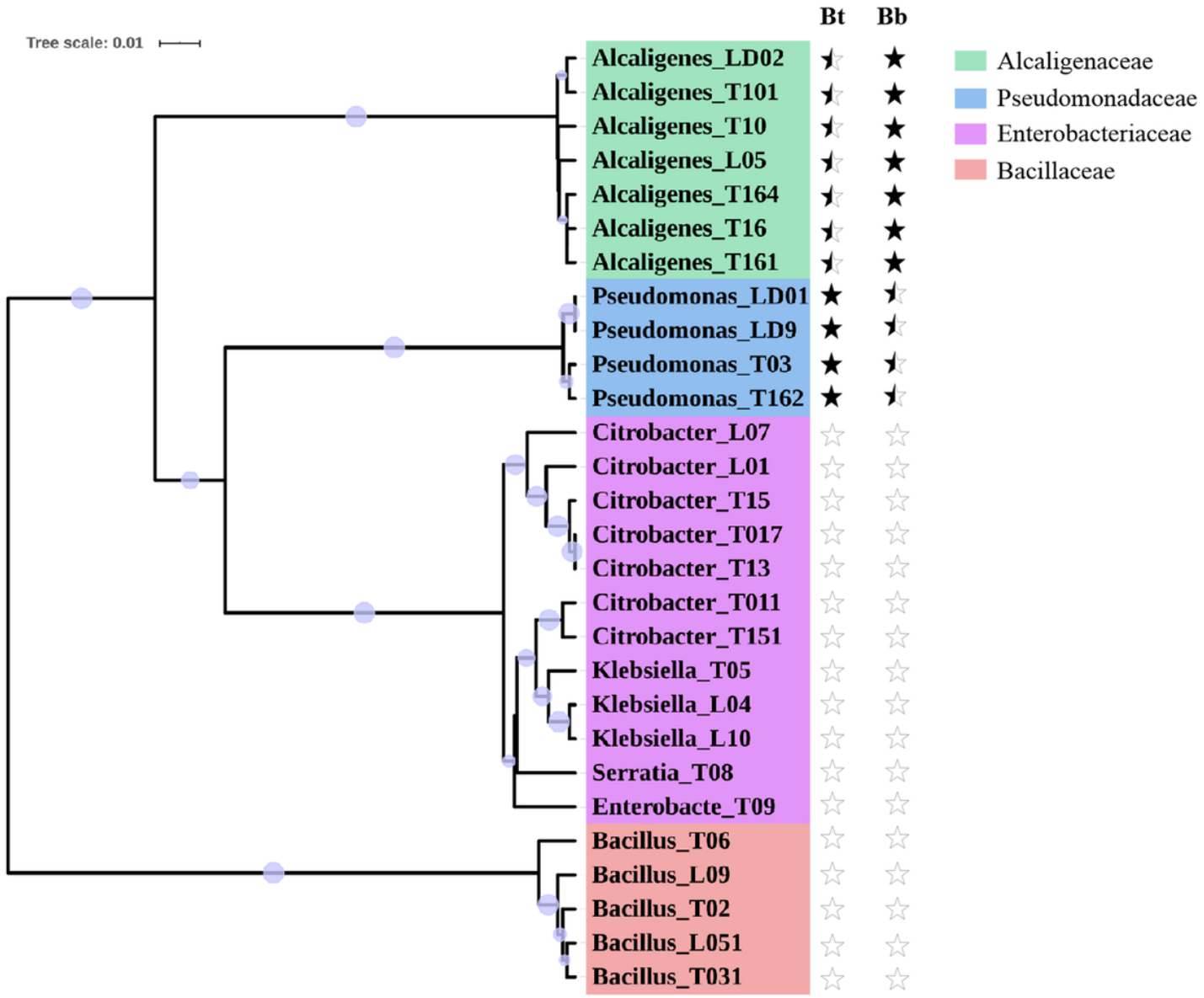

Figure 4

Phylogenetic analysis of 28 cultivable isolates. The tree is constructed based on the 16S rRNA gene sequences of each strain. Bootstrap values over 0.5 (1000 replications) are indicated using filled purple circles on the branch. Strain ID prefixed with “LD” indicates egg surface (E) isolates, strain ID prefixed with “L” indicates egg cocoon (C) isolates, and strain ID prefixed with “T” indicates bulk soil (B) isolates. Different label background colors represent different clades at the family level. The antimicrobial activity against “Bt” or “Bb” strains is indicated using pentagram symbols, filled black pentagrams represent strong antimicrobial activity, partially filled pentagrams represent weak antimicrobial activity, and open pentagrams represent no antimicrobial activity.

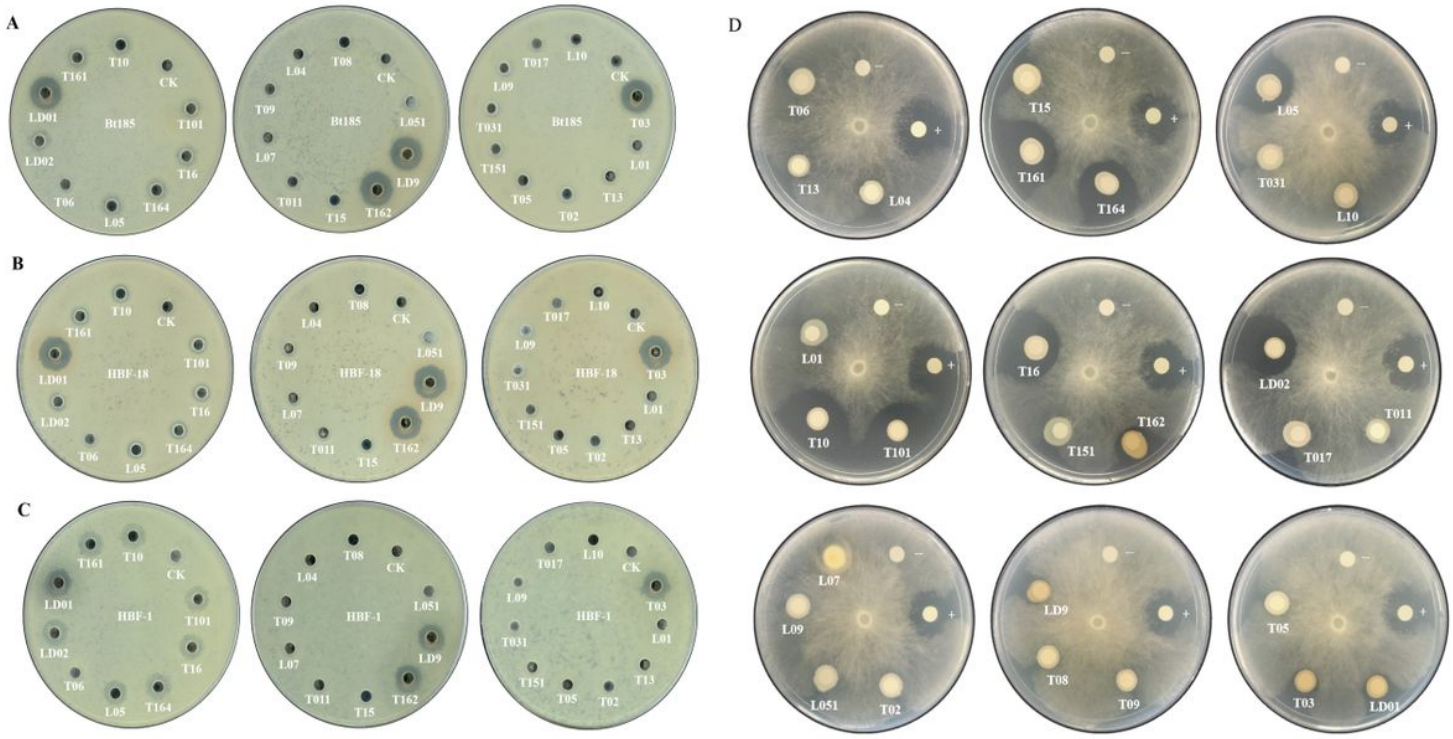

Figure 5

Effect of 28 cultivable isolates against the scarab-specific Bt strain Bt185 (A), HBF-18 (B), HBF-1 (C), and Bb strain (D). CK in (A), (B), and (C) and symbol "–" in (D) signifies negative control. The symbol "+" in (D) signifies positive control. Strain ID prefixed with "LD" indicates egg surface isolates, strain ID prefixed with "L" indicates egg cocoon isolates, and strain ID prefixed with "T" indicates bulk soil isolates.

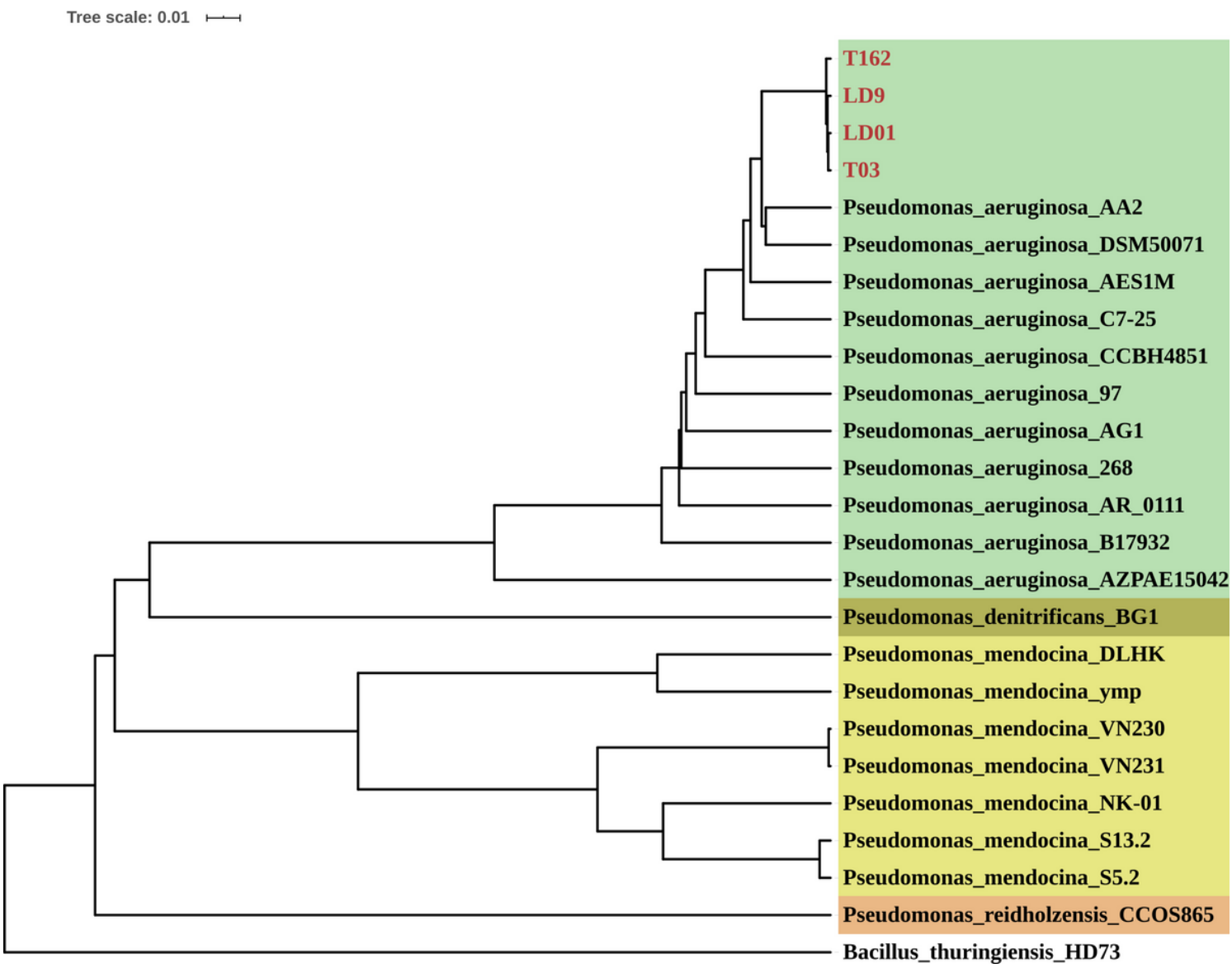

Figure 6

Phylogenetic analysis of four Pseudomonas strains (LD01, LD9, T03, and T162). This tree is constructed based on the whole genome of each strain. Different label background colors represent different clades at the species level. Bt strain Bacillus_thuringiensis_HD73 is used as the outgroup.

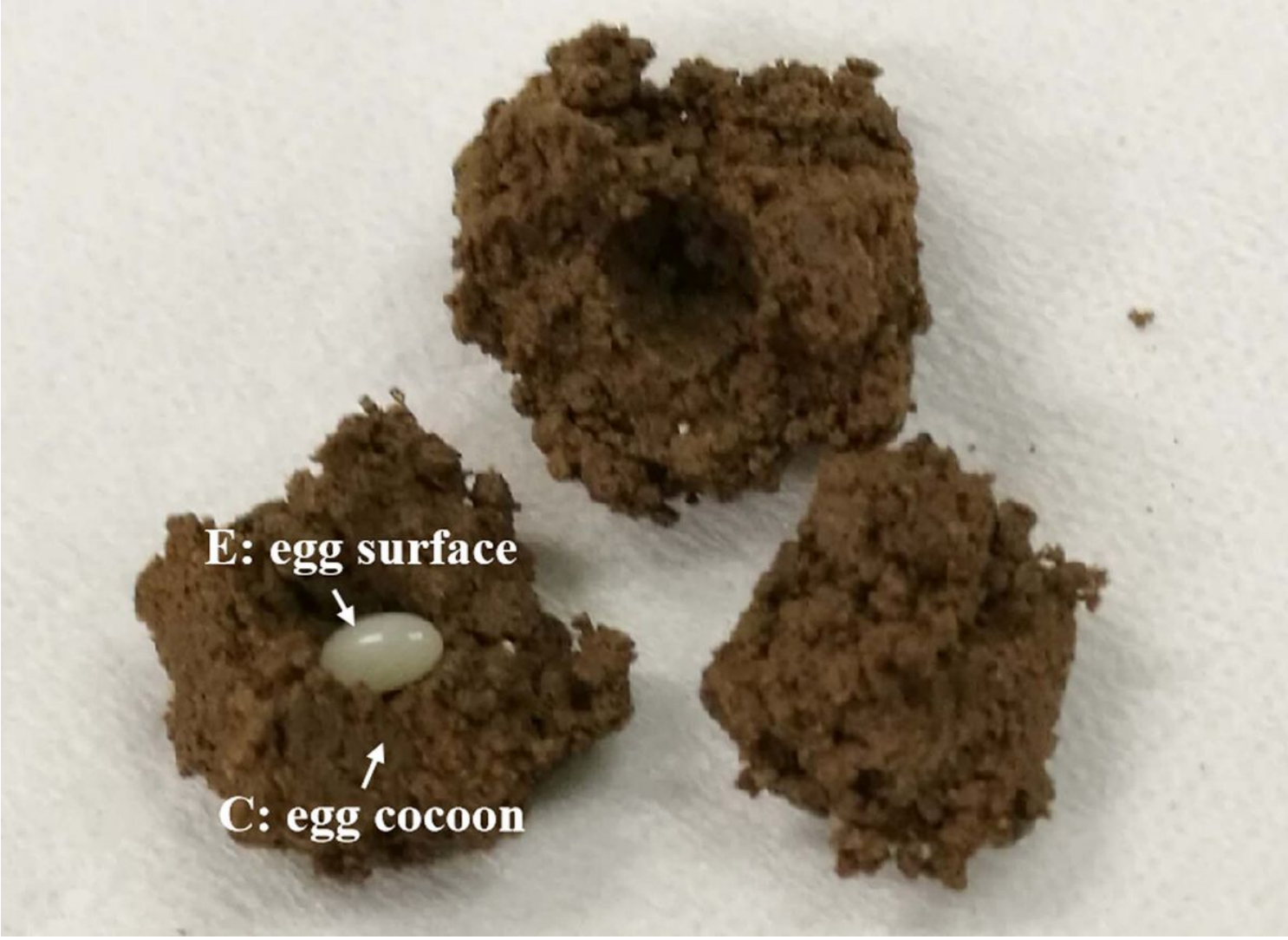

Figure 7

Egg cocoon (C) samples and egg surface (E) samples were collected as shown in the figures above, and bulk soil (B) samples were collected from the soil about 10 cm away from the egg surface.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation.pdf