# PLANT HOST INDUCED VARIATION OF ENDOSYMBIONTS ASSOCIATED WITH *KUSMI* LAC INSECTS

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**Abstract**
*Kerrialacca* (Kerr), a sap sucking insect, is widely grown commercially on *Butea monosperma* (*Palas*), *Schleicheraoleosa* (*Kusum*), *Ziziphusmauritania*(*Ber*) and *Flemingiasemialata* in India. Lac insects secrete resin, wax and dye for self-protection, which have numerous applications in different fields. Lac cultivation is one of the major sources of income for the tribal community of the country. *Kusmi* lac insects is specific to India and contribute good quality and quantity lac. Lac insects live on nutrient-poor diets of phloem sap; hence to compensate for other important nutrients they have evolved obligate associations with microorganisms. Endosymbionts are supposed to have various roles in insect development and physiology. Some bacteria consociates with lac insects have been identified and understood, but several remain unknown. Our objective is to identify the diversity and variation of endosymbiotic bacteria in *kusmi*lac insects *grown on kusum, ber*and*semialata*using molecular methods. We followed culturing method to isolate bacterial endosymbionts from *kusmi* lac insects from different host plants. 16S rDNA of the bacterial isolates were amplified and sequenced for their identification, which could be used to decipher their role in lac insects. This study shows ten different bacteria to be associated with lac insects and differences in the bacterial species from lac insects grown on different host plants. Our study would contribute to a broader understanding of the diversity of endosymbionts and their symbiotic associations with lac insects.
**Key words:** endosymbiont, lac insect, *kusum*, *ber* and *semialata*

## Introduction

Insectsareconsideredtobethemostsuccessfulanddiversegroupoforganisms,withabout 5.5 million species reported (Stork, 2018).Newinsectspeciesarebeingidentifiedatarateofabout5,000species peryear (Morgan,2010).Their adaptability to various habitats is one of the reasons for their success which is mainly attributed by their microbial symbionts. Like vertebrates, insects also harbour symbiotic bacteria on the integument, in the digestive tract and in some unique structures within their body (Chen *et al.* 2000; Fukatsu*et al.* 2000). Many types of bacteria have been identified from different orders of insects including hemiptera, lepidoptera, diptera and isoptera.The role of endosymbionts in insects include digestion (of recalcitrant food), nutrition (supplementation of vitamins and other essential nutrients), protection (production of anti-fungal agents, detoxification of pesticides), resistance (against predators, parasites and pathogens), inter- and intra-specific communication (pheromone production), increasing efficiency as disease vectors, host insect morphogenesis andtemperature tolerance (Dillon and Dillon, 2004).Growth and development of many insects is dependent upon these microbes as theycontribute essentially in insect physiology.

Symbiosis with bacterial community is obligatory in insects whose diet is imbalanced such as vertebrate blood (by mosquitoes), phloem sap (by sap sucking insects) and wood (by termites). Most of the understanding between sap sucking insect host and endosymbionts arise from the studies involving the aphids and *Buchnera*spp which provide amino acids and vitamins to their host (Douglas, 1989). Lac insects widely used for their resin is a sap sucking insect. The Indian lac insect, *Kerrialacca* (Kerr) (Hemiptera: Coccoidea: Kerridae) has a wider host range, though commercially cultivated on few hosts such as *Palas* (*Buteamonosperma*), *Kusum* (*Schleicheraoleosa*), *Ber*(*Zizyphusmauritiana*) and *Flemingiasemialata (semialata).Kusmi* strain of lac insects is specific to India and yields high quantity and good quality resin.

Since lac insects are sap feeding insects, it is envisaged that the symbiotic bacteria must contribute towards nutrients such as amino acids and vitamins which are not obtained from phloem sap.The study on endosymbionts of lac insects is limited to few earlier works. Presence of microbial flora in lac insects is considered beneficial during rainy season crop for higher lac yield. Few bacteria such as *Micrococcus*spp, *Clostridium*sp and *Bacillus subtilis* were reported from lac insects (Sharma and Jaiswal, 2011). Vashishtha*etal*., 2011 had found that lac insects are associated with *Wolbachia* and yeast like symbionts (YLS) and predicted roles such as biased sex ratio and nutrient supplementation for them respectively. Few bacteria specific for male and female lac insects were identified by Shamim*et al*., 2017. However, no study has undertaken so far to document the bacterial species associated with lac insects from different host plants. Hence, an attempt was made to isolate and identify the bacterial species associated with *kusmi* lac insects grown on *kusum, ber* and *semialata*. We have followed culture based method to isolate bacteria from lac insects and 16S rRNA PCR based molecular method to identify them.

## Materials and Methods

### Sample collection and culturing of endosymbionts

The present study was carried out at Lac Production Division, ICAR-Indian Institute of Natural Resins and Gums, Ranchi, Jharkhand.Adult female lac insects of *kusmi* strain after fertilization were collected from different hosts such as *kusum*, *ber* and *semialata*and surface sterilized with 70% ethanol for about 15 minutes followed by a washing with 0.1% mercuric chloride for 10 minutes in laminar air flow chamber and then washed with sterile water for 3-5 times. The insects were homogenized in sterile nutrient brothand were plated onnutrient agar plates and kept for incubation at 37ºC for 24 to 48 hours.Wash water was also plated onnutrient agar platesto identify and confirm the surface contaminants. All the bacterial cultures were re-streaked to isolate single colonies and preserved as glycerol stocks. The morphology of the bacteria was examined using visual investigation and a light microscope.

### DNA isolation and PCR

Genomic DNA was isolated by TE method. Bacterial cultureswere grown overnight in a nutrient broth, cells were pelleteddown and re-suspended in TE buffer [100 mMTris and 10 mM EDTA (pH 8.0)] containing 10% SDS and 20 µg/ml proteinase K and incubated for an hour at 65°C. An equal volume of phenol: chloroform:isoamyl alcohol (25:24:1) was added and mixed well by inverting the tubes until the phases were completely mixed. After phase separation, the tubes were centrifuged at 12,000 x g for 10 min. The aqueous phase was collected in a separate tube and ethanol precipitated to pellet the DNA. The pellet was suspended in 200 µl of TE buffer (10 mMTris [pH 8.0], 1 mM EDTA). After measuring the absorbance of DNA at 260 nm, DNA was used for PCR. PCRs were performed to amplify 1.3 to 1.5 kb of the 16S rRNA gene from all the DNA samples by using universal 16S rRNA primers. The bacterial universal primers; 8F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' ACGGCTACCTTGTTACGACTT 3') were used. The PCR mixes of 25 µl contained, 25 ng of template DNA, 0.25 mM of each dNTP (FermentasInc, MD, USA), 10 pico moles of each primer, 1 unit of *Taq* DNA polymerase (Fermentas Inc., MD, USA). All the PCR reactions were carried out in a thermal cycler (Sensoquest, Germany) programmed with the following cycling condition. Initial denaturation of template DNA was carried out at 95ºC for 3 min followed by 35 cycles programmed for denaturation step at 95 ºC for

30sec, primer annealing step at 57.3 ºC for 30 sec, and DNA extension step at 72 ºC for 1 min. The final extension of the PCR products was carried out at 72 ºC for 10 min.

**Sequencing and Analysis**

The PCR products were sequenced using16S rRNA primers in a sequencing reaction on ABI 3130 automated sequencer (Applied Bio systems Inc., Carlsbad, CA,USA) at the sequencing facility of the Chromous Biotech, Bengaluru. Sequence analyses were performed with Geneious (6.1.8) software (Kearse *et al.,* 2012)in order to verify sequence quality and good sequences having at least 25 phred quality score were used. The ends of sequences were trimmed, aligned to generate consensus following *de novo* assembly. Preliminary identifications of the 16S rRNA gene sequences were done usingBLASTn (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) against 16S ribosomal RNA sequence (Bacteria and Archaea) database at NCBI.Sequences were subsequently submitted to GenBank ribosomal RNA database. Presence of chimeras were checked using DECIPHER v.2.8.1 (Wright *et al*., 2012).

**Results and Discussion**

Almost all insects have endosymbionts for their normal growth and development (Munson*et al*., 1992). Loss of these microorganisms often results in abnormal development and reduces survival of the insect host (Fukatsu and Hosokawa 2002). Identification of endosymbionts in insects is very much essential to decipher their roles in insects. The interaction between endosymbionts and insects are highly responsible for the adaptation of insects to particular ecosystem. In this study we have isolated and identified different bacterial endosymbionts from lac insects by 16S rDNA sequencing. A total of 29 bacterial samples were successfully cultured from lac insects collected from different host plants.

**16S rDNA Analysis**

The bacterial isolates were identified based on 16S rDNA sequencing. The PCR products were sequenced and sequence similarity check was conducted using BLASTn against 16S ribosomal database (Bacteria and Archaea). Organisms showing maximum similarity (97-100%) with the given sequences were considered. Chimera detection results showed that out of 29 sequences, 24 sequences were not deciphered as a chimera and 5 sequences as chimera, or indecipherable sequence (BH4, KH2, KH5, SH1, and SH2). 16S rDNA sequencing is a universal technology yielding unambiguous and reproducible data even for unusual and slow growing isolates (Woo *et al*., 2008). 16S rDNA sequences of the isolates were submitted to GenBank and the accession numbers obtained were from MH714881 to MH714909.

From 29 different bacterial isolates, 10 different bacteria were identified (Table1). *Bacillus kochii, Bacillus oceanisediminis, Bacillus amyloliquefaciens*, *Bacillus nakamurai*and *Enterobacter cloacae* were observed on *kusmi* lac insects collected from *kusum. Klebsiella quasipneumoniae subsp. similipneumoniae, Citrobacter a malonaticus*, *Providencia vermicola* and *B. nakamurai*were found in bacteria isolated from lac insects collected from *ber.Enterobacter ludwigii, B. nakamurai*and *Enterobacter cancerogenus* were found in lac insects collected from*semialata*. It was found that there is difference in bacterial endosymbionts of lac insects grown on different host plants. Host plants influencing the variation of insect endosymbionts is a normal phenomenon and has been reported earlier that facultative symbionts are closely related to the host plant species (Ferrari *et al*., 2012). Host plants influence the population size (Zhang *et al*., 2016) and frequency of the symbionts present in aphids (Ferrari *et al*., 2012). For example, *Regiella insecticola* emerge frequently in pea aphid collected from *Trifolium* whereas *Serratiasymbiotica* with aphids collected from *Cytisus*, *Pisum* and *Vicia* (Ferrari *et al*., 2012). Identification of bacteria in this study is solely based on 16S rDNA sequence. In few cases either sequence length or similarity index is less, wherein chances are there that the identity may change in future.

In most of the cases, different species of *Bacillus* and *Enterobacter* were found. *Bacillus* is a very common genus found in different types of insects, which include *Bacillus subtilis, Bacillusthuringiensis,Bacillus cereus, Bacillussphaericus, Bacilluspopillae,Bacilluscirculans, Bacillusmegaterium, Bacilluslentimorbus a*nd *Bacilluspolymyxa* (Incee*tal*., 2008).Shamim and co-workers (2017) have also identified *Bacillus*spp from lac insects grown on *Flemingiamacrophylla*.*B. nakamurai*the most frequent bacteria identified from this study was originally isolated from soil and known to produce black pigment (Dunlap *et al*., 2016). Since *B. nakamurai* was found in all three types of lac insects studied, it must have some very important function in lac insects, which is essential for their survival. However, further studies are required to address this aspect.*B. amyloliquefaciens* is known to control plant pathogens due to its antifungal activity. *B. amyloliquefaciens* isolated from feces of *Allomyrinadichotoma* larvae showed antifungal activity due to lipopeptide production (Nam *et al.*, 2016). Hence antifungal activity may be anticipated for the*B. amyloliquefaciens* strain present in lac insects.

Bacteria such as *Enterobacter* spp. *K.*quasi*pneumoniae*, *C.amalonaticus*, *Providenciavermicola*belong toEnterobacteriacea family. All these Enterobacters might have come from lac insect gut. Molecular phylogenetic analysis of 16S rRNA genes also demonstrated that most insect symbionts belong to the proteobacteria, primarily within the γ–subdivision (as reviewed in Moran and Telang, 1998). Similarly, in our study as well, *Klebsiella*, C*itrobacter* and *Providencia* were obtained, which belong to γ–proteobacteria. Other species of *Providencia*, *P. burhodogranariea* and *P. sneebia* have been discovered in the hemolymph of *Drosophilamelanogaster* (Ryan and Ray, 2004).*E. cloacae* may produce some metabolic by products like vitamins useful to the larvae (Kuzina*et al*., 2001). *E. cloacae* produces a strong antifungal compound (ammonia) inhibitory to many fungi (Howell *et al*., 1998). Since the ecosystem of lac insect is rich in fungi, *E. cloacae* may help in evading them or play role in nutrient supplementation. *E.cancerogenus*, specifically present in *semialata* was found to produce bio emulsifier, degrade xenobiotics, and resist alkalis and antibiotics (Wei *et al*., 2013) and hence protection role may be anticipated for this bacteria within lac insects.

In the present study, bacteria were isolated from lac insects grown on different host plants because it was assumed that the host factors play a major role in deciding the nature of bacteria dwells on insects. Variation also depends on the intake of sap on which lac insects feed, because nutrition plays a major role in deciding the microbial flora of an organism. Under the given experimental condition, the number of bacteria in lac insect from *kusum* was more than inlac insects from *semialata*and *ber*. Difference in bacterial number and types (from different host plants) may be due to weather conditions like temperature, humidity, moisture, wind, rainfall, and host endophytes *etc*. We could hypothesize roles like nutrition supplementation and protection against foreign agents for these endosymbionts in lac insects. However, further detailed studies are required to prove this hypothesis.

Higher diversity of endosymbionts is expected to be present in lac insects. Present study is a first attempt to explore the diversity of endosymbionts of lac insects from different lac hosts based on culture method. However, much more uncultivable bacteria and also stage dependent and strain dependent endosymbionts may be anticipated to be present in lac insects. Culture independent methods such as metagenomics would reveal more number of endosymbionts in lac insects. Future studies need to be done in these perspectives.

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**Table 1. Details of bacterial endosymbionts isolated from lac insects grown on different host plants**

| S. No | Host plant | Closest relative and its Accession No. | Accession No | %Identity |
|---|---|---|---|---|
| KB1 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714886 | 99% |
| KB2 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714887 | 99% |
| KB3 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714888 | 99% |
| KB4 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714889 | 99% |
| KB6 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714890 | 99% |
| KB7 | *Kusum* | *Bacillus kochii* NR_117050.1 | MH714891 | 99% |
| KB9 | *Kusum* | *Bacillus oceanisediminis* NR_117285.1 | MH714892 | 99% |
| KB10 | *Kusum* | *Bacillus amyloliquefaciens* NR_117946.1 | MH714893 | 99% |
| KB11 | *Kusum* | *Bacillus amyloliquefaciens* NR_117946.1 | MH714894 | 100% |
| KB12 | *Kusum* | *Bacillus amyloliquefaciens*NR_117946.1 | MH714895 | 99% |
| KH1 | *Kusum* | *Bacillus nakamurai* NR_151897.1 | MH714902 | 99% |
| KH2 | *Kusum* | *Bacillus nakamurai* NR_151897.1 | MH714882 | 99% |
| KH3 | *Kusum* | *Bacillus nakamurai* NR_151897.1 | MH714903 | 99% |
| KH4 | *Kusum* | *Bacillus nakamurai* NR_151897.1 | MH714904 | 99% |
| KH5 | *Kusum* | *Bacillus nakamurai* NR_151897.1 | MH714883 | 99% |
| KH6 | *Kusum* | *Enterobacter cloacae* NR_117679.1 | MH714905 | 98% |
| BH1 | *Ber* | *Klebsiellaquasipneumoniae* subsp. *similipneumoniae* NR_134063.1 | MH714896 | 99% |
| BH2 | *Ber* | *Klebsiellaquasipneumoniae* subsp. *similipneumoniae* NR_134063.1 | MH714897 | 97% |
| BH3 | *Ber* | *Bacillus nakamurai* NR_151897.1 | MH714898 | 98% |
| BH4 | *Ber* | *Citrobacteramalonaticus* NR_104823.1 | MH714881 | 99% |
| BH5 | *Ber* | *Citrobacteramalonaticus* NR_104823.1 | MH714899 | 99% |
| BH6 | *Ber* | *Providenciavermicola*NR_042415.1 | MH714900 | 99% |
| BH7 | *Ber* | *Citrobacteramalonaticus* NR_104823.1 | MH714901 | 98% |
| SH1 | *Semialata* | *Enterobacterludwigii* NR_042349.1 | MH714884 | 99% |
| SH2 | *Semialata* | *Enterobactercancerogenus* NR_116756.1 | MH714885 | 99% |
| SH3 | *Semialata* | *Bacillus nakamurai* NR_151897.1 | MH714906 | 99% |
| SH4 | *Semialata* | *Bacillus nakamurai* NR_151897.1 | MH714907 | 99% |
| SH5 | *Semialata* | *Bacillus nakamurai* NR_151897.1 | MH714908 | 99% |

| | | | | |
|---|---|---|---|---|
| SH6 | *Semialata* | *Bacillus nakamurai* NR_151897.1 | MH714909 | 99% |

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