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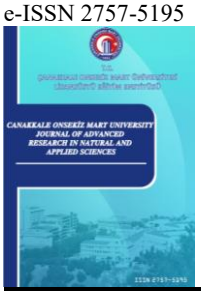

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Culturable Bacterial Communities Related to Different Larval Stages of *Sanys irrosea* (Guenee, 1852) (Lepidoptera: Noctuidae)

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Research Article

Abstract − Many bacterial species are frequently associated with insects in symbiotic, mutualistic, or parasitic relationships. Symbiotic bacteria living in mostly insect gut have many roles in insect's biology such as nutrition, development, sex determination and evolution. Therefore, studying of symbiotic bacteria in insects is very important to elucidate their roles in their hosts biology. In this study, we purposed to isolate and identify the culturable bacterial species in internal organs (mostly gut parts) of *Sanys irrosea* (Guenee, 1852) (Lepidoptera: Noctuidae) which was selected as model organism. The bacterial flora of different development stages of *S. irrosea* was studied by culture dependent techniques and the isolated bacteria was identified by 16S rRNA sequencing and phylogenetic analysis. A total of 22 bacterial isolates were obtained from different instar larvae of the insect and were identified. Among the identified bacterial species, *Staphylococcus*, *Micrococcus* and *Bacillus* species were dominant. In addition, some potential slug, human and plant pathogenic bacteria (*Moraxella osloensis*, *Kocuria rosea* and *Clavibacter michiganensis*) were isolated. The results were discussed with respect to the bacterial composition of *S. irrosea* regarding effects of bacterial diversity on the larval development of the insect. Results obtained from this study should be beneficial for future studies to understand roles of bacteria in the larval development of Lepidopteran insects.

Keywords − Bacteria, insect, symbiosis, 16S rRNA

1. Introduction

Bacteria are prokaryotic microorganisms which have different shapes, metabolism and lifestyle and they can survive in different environments from soil to water. They can also live with different organisms such as plants and animals in symbiotic and parasitic relations (Madigan, Martinko & Parker, 2003). They have also some genes and enzymes to sensitize some essential vitamins (such as cobalamin) which are necessary for nearly all animal life (Moore & Warren, 2012). Most of the bacteria in the human and various animal bodies including insects are in the intestinal (gut) system. Many of these bacteria are symbiotically related with their hosts and beneficial (Scudder, 2009; Engel & Moran, 2013). Insects are the biggest animal group on earth, including more than approximately 2 million species. They can be found almost in every ecosystem in the world and can sometimes be seen in very intense populations (Basset et al., 2012; Novotny et al., 2002). These creatures which have a large amount of species and are found in large quantities have been successful in their evolutionary histories partly by dint of beneficial microorganisms living with them (Engel & Moran, 2013). Based on today's information, it can be said that these symbiotic microorganisms are responsible for various functions (making useful nutrient-poor diet, aiding digestion of insect's food compounds, protecting against their enemies and determining mating and reproductive systems) in insects (Douglas, 2015). Especially, some studies showed that bacterial communities within insects can affect the developments stages of insects (Souza

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et al., 2019; Peterkova-Koci, Robles-Murguia, Ramalho-Ortigao & Zurek, 2012). Therefore, it is important to study insect-microbial relations to clarify roles of these microorganisms in insect's life.

Therewithal, some microorganisms such as viruses, bacteria, nematodes, fungi, and protozoans can cause infection diseases in various insects (Lacey et al., 2015). These insect pathogenic microorganisms are sometimes harmful in terms of beneficial insects such honeybee and silkworm and sometimes useful for harmful insects (also called pests) in agriculture and forestry. Within these microbial control agents, bacteria (especially *Bacillus thrungiensis*) have special importance since they are the most commercially used and produced in terms of controlling of important pest species (Ben-Dov, 2014). Apart from *B. thuringiensis*, there are many bacterial species (such as *Lysinibacillus sphaericus*, *Brevibacillus laterosporus*, *Serratia* spp. and, *Pseudomonas entomophila*) which are pathogenic to insects (Ruiu, 2015).

The first step is to isolate and characterize bacterial species in insect's gut to elucidate their functions in host's development and biology. In accordance with this purpose, up to now, many studies have been carried out to isolate and identify various bacteria from various insect species (Sevim, Çelebi & Sevim, 2012; Liu et al., 2016; Anand et al., 2010). According to many studies, molecular characterization techniques (especially 16S rRNA sequencing) are the most reliable techniques for identifying bacterial species (Janda & Abbott, 2007). In this study, we purposed to isolate and identify bacterial species from different development stages of *Sanys irrosea*, which was selected as model organism, based on the culture-dependent technique and to characterize them by 16S rRNA gene sequence analysis. This insect was selected as model organism for moths to study the bacterial diversity since it has a wide and intense distribution in the study region. The attained results from here could be beneficial for future and further studies to elucidate the roles of these bacteria in the insect biology, especially Lepidopteran insects.

2. Materials and Methods

2.1. Collection of *Sanys irrosea* larvae

Sanys irrosea larvae were collected from Kırşehir city (steppe fields) in Turkey between May-June 2019. The collected larvae were put into plastic boxes (30 × 25 cm) with leaves of the plant on which they feed and brought to the laboratory. After that, the larvae were separated based on their sizes into different developments stages from first to fifth instar. They were fed with the collected plant leaves in the laboratory for two days and healthy larvae without disease were selected and used for bacterial isolation.

2.2. Molecular identification of larvae

Recently, molecular identification techniques such as DNA sequencing are being frequently used for many insects due to difficulties and limitations in morphological taxonomy (Campbell, Lawrence, Hudspath & Gruwell, 2014). Also, one of the most important parts for solution in growing problems with many pest species and the studying of insect-microbe relations require complete and accurate species identification. Therefore, in this study, we used the partial sequence of cytochrome oxidase (subunit I) (*COI*) gene (598 bp long) for identification of the collected larvae samples. Total genomic DNA from larvae were extracted using QIAGEN DNeasy Blood & Tissue kit (Hilden, Germany) based on the manufacturer's recommendations. For isolation, head parts of the larvae were used. The isolated DNAs were stored at -20 °C until use in PCR amplification. After that, approximately 620 bp fragment of *COI* gene was amplified by PCR. The primer pair of LCO1490-5'-GGTCAACAAATCATAAAGATATTGG-3' as forward and HCO2198-5'-TAAACTTCAGGGTGAC-CAAAAAATCA-3' as reverse were used in PCR (Folmer, Black, Hoeh, Lutz & Vrijenhoek, 1994). The PCR was performed in 50 µl reaction volume as follow: 1 µl genomic DNA, 5 µl 10 × PCR buffer, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA Polymerase enzyme, 0.25 mM opposing primers and 200 mM of each dNTP. The final volume was completed to 50 µl by sterile ddH_2O. Thermal cycles were as follow; after first denaturation at 96 °C for 5 min, 95°C for 1 min, 56 °C for 45 s and 72 °C for 1 min as 36 cycles and 72 °C for 10 min for final extension. After PCR, the obtained products were run on 1 % agarose gel containing ethidium bromide for 15 min at 90 V and viewed under UV light. After the correct PCR bands were seen on the gel, one of them was purified, quantified, and sent to Macrogen (the Netherlands) for sequencing. Amplification primers for *COI* gene were also used for sequencing.

2.3. Bacterial isolation

The field collected larvae of *S. irrosea* were firstly separated into different development stages (instar) from first to fifth instar and waited for 2-3 days in the laboratory to select healthy larvae for the bacterial isolation. After selection of the healthy larvae, ten larvae for each instar were used for bacterial isolation. Firstly, the larvae were separately immersed in 70% ethanol for 3 min for surface sterilization and then, washed twice with sterile dH_2O. The surface sterilized larvae were separately (based on different instar) homogenized in 3 ml nutrient broth using a tissue grinder and filtered through two layers of sterile cheese cloth to remove insect debris. After that, five homogenates (for each instar) were diluted with sterile nutrient broth from 10^{-1} to 10^{-8}. 100 µl from each dilution was taken and spread on nutrient agar (NA) by the spread plate method and incubated at 30 °C for two days in dark. After this, the total colony on each countable petri was counted by eye and the number of bacteria for per larva was calculated as colony forming unit (cfu). Later, each different colony was selected according to their shape, type, color, and morphology. The selected colonies were purified and stored in 20% glycerol at - 20°C for further identification studies.

2.4. 16S rRNA gene sequencing

The stock cultures for each bacterial strain were plated on nutrient agar by the streak plate method to obtain a single colony. A single colony for each strain was inoculated into 3 ml of nutrient broth (NB) and incubated in a rotary shaker at 200 rpm overnight. Later, genomic DNA extraction was done using these cultures. Genomic DNAs were extracted using PureLink™ Genomic DNA Mini Kit (Invitrogen). 16S rRNA gene regions belonging to each bacterial strain were amplified by the universal primer pair of 27F (5'- AGAGTTT-GATCMTGGCTCAG-3') as forward and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') as reverse (Macrogen). The PCR was performed in 50 µl reaction volume including 1 µl genomic DNA, 5 µl 10 x PCR buffer, 1.5 mM MgCl2, 1.25 U *Taq* DNA Polymerase, 0.25 mM forward and reverse primer for each and 200 mM of each dNTP. The final volume was completed by sterile ddH_2O to 50 µl. Thermal cycles were as follow; after first denaturation at 96 °C for 5 min, 95°C for 1 min, 55 °C for 1 min and 72 °C for 1,5 min as 36 cycles and 72 °C for 10 min for final extension. After PCR, the obtained products were run on 1 % agarose gel containing ethidium bromide for 15 min at 90 V and viewed under UV light. After the correct PCR bands were seen on the gel, all were sent to Macrogen (the Netherlands) for sequencing. The primer pair 518F (5′-CCAGC AGCCGCGGTAATACG-3′) and 800R (5′-TACCAGG GTATCTAATCC-3′) were used for sequencing (Macrogen).

2.5. Data analysis

The gene sequences were edited and aligned with BioEdit version 7.2.5 (Hall, 1999). The edited sequences were used for Blast search in GenBank to compare each sequence with their closely related insect or bacterial species and percent (%) similarity values were calculated (Altschul, Gish, Miler, Myers & Lipman, 1990; Benson et al., 2012). In addition, these gene sequences were used for phylogenetic analysis using MEGA-X software (Kumar, Stecher, Li, Knyaz & Tamura, 2018). For larvae, the partial sequence of *COI* gene (approximately 598 bp) was used to compare reference species in the study of (Mutanen, Wahlberg & Kaila, 2010). For bacterial strains, the 16S rRNA gene sequences were compared with their most closely related bacterial species based on Blast search in GenBank. For phylogenetic relationships, the neighbor-joining method with p-distance analysis packed in MEGA-X was used. The strength of the internal branches in the final tree was statistically evaluated by bootstrap analysis based on 1.000 pseudoreplicates using MEGA-X.

2.6. GenBank accession numbers of the bacterial isolates

GenBank accession numbers for 16S rRNA gene sequences belonging to the bacterial isolates are from MT537942 to MT537963.

3. Results and Discussion

The collected larvae were identified by the partial sequence of *COI* gene (approximately 598 bp). Based on the Blast search, *COI* sequence (598 bp) of the collected larvae was found to be similar with *Peactes fuscescens voucher* 11-SRNP-23183 with 90.62 % and *S. irrosea* DHJ04 with 90.10%. Since this similarity was very low

for species identification, we performed a phylogenetic analysis using reference species mentioned in the study of (Mutanen et al., 2010) to perform more correct species identification. Based on the phylogenetic analysis, the larva was identified as *Sanys irrosea* (Guenee, 1852) (Lepidoptera: Noctuidae) (Figure 1).

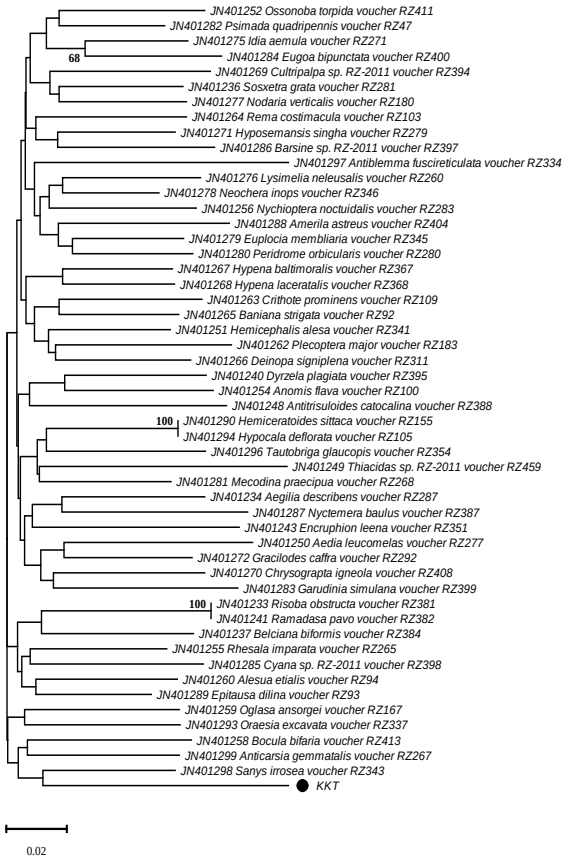

Figure 1. The dendrogram generated using Neighbor Joining (N-J) algorithm with p-distance analysis.

The tree was generated using the partial sequence (598 bp) of *COI* gene. *Sanys irrosea* sample which is marked with solid black dot was compared with the reference insect samples used in the study of Mutanen et al. (2010). The statistical accuracy of the tree was evaluated by bootstrap confidence analysis according to 1.000 repetitions and bootstrap values of 70% or higher were specified in the tree. The scale located under the tree shows the degree of dissimilarity.

Bacteria grown in petri dishes, where bacterial colonies can be evaluated separately, were counted and the number of culturable bacteria per larva was calculated as follows. The numbers of total bacteria in first, second, third, fourth and fifth instar larvae were determined as 1.2×10^7 bacteria/larva, 1.9×10^7 bacteria/larva, 2.1×10^8 bacteria/larva, 3.6×10^8 bacteria/larva, and 0.6×10^9 bacteria/larva, respectively.

A total of 22 culturable bacterial isolates were obtained from different development stages of *S. irrosea*. Among them, six isolates from first instar, six isolates from second instar, two isolates from third instar, three isolates from forth instar, and five isolates from fifth instar larvae were isolated. All bacterial isolates were identified at species or genus level using 16S rRNA sequence analysis. Blast results of 16S rRNA genes are given in Table 1.

Table 1.
Percentage identities of *S. irrosea* bacterial isolates with their the most closely associated bacteria in GenBank according to the Blast search of 16S rRNA gene sequences

Instar	Strain	Species	GenBank ID	Query coverage (%)	Percent (%) Similarity
First Instar	KT1.1	*Micrococcus yunnanensis SJU9*	MN511766	99%	99.57%
		Micrococcus sp. NJS11	MN833053	99%	99.64%
		Micrococcus yunnanensis 190306H2421	MT225720	99%	99.50%
	KT1.2	*Staphylococcus haemolyticus B-16*	KC139451	99%	99.57%
		Staphylococcus haemolyticus N15	KX507089	99%	99.57%
		Staphylococcus sp. CLC-F26	MH518208	100%	99.50%
	KT1.3	*Micrococcus sp. 185*	EU714334	98%	99.71%
		Micrococcus luteus 10240	CP041689	99%	99.71%
		Micrococcus luteus NCCP 16831	CP043842	99%	99.71%
	KT1.4	*Acinetobacter lwoffii O27*	MG594818	99%	99.57%
		Acinetobacter sp. NMS3	MN515076	99%	99.57%
		Acinetobacter lwoffii WST 5	DQ289068	99%	99.57%
	KT1.5	*Bacillus anthracis 7-6*	JX867748	99%	99.93%
		Bacillus sp. SRG13	MK743992	99%	99.93%
		Bacillus albus XM4	MT023381	99%	99.93%
	KT1.6	*Arthrobacter agilis AU D4.2*	KY775493	100%	97.94%
		Arthrobacter sp. ITT16	FR667186	100%	97.94%
		Arthrobacter agilis UMCV2	CP024915	100%	97.80%
Second Instar	KT2.1	*Staphylococcus capitis IAE36*	MK414980	100%	99.19%
		Staphylococcus capitis BQEN3-03	FJ380955	100%	99.19%
		Staphylococcus capitis BBN3T-04d	FJ357614	100%	99.19%
	KT2.2	*Staphylococcus sp. PaD1.45b1*	GQ406605	99%	99.79%
		Staphylococcus sp. PaH2.43b	GQ391961	99%	99.79%
		Staphylococcus epidermidis BBEN-01d	FJ357583	99%	99.79%
	KT2.3	*Staphylococcus capitis BBN3T-04d*	FJ357614	99%	99.86%
		Staphylococcus capitis BBN3P-01d	FJ357608	99%	99.79%
		Staphylococcus epidermidis BQN1N-02d	FJ380964	99%	99.79%
	KT2.4	*Bacillus simplex EH12*	MN750767	99%	100%
		Bacillus thuringiensis EGI94	MN704417	99%	100%
		Bacillus simplex EGI87	MN704413	99%	100%
	KT2.5	*Micrococcus sp. N36(2010)*	HQ188562	99%	98.12%
		Micrococcus yunnanensis KA-20	KX108873	99%	98.12%
		Micrococcus sp. Actino-13	MH671510	99%	98.05%
	KT2.6	*Clavibacter sp. PDD-59b-50*	KR922173	99%	98.77%
		Clavibacter michiganensis PDD-57b-26	KR922121	100%	98.70%
		Clavibacter michiganensis Cmm VT3	HQ144242	100%	98.70%
Third Instar	KT3.1	*Staphylococcus hominis CU1-6*	MT373476	99%	98.75%
		Staphylococcus hominis K23	KU922442	99%	98.75%
		Staphylococcus hominis H45	KU922315	99%	98.75%
	KT3.2	*Uncultured Staphylococcus sp. clone TJ-3*	JQ858218	99%	99.93%
		Staphylococcus haemolyticus SR4-27	MN421506	99%	99.86%

		Staphylococcus haemolyticus M2	KC182061	99%	99.86%
Fourth Instar	KT4.1	*Moraxella sp. CRE4*	MT380814	100%	99.64%
		Moraxella osloensis NT4	MK571189	100%	99.64%
		Moraxella osloensis NT4	MK571171	100%	99.64%
	KT4.2	*Micrococcus yunnanensis SJU9*	MN511766	99%	99.86%
		Micrococcus yunnanensis L7-617	JQ659453	99%	99.86%
		Micrococcus sp. T7	MN049740	99%	99.86%
	KT4.3	*Kocuria rosea RR75*	MK532258	100%	98.22%
		Kocuria rosea PGRS5	MH489032	100%	98.22%
		Kocuria sp. H200-662	MG754440	100%	98.22%
Fifth Instar	KT5.1	*Micrococcus sp. DMO-7*	MT294696	100%	99.49%
		Micrococcus luteus 1910ICU142	MT225650	100%	99.49%
		Micrococcus yunnanensis QT410	MT033093	100%	99.49%
	KT5.2	*Uncultured Staphylococcus sp. TJ-3*	JQ858218	100%	99.72%
		Staphylococcus haemolyticus SR4-27	MN421506	100%	99.65%
		Staphylococcus haemolyticus BQN1L-01d	FJ380961	100%	99.65%
	KT5.3	*Bacillus aerius CJLT*	JN852814	99%	99.29%
		Bacillus licheniformis YC1-A	HQ634208	99%	99.29%
		Bacillus licheniformis SIITMB5	MG892780	99%	99.22%
	KT5.4	*Staphylococcus sp. PGT-LC*	KY490691	100%	99.79%
		Staphylococcus hominis OsEnb_ALM_C9	MN889343	99%	99.79%
		Staphylococcus sp. CIFRI PTSB-29	JF784037	100%	99.72%
	KT5.5	*Staphylococcus sp. ST5-08*	KF891400	100%	99.79%
		Staphylococcus hominis OsEnb_ALM_C9	MN889343	99%	99.79%
		Bacterium MTL7-24	MH151280	99%	100%

According to the BLAST search and phylogenetic analysis, the bacterial isolates were identified as *Micrococcus yunnanensis* KT1.1, *Staphylococcus haemolyticus* KT1.2, *Micrococcus luteus* KT1.3, *Acinetobacter lwoffii* KT1.4, *Bacillus* sp. KT1.5, *Arthrobacter agilis* KT1.6, *Staphylococcus capitis* KT2.1, *Staphylococcus* sp. KT2.2, *Staphylococcus capitis* KT2.3, *Bacillus* sp. KT2.4, *Micrococcus* sp. KT2.5, *Clavibacter michiganensis* KT2.6, *Staphylococcus hominis* KT3.1, *Staphylococcus hemolytic* KT3.2, *Moraxella osloensis* KT4.1, *Micrococcus yunnanensis* KT4.2, *Kocuria rosea* KT4.3, *Micrococcus* sp. KT5.1, *Staphylococcus haemolyticus* KT5.2, *Bacillus* sp. KT5.3, *Staphylococcus* sp. KT5.4 and *Staphylococcus* sp. KT5.5 (Figure 2).

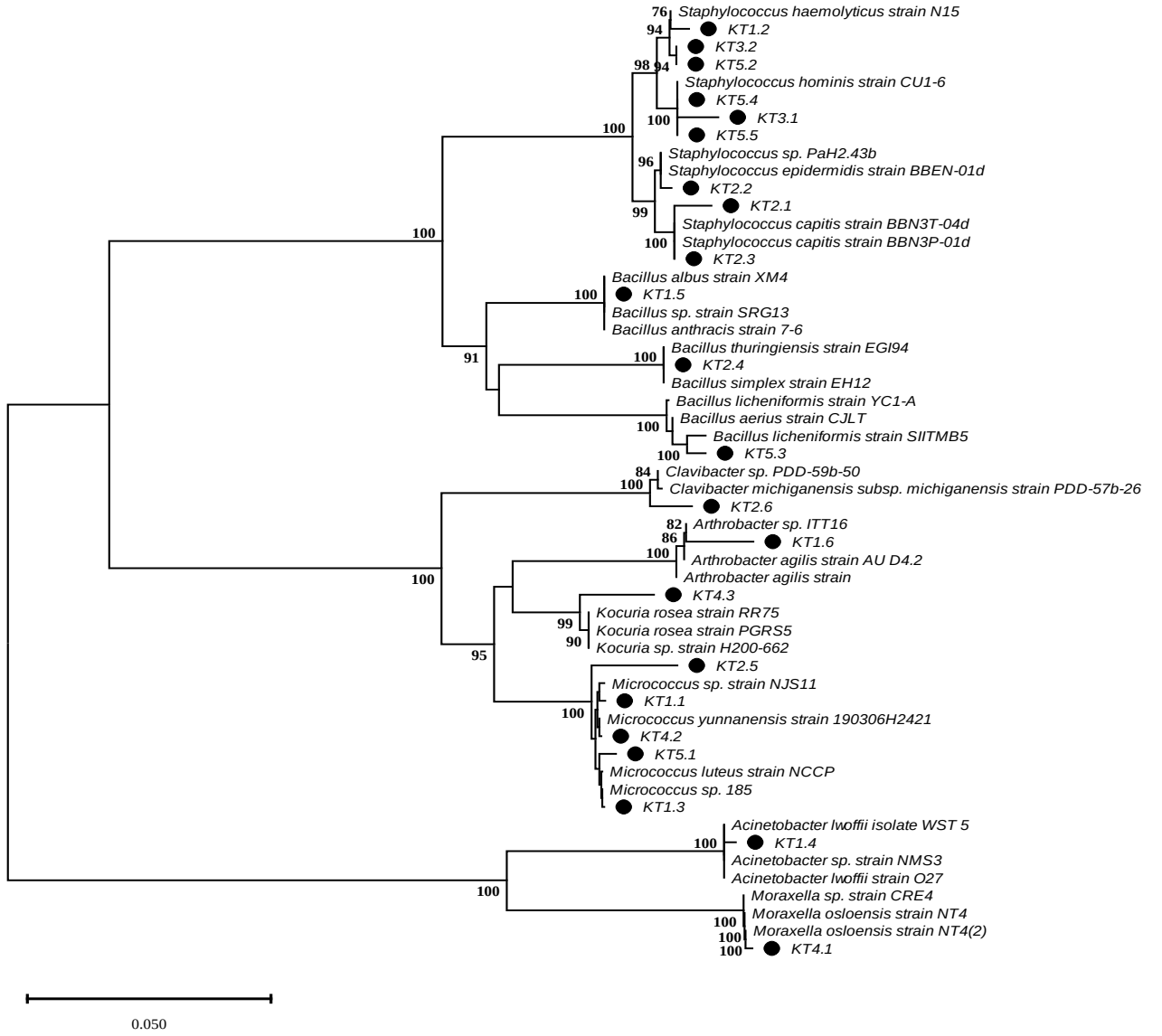

Figure 2. The dendrogram generated using Neighbor Joining (N-J) algorithm with p-distance analysis.

The tree was generated using the partial sequence (approximately 1.500 bp) of 16S rRNA gene. The bacterial isolates used in this study which are marked with solid black dot were compared with the reference species or strains taken from GenBank based on their percent identities with the bacterial isolates. The statistical accuracy of the tree was evaluated by bootstrap confidence analysis according to 1.000 repetitions and bootstrap values of 70% or higher were specified in the tree. The scale located under the tree shows the degree of dissimilarity.

Insects are the most crowded animal group in Arthropoda phylum in terms of class, taxon and species number and they have the biggest number of species in the world. Their evolutionary success partly depends on their interaction with many prokaryotic and eukaryotic microorganisms (Scudder, 2009). These microorganisms which live inside insects are responsible for many important features in insect's life cycle such as enzymatic degradation of nutrients, synthesis of essential metabolites like vitamins, protection of the hosts against biotic and abiotic factors and regulation of development and reproduction of insects (Douglas, 2014). For Lepidopteran insects, there are some evidence that instar-specific bacterial communities should be available for some insects (Chen et al., 2016). Moreover, insect symbiotic bacteria are good candidate for manipulating them using genetic engineering techniques to protect beneficial insects against their enemies and to combat with harmful insects (pests). Based on all this, it is important to study and identify bacterial species that live in insects.

In this study, we found that the gut bacteria (or microbiome) of *S. irrosea* showed great variability consisting of different members of Bacillaceae, Staphylococcaceae, Micrococcaceae, Moraxellaceae and Microbacteriaceae families. Many of the isolated bacteria in this study such as *Staphylococcus*, *Micrococcus*, *Bacillus*, *Acinetobacter* and *Arthrobacter* have been isolated from many insects including Lepidopteran pests (Sevim et al., 2012; Minard et al., 2013). Within these bacteria, *Bacillus* species (*Bacillus* sp. KT1.5, KT2.4 and KT5.3) draw an attention since bacterium *B. thuringiensis* (Bt) is located in this genus and widely used as biological insecticides against many pest's species worldwide (de Maagd, Bravo & Crickmore, 2001). However, it is very hard to distinguish this species from other *Bacillus* members based on 16S rRNA sequence analysis. Therefore, it should be interesting to identify these isolates at species level, to search crystal proteins (if there is) and to test them against a numbers of insect pests to determine their biocontrol potential.

We isolated two interesting bacteria (*Moraxella osloensis* KT4.1 and *Kocuria rosea* KT4.3) regarding insect-bacterium relation. Both species were isolated from fourth instar larvae. *M. osloensis* is known as a bacterial symbiont of *Phasmarhabditis hermaphrodita* which is a slug-parasitic nematode, and this bacterium is transported to the shell cavity of the slug via the nematode and kills the host (Crawford, Hutton & Chapman, 1975). The members of *Kocuria* genus includes gram-positive bacteria that normally inhabit skin and mucous membrane of human and many animals. But there are some evidences that these bacteria might be related to human infections with weakened immune systems (Kandi et al., 2016). In terms of insect-bacteria associations, these two bacteria seem to be novel for further investigations. Especially, due to pathogenic properties of these bacteria, it should be interesting to study a potential transmission of these bacteria to other animals or humans.

In this study, we also isolated a *Clavibacter michiganensis* subsp. *michiganensis* KT2.6 which is a plant pathogenic bacterium causing a bacterial wilt and canker in tomatoes (Gartemann et al., 2003). It has been known that some bacterial pathogens colonizing and living in the vascular systems of plants (phloem or xylem) can be transmitted by several insects such as whiteflies, aphids, and leafhoppers (Perilla-Henao & Casteel, 2016). That means some insects can serve a vector of important plant pathogenic bacteria. Therefore, studying vector-pathogen-host (etc. insects) is a crucial issue to understand the epidemiology of plant diseases. There are some evidences that some plant pathogenic subspecies of *Clavibacter michiganensis* can be transmitted from plant to plant via insect vectors such as Colorado potato beetles and the green peach aphid (Christie, Sumalde, Schulz & Gudmestad, 1991). In addition, nematodes *Dylenchus dipsaci* can serve a vector of *C. michiganensis* causing bacterial wilt in alfalfa (Hawn, 1971). Therefore, it should be interesting to study a vector potential of *S. irrosea* with respect to C. *michiganensis*, at least for non-agricultural plants.

We isolated different bacterial species from different development stages (instars) of *S. irrosea*. Based on our results, we did not determine a clear pattern regarding changes in gut microbiota diversity for different instars. In the literature, it has been demonstrated that although development stages do not affect the bacterial community in some insect species, bacterial diversity or communities can change in other insects such as *Spodoptera littoralis* (Boisduval, 1833) according to the developmental stages (Chen et al., 2016; Mereghetti, Chouaia & Mantagna, 2017). Based on these, it can be said that there is no general rule regarding bacterial diversity or composition could be changed according to the developmental stages of insects.

4. Conclusion

We determined a bacterial diversity in different developmental stages (instars) of *S. irrosea* using the culture-dependent technique and showed that there is no specific pattern with respect to the bacterial diversity in different instars.

Author Contributions

Ali Sevim: Conception/design of study, data acquisition, data analysis and interpretation, drafting manuscript, critical revision of manuscript.
Elif Sevim: Data acquisition, data analysis and interpretation.

Conflicts of Interest

The authors declare no conflict of interest.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol, 215(3),* 403-10. doi: https://doi.org/10.1016/S0022-2836(05)80360-2

Anand, A. A., Vennison, S. J., Sankar, S. G., Prabhu, D. I., Vasan, P. T., Raghuraman, T., Geoffrey, C. J., & Vendan, S. E. (2010). Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. *Journal of Insect Sci, 10,* 107. doi: https://doi.org/10.1673/031.010.10701

Basset, Y., Cizek, L., Cuenoud, P., Didham, R. K., Guilhaumon, F., Missa, O., Novotny, V., Odegaard, F., Roslin, T., & Leponce, M. (2012). Arthropod diversity in a tropical forest. *Science, 338(6113),* 1481-1484. doi: 10.1126/science.1226727

Ben-Dov, E. (2014). *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. *Toxins (Basel), 6(4),* 1222-1243. doi: https://doi.org/10.3390/toxins6041222

Benson, D. A., Karsch-Mizrachi, I., Clark, K., Lipman, D. J., Ostell, J., & Sayers, E. W. (2012). GenBank. *Nucleic Acids Res, 40(Database issue),* D48-53. DOI: https://doi.org/10.1093/nar/28.1.15

Campbell, A. M., Lawrence, A. J., Hudspath, C. B., & Gruwell, M. E. (2014). Molecular Identification of Diaspididae and Elucidation of Non-Native Species Using the Genes 28s and 16s. *Insects, 5(3),* 528-538. doi: https://doi.org/10.3390/insects5030528

Chen, B., The, B. S., Sun, C., Hu, S., Lu, X., Boland, W., & Shao, Y. (2016). Biodiversity and Activity of the Gut Microbiota across the Life History of the Insect Herbivore *Spodoptera littoralis*. *Sci Rep, 6,* 29505. doi: https://doi.org/10.1038/srep29505

Christie, R. D., Sumalde, A. C., Schulz, J. T., & Gudmestad, N. C. (1991). Insect transmission of the bacterial ring rot pathogen. *Am Potato J, 68(6),* 363-372. doi: https://doi.org/10.1007/BF02853617

Crawford, R. L., Hutton, S. W., & Chapman, P. J. (1975). Purification and properties of gentisate 1,2-dioxygenase from *Moraxella osloensis*. *J Bacteriol, 121(3),* 794-9. doi: https://doi.org/10.1128/jb.121.3.794-799.1975

de Maagd, R. A., Bravo, A., & Crickmore, N. (2001). How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Gen, 17(4),* 193-199. doi: https://doi.org/10.1016/S0168-9525(01)02237-5

Douglas, A. E. (2014). The molecular basis of bacterial-insect symbiosis. *J Mol Biol, 426(23),* 3830-38377. doi: https://doi.org/10.1016/j.jmb.2014.04.005

Douglas, A. E. (2015). Multiorganismal insects: diversity and function of resident microorganisms. *Ann Rev Entomol, 60,* 17-34. doi: https://doi.org/10.1146/annurev-ento-010814-020822

Engel, P., & Moran, N. A. (2013). The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Rev, 37(5),* 699-735. doi: https://doi.org/10.1111/1574-6976.12025

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol, 3(5),* 294-9.

Gartemann, K. H., Kirchner, O., Engemann, J., Gräfen, I., Eichenlaub, R., & Burger, A. (2003). *Clavibacter michiganensis* subsp. *michiganensis*: First steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium. *J Biotechnol, 106(2),* 179-191. doi: https://doi.org/10.1016/j.jbiotec.2003.07.011

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser, 41,* 95-98.

Hawn, E. J. (1971). Mode of Transmission of *Corynebacterium insidiosum* by *Ditylenchus dipsaci*. *J Nematol, 3(4),* 420-1.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol, 45(9),* 2761-4. doi: https://doi.org/10.1128/JCM.01228-07

Kandi, V., Palange, P., Vaish, R., Bhatti, A. B., Kale, V., Kandi, M. R., & Bhoomagiri, M. R. (2016). Emerging Bacterial Infection: Identification and Clinical Significance of *Kocuria* Species. *Cureus, 8(8),* e731. doi: https://doi.org/10.7759/cureus.731

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol Biol Evol, 35(6),* 1547-1549. doi: 10.1093/molbev/msy096

Lacey, L. A., Grzywacz, D., Shapiro-Ilan, D. I., Frutos, R., Brownbridge, M., & Goettel, M. S. (2015). Insect pathogens as biological control agents: Back to the future. *J Invertebr Pathol, 132*, 1-41. doi: https://doi.org/10.1016/j.jip.2015.07.009

Liu, W., Li, Y., Guo, S., Yin, H., Lei, C. L., & Wang, X. P. (2016). Association between gut microbiota and diapause preparation in the cabbage beetle: a new perspective for studying insect diapause. *Sci Rep, 6*, 38900-38900. doi: https://doi.org/10.1038/srep38900

Madigan, M. T., Martinko, J. M., Parker. J., & Brock, T. D. B. (2003). Brock Biology of Microorganisms. New York, NY: Prentice Hall.

Mereghetti, V., Chouaia, B., & Montagna, M. (2017). New Insights into the Microbiota of Moth Pests. *Int J Mol Sci, 18(11)*, 2450. Doi: https://doi.org/10.3390/ijms18112450

Minard, G., Tran, F. H., Raharimalala, F. N., Hellard, E., Ravelonandro, P., Mavingui, P., & Moro, C. V. (2013). Prevalence, genomic and metabolic profiles of *Acinetobacter* and *Asaia* associated with field-caught *Aedes albopictus* from Madagascar. *FEMS Microbiol Ecol, 83(1)*, 63-73. doi: https://doi.org/10.1111/j.1574-6941.2012.01455.x

Moore, S. J., & Warren, M. J. (2012). The anaerobic biosynthesis of vitamin B12. *Biochem Soc Trans, 40(3)*, 581-6. doi: https://doi.org/10.1042/BST20120066

Mutanen, M., Wahlberg, N., & Kaila, L. (2010). Comprehensive gene and taxon coverage elucidates radiation patterns in moths and butterflies. *Proc Roy Soc B, 277(1695)*, 2839-2848. doi: https://doi.org/10.1098/rspb.2010.0392

Novotny, V., Basset, Y., Miller, S. E., Weiblen, G. D., Bremer, B., Cizek, L., & Drozd, P. (2002). Low host specificity of herbivorous insects in a tropical forest. *Nature, 416(6883)*, 841-844. doi: https://doi.org/10.1038/416841a

Perilla-Henao, L. M., & Casteel, C. L. (2016). Vector-Borne Bacterial Plant Pathogens: Interactions with Hemipteran Insects and Plants. *Front Plant Sci, 7*, 1163-1163. doi: https://doi.org/10.3389/fpls.2016.01163

Peterkova-Koci, K., Robles-Murguia, M., Ramalho-Ortigao, M., & Zurek, L. (2012). Significance of bacteria in oviposition and larval development of the sand fly *Lutzomyia longipalpis*. *Parasites Vectors, 5*, 145. Doi: https://doi.org/10.1186/1756-3305-5-145

Ruiu, L. (2015). Insect Pathogenic Bacteria in Integrated Pest Management. *Insects, 6(2)*, 352-67. doi: https://doi.org/10.3390/insects6020352

Scudder, G. G. E. (2009). The importance of insects. Foottit R G, Adler PH, editors. Insect Biodiversity (pp. 7-32). Oxford: Blackwell Publishing Ltd.

Sevim, E., Çelebi, Ö., & Sevim, A. (2012). Determination of the bacterial flora as a microbial control agent of *Toxoptera aurantii* (Homoptera: Aphididae). *Biologia, 67(2)*, 397-404. doi: https://doi.org/10.2478/s11756-012-0022-0

Souza, R. S., Virginio, F., Riback, T. I. S., Suesdek, L., Barufi, J. B., & Genta, F. A. (2019). Microorganism-Based Larval Diets Affect Mosquito Development, Size and Nutritional Reserves in the Yellow Fever Mosquito *Aedes aegypti* (Diptera: Culicidae). *Front Physiol, 10*, 152. doi: https://doi.org/10.3389/fphys.2019.00152