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Research Article

Aerobic Bacterial Community of the Rice Striped Stem Borer: A Step towards Finding Bacterial Candidates for Paratransgenesis and RNAi Control

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Abstract

It is recognized that insects have close associations with a wide variety of microorganisms, which play a vital role in the insect's ecology and evolution. The rice striped stem borer, *Chilo suppressalis*, has economic importance at the global level. With the development of insecticide resistance, it is widely recognized that control of this pest is likely to need new tools that are not available today. Here, we focus on the aerobic bacterial community of the pest, to seek candidates for paratransgenesis or RNAi biocontrol of *C. suppressalis*. Culture-dependent PCR-direct sequencing was used to characterize the midgut bacterial communities of *C.suppressalis* at different life stages, collected in northern Iran, both from rice plants and from weeds on which the insect

feeds. Our results show that the predominant genera of the Firmicutes and Proteobacteria phyla were *Bacillus*, *Enterobacter* and *Klebsiella* in all life stages, and they displayed differences in abundance. The high levels of *B. subtilis* and *Enterobacter* found in *C. suppressalis* suggests their potential utility for paratransgenesis and RNAi approaches to control this pest.

Keywords: *Chilo suppressalis*; Paratransgenesis; RNAi; Bacteria; 16SrRNA; Iran

1. Introduction

Classified amongst various stem borers in the order Lepidoptera, the striped stem borer, *Chilo suppressalis* (Walker), in the family Crambidae, is an important insect pest of rice in southern Europe and Asia, including Iran [1-5]. It infests rice plants from the seedling stage to maturity and may cause dead hearts and whiteheads during the vegetative and reproductive stages, respectively. In addition to rice, this insect harms other crops and weeds, including water bamboo, water oat, corn, and sugarcane [6, 7]. Current efforts to control the rice striped stem borer rely heavily on the application of chemical insecticides; however, this pest still causes economically important damage to crops in many countries [8]. The emergence of pest strains resistant to insecticides, the impact of insecticides on the environment, the toxicity to animals and humans, and the costs to farmers have led to renewed calls for the search for, and development of, new sustainable and cost-effective pest control tools.

Paratransgenesis and RNA interference (RNAi) are among possible alternative control strategies, where commensal or symbiont bacteria found in the gut of the insect pest are engineered to reduce insect fitness and to suppress the pest population [9-15]. The principal and essential step in paratransgenesis is the identification of suitable bacteria in the insect. The characteristics required for a candidate include being non-pathogenic to humans, host plants, and non-target animals; being a dominant species within the insect microflora; being cultivable in cell-free media, being malleable to transformation with foreign DNA, and having a wide distribution [16, 17]. However, so far, most of the research on paratransgenesis or RNAi has been focused on vectors of human diseases, and only a few crop pests have been studied. Insect gut microbial communities, including bacterial species, have been investigated in various insects, including blood-sucking bugs [18], tsetse flies [19], mosquitoes [20-23], American cockroaches [24], sand flies [25-28], honeybees [29-31], and desert locusts [32]. The potential of the paratransgenesis approach against crop pests has been demonstrated in the Glassy-winged sharpshooter [33-34] and in honeybees [15].

Although *C. suppressalis* is the most important rice pest in the world, the microbial diversity associated with this insect pest has been little studied, with only two reports on the microbial diversity associated with the level of insecticide resistance among various populations of stem borer in China [6, 35]. In response to the lack of knowledge about these bacterial communities, and in the hope of finding bacterial candidates for paratransgenesis and RNAi in *C. suppressalis*, this study was conducted to assess the composition of aerobic gut bacterial communities of the insect at different life stages, from two different geographical regions in northern Iran. The

gut bacteria were identified by culture-dependent isolation, followed by PCR-direct-sequencing of the 16S gene. The commensal bacterial communities of rice and sorghum in these geographic regions was also investigated, to examine any possible relationship between host plants and the microbiotal diversity of *C. suppressalis*. This information is important for the better understanding of symbiotic or commensal relationships between the bacteria and stem borer, the mechanisms that determine gut microbiota composition, and the potential for introduction of candidates for paratransgenesis or RNAi approaches against the pest in the study areas.

2. Materials and Methods

2.1 Sample collection

To cover the different life stages (larvae, pupae, and adult), the *C. suppressalis* populations were sampled in May-October 2018 (i.e. during the rice-growing season) from 18 rice fields in different regions of Mazandaran and Gillan provinces (Table 1). Sampling from rice and weeds was performed based on the distribution of rice fields in both provinces. Adults were captured using light traps, whereas the larvae and pupae were collected from the stems of the infested plants.

Each province was divided into three: eastern, central, and western parts, and in each part, where possible, three collection sites were selected based on their distances from the Caspian Sea. The specimens collected in the field were taken immediately to an experimental laboratory, where each sample was identified and used for isolation of bacteria from their alimentary canals.

2.2 Isolation of bacteria

The *C. suppressalis* specimens were immediately microdissected individually on sterile glass slides in a microbiological safety cabinet. First,the specimens were surface sterilized with bleach (10% sodium hypochlorite) for 5 min, and then rinsed with distilled water and air-dried. The gut of each specimen was then micro-dissected and homogenized in 1ml of sterile PBS by shaking with glass beads in a sterile tube.

To reduce the risk of laboratory-derived contamination, we used sterile workstations with sterile gloves, pipette tips with filters, and PCR grade RNAse-free water, and the experiments were performed under laminar flow hoods. The homogenized gut was transferred to screw-topped test tubes containing 5 ml of brain heart infusion (BHI) broth and incubated at 37°C for 24–48 h in aerobic conditions. Cloudy test tubes were considered as positive specimens. The grown bacteria were serially diluted or streaked on BHI agar plates and subcultured several times on the agar plates in the same conditions to achieve individual purified colonies. Test tubes containing BHI broth were opened near the dissection area under sterile conditions during the micro-dissection processes. Individual colonies were selected and used for further molecular identification.

The richness of each bacterial family was calculated, based on the total number of 16S sequences. The water used for the final rinsing of the cuticles was used as negative controls and plated in parallel. Gloves were changed frequently to avoid RNAse/DNAse contamination. Surface sterilization

of the workstation was performed with bleach (10% sodium hypochlorite) followed by alcohol (70%) before and after each experiment. Also, instruments were autoclaved before and after handling each sample, and we avoided talking, sneezing, and coughing, and touching areas where DNA might exist.

2.3 16S rRNA gene amplification

DNA extraction from individual colonies was carried out using the phenol/chloroform DNA extraction method, as previously described [26]. Nearly 1500 bp of the bacterial 16S rRNA gene, including the less variable V1 and V2 regions and the highly variable V3-V5 regions were amplified using the universal primers 16suF (5'-GAG TTT GAT CCT GGC TCA G-3') and 16suR (5'-GTT ACC TTG TTA CGA CTT-3') [36]. Polymerase chain reaction (PCR) amplification was carried out using a Maxime PCR PreMix Kit (i-Taq) in 20µl reaction mixtures containing 1µl of each primer at 10µM concentration and 1–2µl (~0.1µg) of extracted genomic DNA.

BHI agar media and ddH2O were used as negative controls. The thermal cycler conditions were set as follows: an initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57.5°C for 40 s and extension at 72°C for 30 s. Final extension was at 72°C for 8 min. The PCR products were separated on a 1% agarose gel containing ethidium bromide and were visualized using a UV transilluminator. The QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the PCR products before sequencing.

To assess the environmental contamination, the cuticles of each specimen were used as an environmental control and were removed from the carcass and subjected to DNA extraction by phenol/chloroform and PCR amplification of 16S rRNA gene, as before. Where the negative control was positive, the specimen was eliminated from further analysis.

2.4 16S rRNA gene sequencing and analysis

First, 16S rRNA amplicons were amplified using each forward or reverse primer, and then sequenced using the Sanger method (Macrogen, Seoul, S. Korea). To compare these sequences with those available in ribosomal databases, eight databases of prokaryotic 16S rRNA gene were used: NCBI (nucleotide collection; http:// blast.ncbi.nlm.ni-h.gov/Blast.cgi), EMBL (http://www.ebi.ac.uk/ena), RDP (http://rdp.cme.msu.edu/seqmatch/ seqma-tch_intro.jsp), EzTaxon-e (http://eztaxon-e.ezbiocloud.net), Greengenes (http://greengenes.lbl.gov/cgibin/nph-index.cgi), DDBJ (http://blast.ddbj.nig.ac.jp/ ?lang=en), leBIBI (http://umr5558-sud-str1.univ-lyon1.fr /lebibi/lebi-bi.cgi), and Blast2Tree (http://bioinfo.unice.fr/ blast) [37, 38]. Sequence homology with available data was assessed, based on the number and quality of nucleotides of the sequence reads using appropriate features of the data, such as cultivable and/or non-cultivable phenotype and type and/or non-type specimens.

The sequences were assigned at the species level, based on either the most common nomenclature within the results of the eight databases or the highest similarity rate.

Nucleotide homology >95% and >98% were considered as lower thresholds at genus and species levels, respectively (https://rdp.cme.msu.edu).

The partial 16S rDNA consensus sequences obtained in this study were annotated in the GenBank database using the 16S ribosomal RNA database (https://submit.ncbi.nlm. nih.gov/subs/genbank). Species assignation of the bacteria was verified by phylogenetic analysis using 16S rRNA gene sequences, based on the neighbor-joining algorithm of MEGA7 Software. Cytoscape Software (http://www.cytoscape.org) was used as a tool for visualizing complex networks between data, to visualize bacterial richness and shared bacteria in the three life stages through the network analysis. Data, as CYS files containing vertices or nodes (representing bacteria and life stages, or hosts, or location) and edges (representing links), were submitted to Cytoscape software v.3.5.1. GraphPad Prism software v.5.00 for Windows (GraphPad, San Diego, USA) and Student's t-test embedded in the software was used for graphical representation and statistical analysis, respectively.

3. Results

In total, 1150 *C. suppressalis* specimens, comprising 671 (58.35%) larvae, 199 (17.3%) pupae, and 207 (24.35%) adults were collected from the study areas. Almost identical numbers of specimens were collected from each of the provinces of Mazandaran and Gillan (573 *versus* 577) (Table 1). All the specimens were processed individually for their midgut bacterial composition.

In total, 250 bacterial colonies, comprising 40 species, were isolated and identified from the midgut of the infected *C. suppressalis* specimens (Tables 2-3). The bacteria in this study were assigned to three phyla, nine families, and thirteen genera. Amongst the detected phyla, the Firmicutes were predominant, with a mean relative frequency of 58.5%, followed by Proteobacteria with a mean relative frequency of 39.0%, and Actinobacteria with a mean relative frequency of 2.5% (Figure 1). The family Bacillaceae was the most predominant group, with mean relative frequencies of 45 % (Tables 2-3).

The most abundant of the genera in the *C. suppressalis* specimens was *Bacillus*, with a mean relative frequency of 35% (Figure 2). The second most abundant genera were *Enterobacter* and *Klebsiella*, each with a mean relative frequency of 12.5% (Figure 2). Among the gut bacteria, *B. subtilis* and *Enterobacter* spp. were found often in the *C. suppressalis* samples. These bacteria are potential candidates for paratransgenesis or RNAi approaches against this important pest.

The frequency and diversity of bacteria in adults and larvae of *C. suppressalis* was very similar (n=17 *versus* n=21); however, there was a huge reduction in the diversity and abundance of bacteria in the pupal stage (n=4) (Figure 3). The most abundant bacteria in the larvae were of the genus *Bacillus* (42.9%), followed by *Klebsiella* (19%), whereas the most abundant bacteria in the adults were *Bacillus* (35.3%) and *Enterobacter* (23.5%).

Bacillaceae were present at high relative abundance in both *C. suppressalis* adults and larvae, with mean relative frequencies of 64.7% and 47.6%,

respectively. However, there were significant differences between the composition of the bacterial communities in larvae and adults (F=4.35839, p=0.001; Tables 2-3), with only *Bacillus cereus* and *B. albus* being common to both (Figure 3). Although there was a slight increase in the abundance of Enterobacteriaceae in adults, the Enterobacteriaceae bacteria showed similar relative abundances in adults and larvae. Of the four bacteria species observed in pupae, only *B. luti* was common to pupae and adults (Figure 3).

The host of the *C. suppressalis* specimens was, almost exclusively, the Rice plant. Although six host weeds (Johnson/Aleppo millet grass, cockspur grass, Bidens, Willow weed/Curlytop knotweed/Ladys thumb, sorghum, and one unknown species) in the study area were investigated, only a few weed plants were infested with *C. suppressalis*. Moreover, the majority of the *C. suppressalis* specimens of these hosts did not harbor bacteria in their midgut. To find the possible origins of the gut microbiome of *C. suppressalis*, we tested the bacteria on the surface of leaves and stems of the plant hosts (rice and weeds) and matched them with those isolated from the *C. suppressalis* guts. Rice plants were found to harbor more bacteria species than the weed plants (8 *versus* 2). Out of the ten bacteria species observed on the plants, eight were common to both the guts and the host plants (Figure 4). Of these eight shared bacteria, five were observed in larvae, two in adults, and one in a pupa (Tables 2,3). The other two bacteria were not observed in the insect guts.

Although Mazandaran province showed more abundant bacterial communities than Gillan, statistical analysis confirmed that there were no significant differences between the microbial abundance of *C. suppressalis* guts in the two provinces (26 *versus* 21) (Figure 5). However, there were significant differences in the microbial composition of the samples from the two provinces (F=1.93071, p=0.041), where only 7 bacterial species were shared between the two locations (Figure 5).

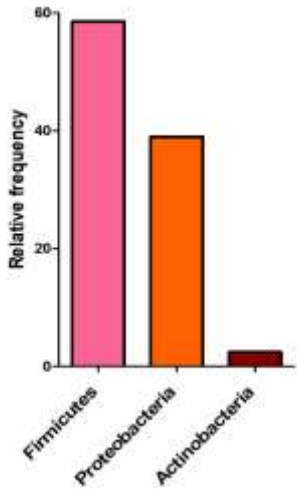

Figure 1: Relative frequency at phylum level of aerobic bacteria grown in BHI media from the gut of *C. suppressalis* samples originating from Mazandaran and Gillan provinces, northern Iran.

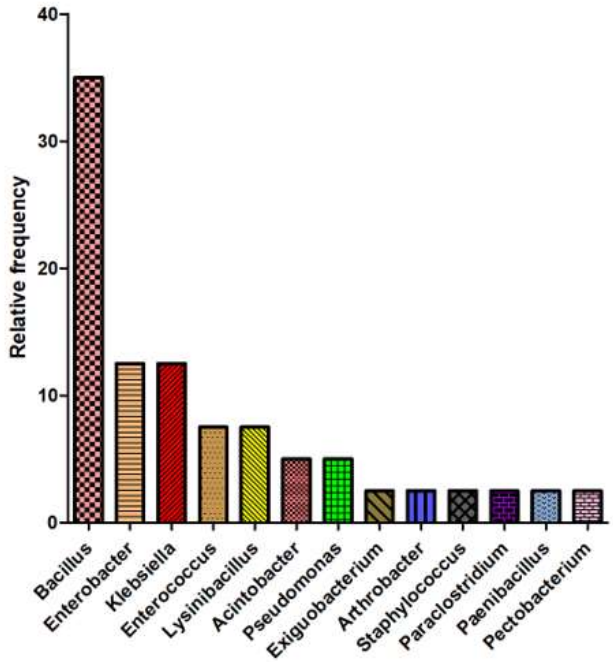

Figure 2: The relative frequency of bacterial genera in the midgut of *C. suppressalis* from northern Iran.

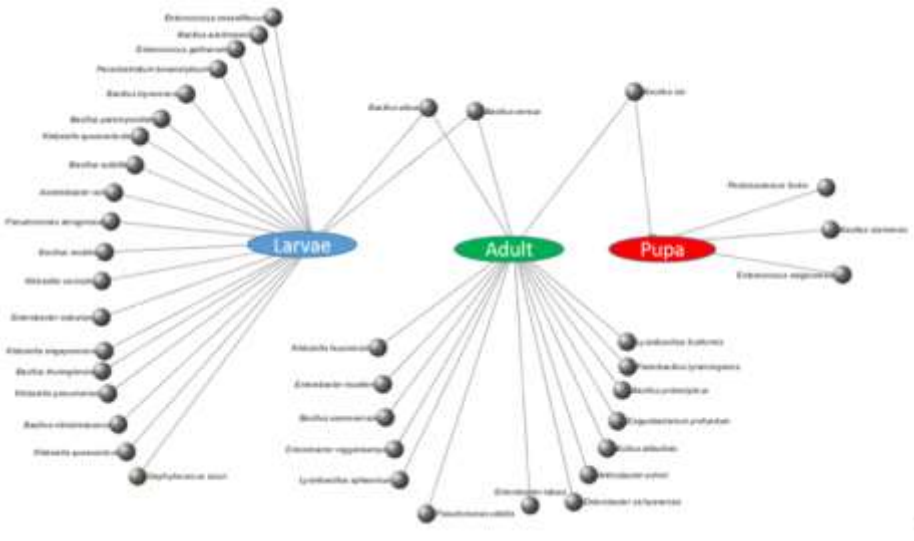

Figure 3: Network analysis showing the shared and non-shared bacteria species isolated from guts of different life stages of *C. suppressalis* in northern Iran.

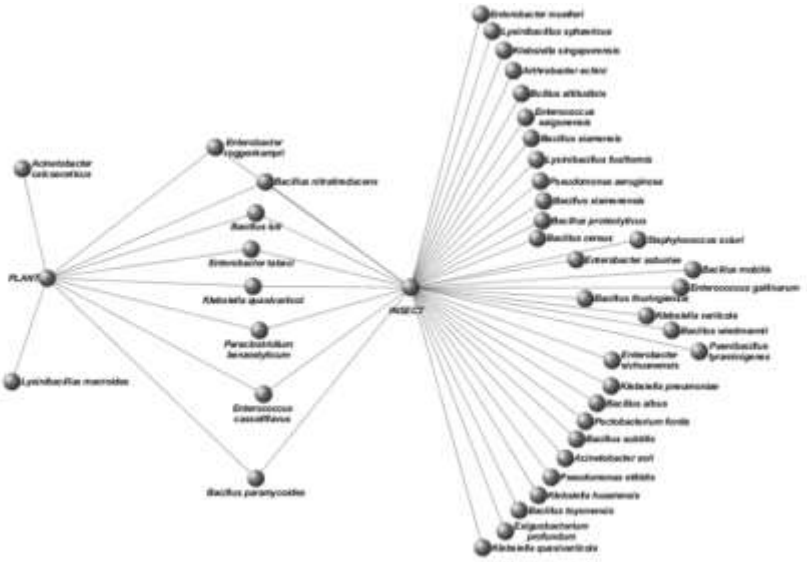

Figure 4: Network analysis showing the shared and non-shared bacteria species isolated from guts of *C. suppressalis* and their host plants in northern Iran.

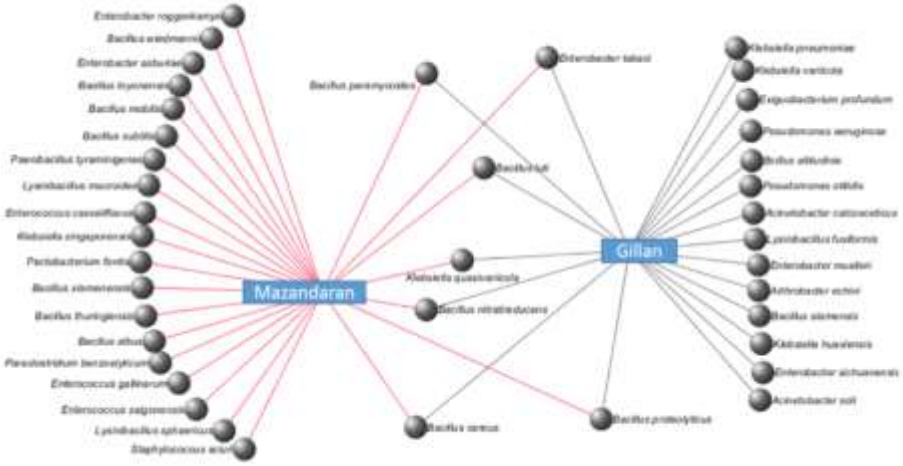

Figure 5: Network analysis showing the shared and non-shared bacteria species isolated from guts of *C. suppressalis* in two provinces in northern Iran.

Province	Location	Latitude & Longitude	No of specimens on				Total
			Rice			Weeds	
			L	P	A	L	
Gillan	Astara	N:37°-16'-82" E:49°-53'-40.9"	11	10	24	21	66
	Talesh	N:37°-42'-59.8" E:48°-57'-10.6"	15	12	23	24	74
	Asalem	N:37°-33'-55.8" E:49°-6'-55.3"	11	10	19	12	52
	Anzali	N:37°-27'-21.5" E:49°-36'-11.6"	12	12	17	30	71
	KHomam HW	N:37°-22'-56.6" E:49°-39'-33.5"	12	10	21	21	64
	Rasht Res Ins	N:37°-12'-17.8" E:49°-38'-29.5"	11	10	11	21	53
	Lahijan	N:37°-13'-7" E:49°-58'-26.5"	13	10	22	25	70
	Navideh	N:37°- 16'- 35.3" E:49°-44'-54.2"	12	10	11	27	60
	Klachai	N:37°-40'-32.6" E:50°-23'-13.3"	13	19	11	24	67
	Subtotal		110	103	159	205	577
Mazandaran	Shirood	N:36°-51'-53.3" E:50°-46-"7.45´	7	11	10	6	34
	Tonekabon RW	N:36 °- 47'-43.7" E:50°-54'-49.2"	10	10	20	32	72
	Chalous RW(Kosksara)	N:36°-37' -55.615" E:51° -27'-24.777"	19	11	10	34	74
	Amol, MohamadAbad	N:36°-28' -30.685" E:52°-27'-48.617"	6	9	10	30	55
	Amol Rice Sta. (Galekash)	N: 36°-21'-48.911" E: 52°-21'-17.892"	10	10	10	32	62
	Amol,	N: 36° -35'30.615"	10	12	9	34	65
	Marzango	E: 52° 28'-38.671"					
	Ghaemshahr, CheftKola	N: 36°- 25' 34.281" E: 52°-48' -44.993"	10	10	11	30	61
	Ghaemshahr, Telar Hotel	N: 36°-28'-56.524" E: 52° -48'-54.242"	12	10	30	33	85
	Jouybar (Sarvkola)	N: 36°-35'-26.104" E: 52°-54'-22.013"	10	13	11	31	65
	Subtotal		94	96	121	262	573
Total			204	199	280	467	1150

L: larva; P: pupa; A: adult.

Table 1: Details of *C. suppressalis* specimens collected in rice fields during the growing season, northern Iran, 2018.

Assigned bacterial spp.	Origin	Plant host	In Dev. Stage	Family	Phylum	Genbank ID
Bacillus xiamenensis	In	NA	A	Bacillacae	Firmicutes	MT176557
Bacillus wiedmannii	In	Rice	L	Bacillacae	Firmicutes	MT355806
Bacillus toyonensis	In	Rice	L	Bacillacae	Firmicutes	MT176558 MT176559
Bacillus thurengiensis	In	Rice	L	Bacillacae	Firmicutes	MT176560
Bacillus proteolyticus	In	NA	A	Bacillacae	Firmicutes	MT176561
Bacillus paramycoides	Pl	Rice	NA	Bacillacae	Firmicutes	MT176562
	In	Rice	L			MT176563
Bacillus nitratireducens	Pl	Weed	NA	Bacillacae	Firmicutes	MT176564
	In		L			MT176565
Bacillus subtilis	In	Rice	L	Bacillacae	Firmicutes	MT176566
Bacillus mobilis	In	Rice	L	Bacillacae	Firmicutes	MT176567
Bacillus luti	Pl	Rice	NA	Bacillacae	Firmicutes	MT176568
	In	Rice	P			MT176569
	In	Rice	P			MT176570
Bacillus cereus	In	Rice	L	Bacillacae	Firmicutes	MT176571
	In	NA	A			MT176574
	In	NA	A			MT176572
	In	NA	A			MT176573
	In	NA	A			MT176575
Bacillus albus	In	Rice	L	Bacillacae	Firmicutes	MT176576
	NA	NA	A			MT176577
Lysinibacillus sphaericus	In	NA	A	Bacillacae	Firmicutes	MT176578
Lysinibacillus macroides	Pl	Rice	NA	Bacillacae	Firmicutes	MT176579
Paraclostridium benzoelyticum	Pl	Rice	NA	Peptostreptococcaceae	Firmicutes	MT176580
	In	Rice	L			MT176582
	In	Rice	L			MT176581
Paenibacillus tyraminigenes	In	NA	A	Flexibacteraceae	Firmicutes	MT355807
Enterococcus saigonensis	In	Rice	P	Enterococcaceae	Firmicutes	MT176583
Enterococcus gallinarum	In	Rice	L	Enterococcaceae	Firmicutes	MT176584
Enterococcus casseliflavus	Pl In	Rice Weed	NA L	Enterococcaceae	Firmicutes	MT176585 MT176586
Staphylococcus sciuri	In	Rice	L	Staphylococcaceae	Firmicutes	MT176587
Enterobacter Tabaco	Pl In	Rice NA	NA A	Enterobacteriaceae	Proteobacteria	MT176588 MT366203
Enterobacter roggenkampii	Pl In	Rice NA	NA A	Enterobacteriaceae	Proteobacteria	MT176589 MT366202
Enterobacter asburiae	In	Rice	L	Enterococcaceae	Proteobacteria	MT176601
Pectobacterium fontis	In	Rice	P	Pectobacteriaceae	Proteobacteria	MT355808
Klebsiella singaporensis	In	Rice	L	Enterobacteriaceae	Proteobacteria	MT355805
Klebsiella quasivariicola	Pl In	Rice Rice	NA L	Enterobacteriaceae	Proteobacteria	MT176590 MT366204

In: insect; Pl: plant; A: adult; L: larva; P: pupa; NA: not applicable; Dev: developmental.

Table 2: Details of the 26 bacterial species (42 strains) isolated from the midgut of *C. suppressalis* or its host plants in Mazandaran province, east coast of Caspian Sea, north Iran.

Assigned bacterial spp.	Origin	Plant host	In Dev. Stage	Family	Phylum	Genbank ID
Bcillus altitudinis	In	*NA*	A	Bacillacae	Firmicutes	MT176591
Bacillus cereus	In	Rice	L	Bacillacae	Firmicutes	MT176592
	In	NA	A			MT366205
Bacillus luti	In	NA	A	Bacillacae	Firmicutes	MT355810
Bacillus nitratireducens	In	Rice	L	Bacillacae	Firmicutes	MT176593
Bacillus paramycoides	Pl	Rice	NA	Bacillacae	Firmicutes	MT176594
Bacillus proteolyticus	In	NA	A	Bacillacae	Firmicutes	MT176595
Bacillus siamensis	In	Rice	P	Bacillacae	Firmicutes	MT176596
Lysinibacillus fusiformis	In	NA	A	Bacillacae	Firmicutes	MT176597
Exiguobacterium profundum	In	NA	A	Bacillacae	Firmicutes	MT355804
Acinetobacter calcoaceticus	Pl	Rice	NA	Moraxellaceae	Proteobacteria	MT176598
Acinetobacter soli	In	Rice	L	Moraxellaceae	Proteobacteria	MT176599
Arthrobacter echini	In	NA	A	Micrococcaceae	Actinobacteria	MT176600
Enterobacter muelleri	In	NA	A	Enterobacteriaceae	Proteobacteria	MT176601 MT366201
Enterobacter sichuanensis	In	NA	A	Enterobacteriaceae	Proteobacteria	MT176602
Enterobacter tabaci/mori	In	NA	A	Enterobacteriaceae	Proteobacteria	MT176603
						MT355803
Pseudomonas aeruginosa	In	Rice	L	Pseudomonadaceae	Proteobacteria	MT355802
Pseudomonas otitidis	In	NA	A	Pseudomonadaceae	Proteobacteria	MT176604
Klebsiella quasivariicola	In	Rice	L	Enterobacteriaceae	Proteobacteria	MT176605 MT176606
Klebsiella variicola	In	Rice	L	Enterobacteriaceae	Proteobacteria	MT355809
Klebsiella pneumoniae	In	Rice	L	Enterobacteriaceae	Proteobacteria	MT176607
Klebsiella huaxiensis	In	NA	A	Enterobacteriaceae	Proteobacteria	MT176608

In: Insect; Pl: Plant; A: Adult; L: Larva, P: Pupa; NA: Not applicable; Dev: Developmental

Table 3: Details of the 21 bacterial species (25 strains) isolated from the midgut of *C. suppressalis* and its host plants in Gillan province, west coast of Caspian Sea, north Iran.

4. Discussion

This study is the first significant survey of the bacterial communities in *C. suppressalis* in Iran. Understanding the microorganisms that accompany insects, especially those of international economic importance, is essential for developing microbial-

based tools for insect pest management, such as paratransgenesis, gene silencing or RNA interference, as well as novel chemical and microbial biopesticides, sterile insect technique (SIT), and incompatible insect technique (IIT) [39]. Here, we report an association between the bacterial communities of *C. suppressalis* and the bacteria of the host plant on which the larvae or adults feed. These results suggest new opportunities to develop paratransgenesis approaches, or combinations of paratransgenesis and RNAi approaches, by manipulation of the *C. suppressalis* microbiome to produce effector molecules with high impacts on the insect fitness contributing to their pest status. The presence of *B. subtilis* and *Enterobacter* in the *C. suppressalis* samples suggests that these bacteria are potential candidates for a paratransgenesis plus RNAi approach against the pest. These bacteria can be cultured, transformed, and readily delivered by the host plant (rice) to the *C. suppressalis* gut. These bacteria have been already used for the production of paratransgenic sand flies [11] and mosquitoes [40].

During this study, we characterized the bacterial community of *C. suppressalis* larvae, pupae, and adults and their plant hosts using culture-dependent isolation, followed by sequencing of the 16S gene. For the first time, to our knowledge, the possible transstadial transmission of bacteria from immature stages to the adult was investigated, as well as the possible acquisition of bacteria from host plants in this insect pest. Delivery of the modified bacteria to the insect gut is one of the most challenging issues of paratransgenesis or RNAi approaches to insect control, and the results of this study show that *C. suppressalis* may acquire bacteria from the host plant on which they feed at the larval stage, and via polluted sugar meals from plant flowers at the adult stage. However, in order to use these bacteria for paratransgenic approaches, it will be important to examine their capacity to efficiently colonize the gut or reproductive organs of *C. suppressalis*, and to express enough effector molecules or dsRNA to inhibit the target gene.

In this study we used culture-dependent isolation, followed by PCR-direct sequencing of the 16S gene to detect bacterial species; this method might have some disadvantages in comparison with high-throughput sequencing techniques (e.g. next generation sequencing: NGS). However, the method we used can potentially exclude slower-growing species and allow the observation of diverse characteristics of the isolated organisms, including the physiological characteristics, such as antibiotic resistance. Moreover, this method facilitates bacterial genome sequencing, and eliminates those bacteria incapable of being propagated on the culture media. Thus, it offers the best way of assessing the validity of the candidate bacteria, as it allows testing of their capacity to accept foreign DNA (genes or plasmids) for paratransgenesis and RNAi. To date, two studies on microbial diversity have been conducted on the larvae of *C. suppressalis* in China, using degeneration gradient gel electrophoresis (DGGE) to clarify the changes of gut microbial diversity before and after treatment with *Bacillus thuringiensis* (Bt) insecticidal proteins [6, 35]. They suggested that the changes in the bacterial abundance in midguts of larval *C. suppressalis* are related to the difference in Bt insecticidal proteins, geographical sites and successive rearing times.

The bacterial communities of the *C. suppressalis* specimens analyzed in our study predominantly consisted of the phylum Firmicutes (58.5%), and, to a lesser extent, the Proteobacteria (39.9%), and Actinobacteria (2.5%). A study of the *C. suppressalis* gut microbiota by Zhang et al (2013) had also found that it was dominated by the Firmicutes, followed by Proteobacteria, Chloroflexi, and Bacteroidetes. However, several studies on the composition of gut microbial communities across the Lepidoptera [41] have shown that most of the gut bacteria detected in butterflies and moths belong to the Proteobacteria phylum (42%), which differs from the *C. suppressalis* gut microbiome. However, those studies also found that the gut microbiome of Lepidoptera is highly variable between and within species; it was suggested that differences in the insect habitat and in the experimental methodologies used in the studies, such as variations in insect diet, the life stage, and the screening procedures (culture-based, culture-independent) may explain this variability. The different bacterial communities of *C. suppressalis* found in Iran and China may reflect the different methodologies, environmental conditions, life stages, and host plants used in the two studies. Therefore, caution is needed when recommending a specific bacterium for paratransgenesis or other symbiont-based control methods, because some bacteria are indigenous only in certain geographic regions and cannot be established in other areas. For example, the Bacillacae and Enterobacteriaceae were dominant in the microbial community of the Iranian *C. suppressalis* samples, differing from the microbial community found in this pest in China, where Enterococcaceae were the most common [6]. However, studies of the microbiome of 30 lepidopteran species indicated that the Bacillaceae and Enterobacteriaceae are two of the most common groups found in moths and butterflies [41]. The Pseudomonadaceae are only the third most widespread group across lepidopteran species and are also found less frequently in *C. suppressalis* specimens. The effect of geographical location on the *C. suppressalis* gut bacterial communities was shown in this study when comparing the provinces of Mazandaran and Gillan. The communities were relatively distinct, even though the host plant (rice) was the same at both sites, probably due to differences in phyllosphere bacteria inhabiting the host plants at each site. This finding is consistent with previous studies indicating that habitat may significantly affect the bacteria associated with lepidopteran species [42, 43].

This study has also shown that the *C. suppressalis* larvae (caterpillars) harbor a lower gut bacterial community than the adults, which agrees with a previous study indicating that microbial symbionts are generally absent or present only in low numbers in guts of lepidopteran larvae [44]. Caterpillars have a simple alimentary canal, lacking the intricate pouch-like structures (diverticula or caeca) that are known to carry bacterial symbionts in other insect taxa [45, 46]. Guts with many pouches might favor the establishment of a strong bacterial community, as occurs in non-lepidopteran insects with extremely rich bacterial gut communities (24, 32, 47-49]. Insects that feed on wood, decomposing matter, or garbage, such as termites, cockroaches, crickets, and some beetles have the most diverse gut bacterial communities [32, 49]. Several other factors, including short gut, highly alkaline conditions (pH

values >10-12), host antimicrobial peptides, rapid food passage, and lining of the midgut epithelium with peritrophic matrix may also hinder microbial colonization in the gut of caterpillars [50-56]. In contrast, some adult butterflies and moths may have high gut microbial loads [57], although many Lepidopteran adults do not feed [44]. It is known that adults of *C. suppressalis* visit rice and other host plants for nectar and pollen [58] that may provide a source of bacteria.

Our results showed that three species of the plant bacteria were present in the guts of *C. suppressalis* adults, suggesting plant hosts as a source of bacterial acquisition in adults of this insect pest. These observations suggest that paratransgenesis or RNAi approaches are more applicable to the adult than to the larval stage of moths, because the bacteria can colonize the adult alimentary canal.

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Declaration of Authorships

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Conflicts of Interest/Competing Interests

The authors declare that they have no conflict of interest.

Ethics Approval

Not applicable

Consent to Participate

Not applicable

Consent for Publication

Not applicable.

Availability of Data and Material

The sequence data generated for this study can be found in GenBank under accession numbers MT176557-MT176608, MT355802-MT355810, and MT366201-MT366204.

Code Availability

Not applicable

Authors' Contributions

AH performed field work and species identification. MAO contributed to conceptualization and management of the project and writing and revising the manuscript. NC and MK performed the experiments, generated sequencing data and performed molecular analyses. FK drafted the manuscript and collaborated in imaging analysis. All authors read and approved the final version of the manuscript.

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