Evaluation Of Mid-Gut Bacteria Present In The Larva Of Rice Moth, Corcyra Cephalonica (Stain.) (Galleriidae: Lepidoptera) Fed On Different Grains Using 16S rDNA Sequence Based Culture Dependent Technique

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Abstract — Corcyra cephalonica (Stainton) (Lepidoptera: Pyralidae) is a factitious host extensively used for rearing egg parasitoids. The present study attempt to investigate mid-gut bacteria present in larva of Corcyra cephalonica (C. cephalonica). The mid-gut bacteria were collected from the C. cephalonica fed on eight different grains were identified and characterized by 16S rDNA sequence based culture dependent method. The results revealed that, 16 bacterial species were present in mid-gut of C. cephalonica larva which are belonging to 11 bacterial genera included in three phyla namely Firmicutes, Proteobacteria and Actinobacteria. Among the three phyla Firmicutes was the most dominant phylum with a record of 7 bacterial species, followed by Proteobacteria with 5 species and Actinobacteria with 4 species. Phylum Firmicutes, was dominated by members of Class Bacilli.

Index Terms— Grains, C. cephalonica, mid-gut bacteria, 16S rDNA sequencing.

1 INTRODUCTION

Corcyra cephalonica (C. cephalonica) is commonly known as rice meal moth or rice moth which belongs to the family of Pyralidae. It is a destructive insect and infects cereals, cereal products, oilseeds, pulses, dried fruits, nuts and spices [1-3]. The adult C. cephalonica is nocturnal, grey in colour and does not feed. Generally the female lays about 100-200 eggs near food source. Eggs hatch after an incubation period of about 5 days [4,5]. The larva however, constructs a feeding tube gallery, consisting of silken web and food particles, to stay, feed and grow inside it. After larval period of 23-25 days fully-grown larvae form dense white cocoons to pupate. Pupae are usually found in food or they may be found between pellets and sacks [6]. Adults emerge from pupae after pupal period of 4-8 days and lives for about a week. One C. cephalonica alone can feed up to 32.9 mg. While feeding, it forms web on the stored grains, thus leads to the quality loss of the stored products [7,8]. The infestation of this stored product pest is limited to the grains in the storage locations like godowns, warehouses, retail shops and local storage areas like houses and the successful establishment of this pest species is attributed to the poor storage facilities [9]. Besides the destructive properties of C. cephalonica, it has some good aspects as well, as it acts as an alternate host for some egg parasitoids which are used for biological control programmes of different destructive pests like sugarcane borers in many countries of the world [10-14]. It is one of the most used factitious hosts and is being utilized in various bio-control research, developmental and extension units for mass production of number of natural enemies in several countries [15,16]. Therefore present study aimed to screen the bacteria present in mid-gut of C. cephalonica larva using 16S rDNA sequence based culture dependent technique.

2 MATERIALS AND METHODS

2.1 Insect sample collection

The infested grains such as rice (Oryza sativa), red rice (Oryza punctata), groundnut (Arachis hypogaea L.), pearl millet (Pennisetum glaucum L.), red sorghum (Sorghum bicolour L.), white sorghum (Sorghum vulgare), sesame black (Sesamum indicum) and almond (Prunus dulcis) were collected from the godowns of Regulated Trade Centre, Villupuram, Tamilnadu, India and white sorghum and dry nuts were collected from local godowns in Madurai, Tamilnadu, India. The actively feeding sixth instar larvae of C. cephalonica were isolated from the infested grains and were used for the determination of gut microbiota study.

2.2 Mid-gut dissection

The collected larvae of C. cephalonica were sacrificed and surface sterilized with 70% of ethanol for 5 min followed by washing in 0.85% normal saline (phosphate buffered saline washing in 0.85% normal saline (phosphate buffered saline...
(PBS)) twice. The mid-gut was dissected out in a sterile condition and placed in 2ml microcentrifuge tube containing 300µl of 0.85% saline (PBS) and homogenized with micro-pestle. The 0.85% saline (PBS) after second wash in all the samples were collected and used as a negative control to check the sterility of the procedure. Scissors, forceps, needle, glasswares, plasticwares, buffers and solutions/reagents used in the dissection process were sterilized in autoclave and UV treatment. About 10 insect specimens were used for single midgut sample preparation and three replicates (positive controls) were maintained.

2.3 Isolation of mid-gut bacteria

The mid-gut homogenates were centrifuged for a brief period of 2min. at 2,000g and the supernatant was collected. 100µl each of the supernatant was spread on Nutrient Agar plate and incubate at 30°C for 48hrs. The resulting bacterial colonies obtained on the spread plate were differentiated according to their colony morphology like shape, size, colour, margin, opacity, elevation etc. and morphologically distinct colonies were selected for repeated subculture on nutrient agar plates until a presumably pure colony was obtained. The pure colonies were transferred to nutrient broth and incubated at 30°C.

2.4 Extraction of Genomic DNA from bacterial samples

1.5 ml of overnight grown bacterial isolates maintained in nutrient broth were transferred to 2 ml of microcentrifuge tube and centrifuged at 10,000g for 2 min and pellet was collected. The same was repeated for another 1.5 ml of culture to harvest enough quantity of cells (100 mg). The pellet was resuspended in 1.5ml of sterile distilled water and centrifuged at 10,000g for 2 min. The pellet was then collected and ground with 300µl of CTAB (cetyltrimethyl ammonium bromide) DNA extraction buffer (1% W/V CTAB: 1.4M NaCl; 10mM EDTA, pH 8.0; 100mM Tris-HCl, pH 8.0; 0.2% V/V 8-mercaptoethanol) in a glass homogenizer. The mixture was emulsified with equal volume of phenol:chloroform (1:1). It was centrifuged at 10,000rpm for 5min. at room temperature. The aqueous phase was collected and mixed with equal volume of chloroform:isoamyl alcohol (24:1). The mixture was then centrifuged at 10,000g for 5min. at room temperature. The aqueous phase collected was then added with equal volume of cold absolute ethanol and the DNA was allowed to precipitate by keeping the tubes in -20°C for overnight. DNA pellets were obtained by centrifugation at 10,000g for 5min. and the ethanol was air-dried. The pellet was dissolved in 50µl of TE buffer (Tris 10mM, pH 8.0 and EDTA 1mM, pH 8.0) and stored at 4°C. The quality of the isolated genomic DNA was tested by agarose gel electrophoresis [17].

2.5 PCR amplification of 16S rDNA

The Universal Primers for the amplification of the 16S rDNA region of approximately 1,550bp were Forward primer 27F: AGAGTTTGATCCTGCGGCTCA[3] and Reverse primer 1492R: GGTTACCTTGTTACGACT [3] used [18]. The primer set used in the PCR reactions resulted in the amplification of the homologous fragments from all the bacterial isolates. The PCR reaction mix was prepared in a total volume of 30ul with 10ng of genomic DNA, a 2.5mM concentration each of dATP, dTTP, dCTP and dGTP, 100ng each of the Forward primer and Reverse primer, 3U of Taq DNA polymerase enzyme and 1X Taq DNA polymerase assay buffer (10X) and the remaining volume with glass distilled water (Bangalore Genei, India). The PCR reactions were conducted MJ Mini-BIO RAD Thermal Cycler. The PCR reaction cycles consisted of initial denaturation for 5 minutes at 94°C, 40 cycles of 94°C for 30 seconds (denaturation), 55°C for 45 seconds (annealing) and 72°C for 30 seconds (extension) and followed by the final extension of 72°C for 10 minutes. The amplicons were run through 1% agarose gel electrophoresis along with 100bp DNA ladder (Bangalore Genei, India) and purified for sequencing process using the DNA elution kit (Bangalore Genei) as per manufacturer’s protocol [19].

2.6 Sequencing of 16S rDNA

The amplified 16S rDNA region of the bacterial samples are sequenced by the dideoxy chain termination method (Sanger et al., 1977), using the Big Dye Terminator Version 3.1” Cycle Sequencing Kit in the ABI 3130 Genetic Analyzer in accordance with the manufacturer’s instructions (Polymer & Capillary Array: POP_7 polymer, 50cm Capillary Array; Analysis protocol: BDTv3-KB-Denovo_v5.2; Data Analysis Software: Seq Scape_v 5.2; Reaction Plate: Applied Biosystem Micro Amp Optical 96-Well Reaction Plate) [20].

2.7 Sequencing of Sequence analysis of 16S rDNA for Bacterial species identification

The sequence of 16S rDNA of the bacterial samples obtained were analysed for the bacterial species identification by carrying out NCBI-BLAST. After identification of the bacterial species, each species have been deposited in the NCBI-GenBank through Blanklt tool [21].

3 RESULTS

As demonstrated C. cephalonica infesting different grains namely rice, red sorghum, sesame black, pearl millet, red rice, groundnut, almond and white sorghum were sacrificed and the mid-guts were isolated for the study of mid-gut microbiota. A total of twenty species of bacteria were recorded in the midgut samples of C. cephalonica infesting eight different grains with an average of 2.5 bacterial species per mid-gut sample (Table 1). In this present study no fungal colony was recorded in the midgut samples of C. cephalonica. The twenty bacterial species represent 11 Genera and 16 species of bacteria (Table 2), which include Staphylococcus saprophyticus, Enterococcus gallinarum, Staphylococcus sp., Alcaligenes faeaalis, Luteimonas sp., Oceanobacillus sp., Kocuria palustris, Brevibacterium sp., Kocuria flava, Brevundimonas sp., Staphylococcus pasteuri, Kyttococcus sp., Staphylococcus warneri, Pseudomonas sp. and Pseudomonas stutzeri belong to the phyla Firmicutes, proteobacteria and Actinobacteria. The C. cephalonica fed on rice (oryza sativa) harbored maximum number of bacteria of five species namely Paenibacillus sp., S. saprophyticus, E. gallinarum, Staphylococcus sp. and A. faeaalis. Three bacterial species was found in the gut of C. cephalonica fed on pearl millet (K. palustris, E. gallinarum and Brevibacterium sp.) and ground nut (S. pasteuri, K. palustris and Kyttococcus sp.). Two species of bacteria was recorded in the midgut of red sorghum (S. saprophyticus and E. gallinarum), sesame black (Luteimonas sp. and Oceanobacillus sp.) red rice (Kocuria flava and Brevundimonas sp.) and white sorghum (Pseudomonas sp. and P. stutzeri). The most common genus of bacteria found in the gut was Staphylococcus which had four species; and Kocuria and Pseudomonas recorded two
species of bacteria each. *E. gallinarum* was the bacterium which was commonly found in the gut of *C. cephalonica* fed with rice, red sorghum and pear millet and *S. saprophyticus* was found in the gut of rice and red sorghum fed *C. cephalonica*. The bacterial genus *Pseudomonas* was exclusively present in the midgut of white sorghum fed *C. cephalonica*. Diet dependent gut flora was clearly exhibited in the present study and no single bacterium was found in all the midgut samples of *C. cephalonica* fed with different grains.

### 4 DISCUSSION

The bacteria species identified from the midgut larval samples of *C. cephalonica* fed on six different grains comprising 11 Genera and 16 species and 3 phyla (Firmicutes, proteobacteria and Actinobacteria). The most dominant species of bacteria is *Staphylococcus saprophyticus* which was present in the mid-gut of fed *C. cephalonica*. From the present study it was observed that the predominant phyla in the mid-gut of larvae of *C. cephalonica* was Firmicutes representing 43.75%. The gut of the insect provides suitable microbiome to many gut-associated microorganisms like bacteria, fungi and viruses, amongst which bacteria dominate in most insect groups [22]. The association between insect and bacteria are significant and the gut of insect species harbor highly diverse bacteria comprising of different Phyla, Class, Order, Family, Genus, species and strains. 16S rDNA gene, which is present in all nucleoid of bacteria have been widely used to determine the diversity of the insect gut bacterial microbiota [23]. Recently 16S rDNA sequence based identification and characterization of insect intestinal bacteria was carried out. They are having well symbiotic association which leads to the enhancement in the nutrition supply, increased physiological activities and behavioral changes in the host insect [24]. Apart from that some bacteria confers resistance of host insect species to particular pesticides and some bacteria plays antagonistic role. The insect pests of household like housefly and cockroach are very common in possessing some pathogenic bacteria in their gut environment and serves as a vector of diseases [25]. The classical way of biochemical based identification of bacterial species, which is usually mentioned as culture dependent method, is cumbersome, laborious and time consuming and needs expertise in bacterial taxonomy, in addition only cultivable bacteria can be identified. The technological advancement in the field of biotechnology made it simple, easy, rapid and uncultivable bacterial species can be identify by sequencing 16S rRNA region and subsequent blast of the query sequence in the NCBI [26]. This culture independent method need less effort in the bacterial species identification as the species is identified purely based on the similarity of the 16S rRNA gene sequences. This method of identification of bacterial species at its current form enables to identify more number of bacterial species due to the pyrosequencing or Next Generation Sequencing [27]. The study on the gut microbiota of the economically important insect pests provide valuable information about the total gut bacterial community and any economically important bacterial species which could be cultured and potent compounds extracted from it.

### 5 CONCLUSION

In the present study, evaluated the presence of mid-gut bacteria in the larva of *C. cephalonica* and it showed 16 bacterial species which are belonging to 11 bacterial genera included in three phyla namely Firmicutes, Proteobacteria and Actinobacteria. Among the three phyla Firmicutes was the most dominant phylum with a record of 7 bacterial species, followed by Proteobacteria with 5 species and Actinobacteria with 4 species. Phylum Firmicutes, was dominated by members of Class Bacilli. None of the single bacterium was found in all the mid-gut samples. Together this study can conclude that, *C. cephalonica* could be serve as factitious hosts and might be utilized in various bio-control research, developmental and extension units for large-scale production of natural enemies. However, further studies such as,
extensive sampling and deep-sequencing are warranted to determine the current findings.

TABLE 2
LIST OF BACTERIAL SPECIES IDENTIFIED

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Grains</th>
<th>Total number of bacterial species/isolates</th>
<th>Bacterial species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Rice (Oryza sativa)</td>
<td>5</td>
<td>Paenibacillus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Staphylococcus saprophyticus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Enterococcus gallinarum</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Staphylococcus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Alcaligenes faexalis</td>
</tr>
<tr>
<td>2.</td>
<td>Red Sorghum (Sorghum bicolor)</td>
<td>2</td>
<td>Staphylococcus saprophyticus</td>
</tr>
<tr>
<td>3.</td>
<td>Sesame black (Sesamum indicum)</td>
<td>2</td>
<td>Luteococcus sp.</td>
</tr>
<tr>
<td>4.</td>
<td>Pearl millet (Pennisetum glaucum)</td>
<td>3</td>
<td>Kocuria palustris</td>
</tr>
<tr>
<td>5.</td>
<td>Red Rice (Oryza punctata)</td>
<td>2</td>
<td>Kocuria flava</td>
</tr>
<tr>
<td>6.</td>
<td>Ground nut (Arachis hypogea)</td>
<td>3</td>
<td>Staphylococcus pasteuri</td>
</tr>
<tr>
<td>7.</td>
<td>Almond (Prunus dulcis)</td>
<td>1</td>
<td>Staphylococcus warneri</td>
</tr>
<tr>
<td>8.</td>
<td>White sorghum (Sorghum vulgare)</td>
<td>2</td>
<td>Pseudomonas sp.</td>
</tr>
</tbody>
</table>

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REFERENCES

