Madras Agric. J., 103 (10-12): 344-348, December 2016
https://doi.org/10.29321/MAJ.10.001046

Molecular Characterization of Endosymbiont in *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst.) Populations of South India.

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Insects have adapted to a wide variety of diets. However, in case of stored grain insect pests, majority of them infest all most all grains, processed food articles, can attack a wide variety of stored foods, seeds materials, dried fruit, drugs, cork, timber and paper products. Such a flexible feeding habits have been brought to insects, at least partially, by the endosymbiont they harbor. To know their (endosymbiont) status in 2 major stored grain insect pests, the molecular characterizations was done in 30 and 35 different geographical populations of *R. dominica* and *T. castaneum*, respectively with FD1 and RP2 (16S rDNA primers) specific primers. Polymerase chain reaction amplification using the respective 16S rDNA primers and sequencing of the respective amplicon revealed the presence of Non-diaspididae associated bacteria in *R. dominica* and gammaproteobacteria in *T. castaneum*. Observed bacterial phyla in these two insects are commonly found in beetles and termites and are reported to involve in digestion process.

Key words: *Rhyzopertha dominica, Tribolium castaneum,* Endosymbiont, Bacteria.

The major grain pests in storage of rice and wheat are the lesser grain borer, *R. dominica* and the red flour beetle, *T. castaneum* (Bell, 2000). They can attack a wide variety of stored foods including cereals, dried fruit, almost all grains, particularly wheat, barley, sorghum and rice, commodities such as seeds, drugs, cork, timber and paper products (Odeyemi *et al.,* 2005). In general, the insects have one of the most successful lifestyles on earth and reason for their success is that, the insects have adapted to a wide variety of diets. Such a flexible feeding habits have been brought to insects, at least partially, by the endosymbiont they harbor. Endosymbiont are frequently observed near an insect's digestive tract, play important roles in the nutrition of host insects. (Ishikawa, 1989). Insect endosymbiont, especially intracellular symbionts, are significant for their hosts not merely as nutritional supplements but also as DNA-containing genetic elements (Ishikawa, 1989). These symbionts are intimately involved in the host's physiology and have been suggested to provide the host with several vitamins, such as riboflavin, pantothenic acid, and biotin and to take part in amino acid metabolism in the host (Nardon and Grenier, 1988).

Important contribution to their host insects is detoxification of the xenobiotics through secretion of enzyme complex by the endosymbiont. The bean bug, *Riptortus pedestris* associated with gut bacterial symbiont of the genus *Burkholderia* in a posterior region of midgut responsible for fenitrothion-degrading which conferred resistance of the host against insecticide (Kikuchi *et al.,* 2012). It has also been suggested that these symbionts stimulate oxidative phosphorylation in mitochondria in the host cell (Heddi *et al.,* 1993). The information on the endosymbionts of stored grain pest is scanty, where as some studies revealed that, the rice weevil, *Sitophilous oryzae* (L.) which is devoid of gut microbes, harbors intracellular bacterial symbiont in the bacteriocyte belongs to the Enterobacteriaceae of the g-proteobacteria (Campbell *et al.,* 1992), and its genome size was estimated to be about 3 megabases (Mb) (Charles *et al.,* 1997). Considering the importance of endosymbionts role in the insects species of major importance, we focused to characterize the endosymbiont harbored by *R. dominica* and *T. castaneum* to know the type of bacterial phyla present in them and for further host nutrition related interaction with these bacterial communities.

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Materials and Methods

Collection and culturing of insects

R. dominica and *T. castaneum* infested grain samples were collected from 30 different locations representing 3 southern states of India *viz.,* Tamil Nadu, Karnataka and Andhra Pradesh. *R. dominca* and *T. castaneum* was mass cultured on whole wheat grain and wheat flour with Brewer's yeast, respectively. About 25 pairs of freshly collected *R. dominica* and *T. castaneum* were released separately in to the containers containing 2 kg of food media. The containers were covered with muslin clothes and

placed in dark to facilitate the maximum oviposition at room temperature of $28^0 \pm 2^0$ C and relative humidity of 60±5 per cent. The uniform aged adults were selected for extraction of DNA for endosymbiont characterization.

DNA isolation

Genomic DNA was isolated from *R. dominica* and *T. castaneum* adults following the CTAB method. The DNA extraction buffer had 100mM Tris. HCl (pH 8), 10mM EDTA, 1.4M NaCl, 2 per cent CTAB and 5 per cent β-mercaptoethanol. The single beetle of *R. dominica* and *T. castaneum* was ground with 200 µl of CTAB buffer, transferred into an eppendorf tube and incubated for 1 h at 65 °C with occasional mixing. The tubes were removed from the water bath and allowed to cool at room temperature. Chloroform: isoamyl alcohol mixture (24: 1, v/v) (0.8 volume) was added and mixed by inversion for 10 min. to form an emulsion. It was centrifuged at 12,000 rpm for 10 min. and the clear aqueous phase was transferred to a new sterile tube. Ice-cold isopropanol (0.7 volume) was added and mixed gently by inversion and it was stored -20° C for overnight. It was then centrifuged at 12,000 rpm for 10 min. to pellet the DNA and the supernatant was discarded. The DNA pellet was washed with 70 per cent ethanol. After washing DNA pellet was air dried and dissolved in 20µl of TE buffer and stored at 4°C. The isolated DNA was checked for its quality by separating in 0.8 per cent agarose gel electrophoresis and quantified by Spectrophotometer ND.1000 (NanoDrop Technologies Inc., USA).

Primers used to characterize the endosymbiont

Primer name	Primers sequence	Reference
FD1	5'-GGA GAG TTA GAT CTT GGC TCA G -3'	Lane *et al*. (1991)
RP2	5'-AAG GAG GGG ATC CAG CCG CA-3'	

Polymerase chain reactions were performed in 25µl volumes in a thermocycler (BIORAD DNA engine). The composition of cocktail mixture (for 23 ml reaction mix) is given below.

Characterization of endosymbionts

DNA from 30 and 35 geographical populations of *R. dominica* and *T. castaneum* were amplified using a set of 16S rDNA primers pairs. Polymerase chain reactions were performed in 25µl volumes in a thermocycler (BIORAD DNA engine). The composition of cocktail mixture (for 23 ml reaction mix) contains 16.50 ml sterile water; 2 ml of template DNA of *R. dominica*; 1 ml of dNTPs (Mixture of dATP, dCTP, dGTP and dTTP); 2.5ml of 10X PCR Buffer; 1ml each of 10µM forward and reverse primer; 0.5ml of Taq polymerase (1.5Units) and 2 ml of BSA 23ml of cocktail mixture was added to each tube containing 2ml of template DNA. The tube content was gently mixed by overtaxing or by repeatedly pipeting up and down. A brief centrifugation is needed to seat the mix in the bottom of the tube. The samples were placed in the thermal cycler using the following PCR conditions: Initial annealing was at (one cycle) 95 °C for 10 min; denaturation at 95 °C for 1 minute. annealing at 55°C for minute depending upon primer, extension for 72 °C for 1 min and these were repeated 38 times and final extension at 72 °C for 10 min 1 cycle and storage at 4°C forever.

Purification of PCR product 16S rRNA fragments

All the amplified 16S rRNA fragments from *R. dominica* and *T. castaneum* samples were mixed in eppendorf tube and to this 500 ml of PCR binding solution were added. This was carried out for separately for all 3 location selected for both insects. The mixture was applied to the column and centrifuged at 13000 rpm for 2 minute. After elution discarded the flow through and placed the column (PCR product/gel-purification spin columns (Bangalore Genei, India)) in a new tube, to this added the 700 ml of wash solution and centrifuged at13000 rpm for 2 minute. Again the column flow was discarded and kept the replaced the column on new tube. To this column, 50 ml of elution buffer was added at the centre of column and centrifuged at 13000 rpm for 1 minute. The quality of eluted PCR product was checked by agarose gel electrophoresis (1.5 %). The remaining purified products were stored at -20 °C for further use. Purified PCR products were cloned in T/A cloning vector pTZ57R/T vector (Fermentas, USA) and transformed in to E. coli (JM109) as per the manufacturer's protocol (Fermentas, USA).

Sequencing

Sequencing was done by using M13F and M13R primers and sequencing reactions were performed using ABI prism terminator cycle sequencing ready reaction kit, and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer in Scigenome laboratories, Cochin.

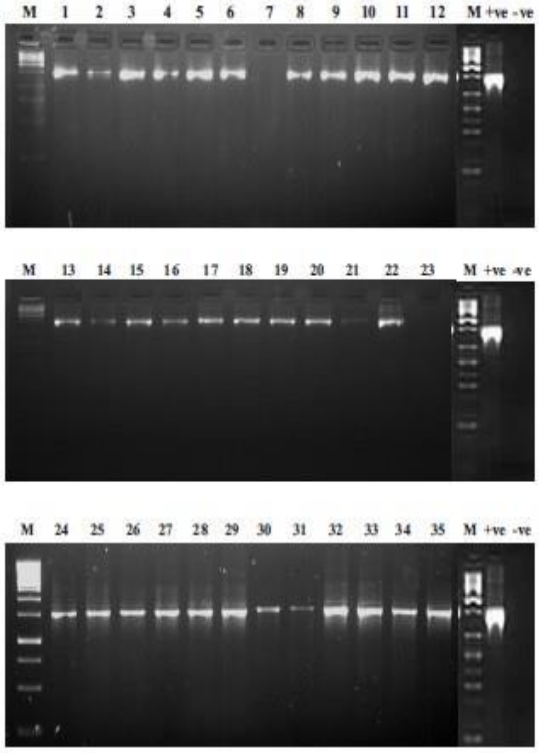
Phylogenetic analysis

The identity of 16S rDNA sequence was established by performing a similarity search against the GenBank database (http://www.ncbi.nih.gov/BLAST). The phylogenetic tree was constructed for positive clones. The 16S rRNA gene sequence of the type strains of the species were obtained from the NCBI and ClustalX programme was used for multiple sequence alignment. The phylogenetic tree was constructed by neighbor-joining method of Saitou and Nei (1987) using MEGA 5.0 software with existing 16S rRNA gene sequences from different eubacteria, obtained from NCBI GenBank database.

Results and Discussion

Analyses of endosymbiont in 30 different populations of *R. dominica*, with specific primers, FD1 5'-GGA GAG TTA GAT CTT GGC TCA G -3' and RP2 5'-AAG GAG GGG ATC CAG CCG CA-3' showed their presence except Mehabubnagar and Nalagonda population (Plate 1) and similarly in 35 different populations of *T. castaenum* showed the

presence of endosymbionts except Namakkal and Mandya region population (Plate 2). The amplicon size was more than 1.5 kb for all the population. The phylogenetic analyses revealed the presence of Non-diaspididae associated bacteria in *R. dominica* and gammaproteobacteria in *T. castaneum* (Figure 1). In both insects, we could able to characterize only 2 group of bacterial communities with specific primer, however, a variety of bacterial phyla are commonly present in insect guts, including *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria,*

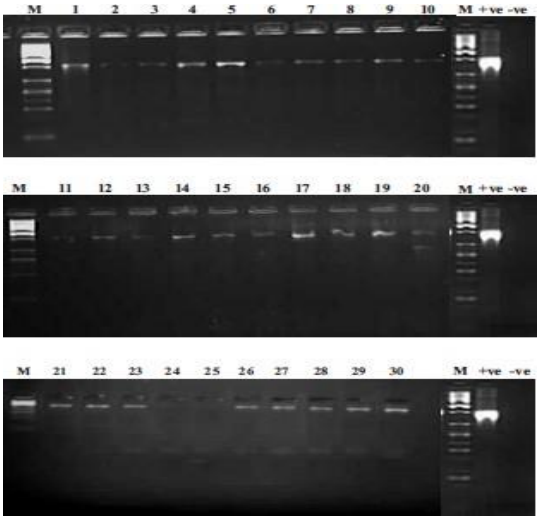


Bacteroidetes, Firmicutes including *Lactobacillus* and *Bacillus* species, *Clostridia, Actinomycetes, Spirochetes, Verrucomicrobia, Actinobacteria* and these community diversity is higher in guts of insects, such as some beetles and termites, which feed on wood or detritus (Colman *et al.,* 2012).

Legends: M- 1kb DNA marker, 1.Coimbatore, 2. Dindigul, 3. Salem, 4. Erode, 5. Villipuram, 6.Tirupur, 7.Namakkal, 8.Karur, 9.Virudhunagar, 10.Madurai, 11. Thanjavur, 12.Tirunelveli, 13.Bangalore, 14. Davanagere, 15 Belagavi, 16. Bidar, 17.Shivamogga, 18. Raichur, 19. Chamarajnagar, 20. Dharwada, 21.Tumkur, 22.Chikkamagalore, 23. Mandya, 24.Hyderabad, 25.West Godavari, 26.East Godavari, 27.Mehabubnagar, 28.Nalagonda, 29.Karimnagar, 30.Medak, 31. Nizamabad, 32. Adilabad, 33. Nellore, 34. Ananthapuram, 35. Warangal

Plate 1. Gel profile of endosymbionts in *T. castaneum* collected from South India with FD1 and RP2 primers

Intracellular symbiosis of microorganisms present in storage pests was studied earlier (Nardon and Grenier, 1988; Campbell *et al.,* 1992). These symbionts were intimately involved in the host's physiology and had been suggested to provide the host with several vitamins, such as riboflavin, pantothenic acid, and biotin and to take part in amino acid metabolism in the host. Our findings are in line with the similar studies on *S. oryzae* recorded the presence of g-proteobacteria.



Legends: M- 1KB DNA marker, 1. Coimbatore, 2.Karaikal, 3.Madurai, 4.Kanchipuram, 5.Salem, 6.Erode, 7.Thanjavur, 8.Tirunelveli, 9.Tirupur, 10.Virudhnagar, 11.Bangaluru, 12.Belagavi, 13.Bidar, 14.Shivamogga, 15.Chamarajnagar, 16.Dharwada, 17.Bijapur, 18.Mandya, 19.Haveri, 20.Chitradurga, 21.Hyderabad, 22.West Godavari, 23.East Godavari, 24.Mehabubnagar, 25.Nalagonda, 26.Karimnagar, 27.Nizamabad, 28.Medhak,29.Guntore, 30.Chittore.

Plate 2. Gel profile of endosymbionts in *R. dominica* collected from South India with FD1 and RP2 primers

The predominance of *Gammaproteobacteria* in the gut of *T. castaneum* was in accordance with the earlier reports of its presence in gut of many insects (Campbell *et al.,* 1992; Charles *et al.,* 1997). Hiroaki and Kentaro, (1996) studied the endosymbiont of anobiid beetles *Stegobium paniceum* (L.) and *Lasioderma serricorne* (Fab.) which possess the intracellular yeast like symbionts *viz., Symbiotaphrina buchneri* and *Symbiotaphrina kochii*, respectively, in the mycetome between the foregut and midgut. Insect symbiotic systems studies with anobiid beetles and cigarette beetle where histological assays indicated the mycetomes were a concentrated source of the total gut enzymes capable of hydrolyzing the esterase substrate 1-naphthyl acetate and tannic acid (Dowd, 1989). The bacterial flora of other stored grain beetles *viz., bruchids* and *Angoumois grain moth* showed the presence of *Bacillus pumilus*, *Staphylococcus* sp. and *Pantoea* sp. and *Staphylococcus succinus*, *Enterococcus* sp.and *Staphylococcus* sp., respectively. (Sevim *et.al.,* 2016). We conclude that, the present study revealed the preliminary information on the bacterial

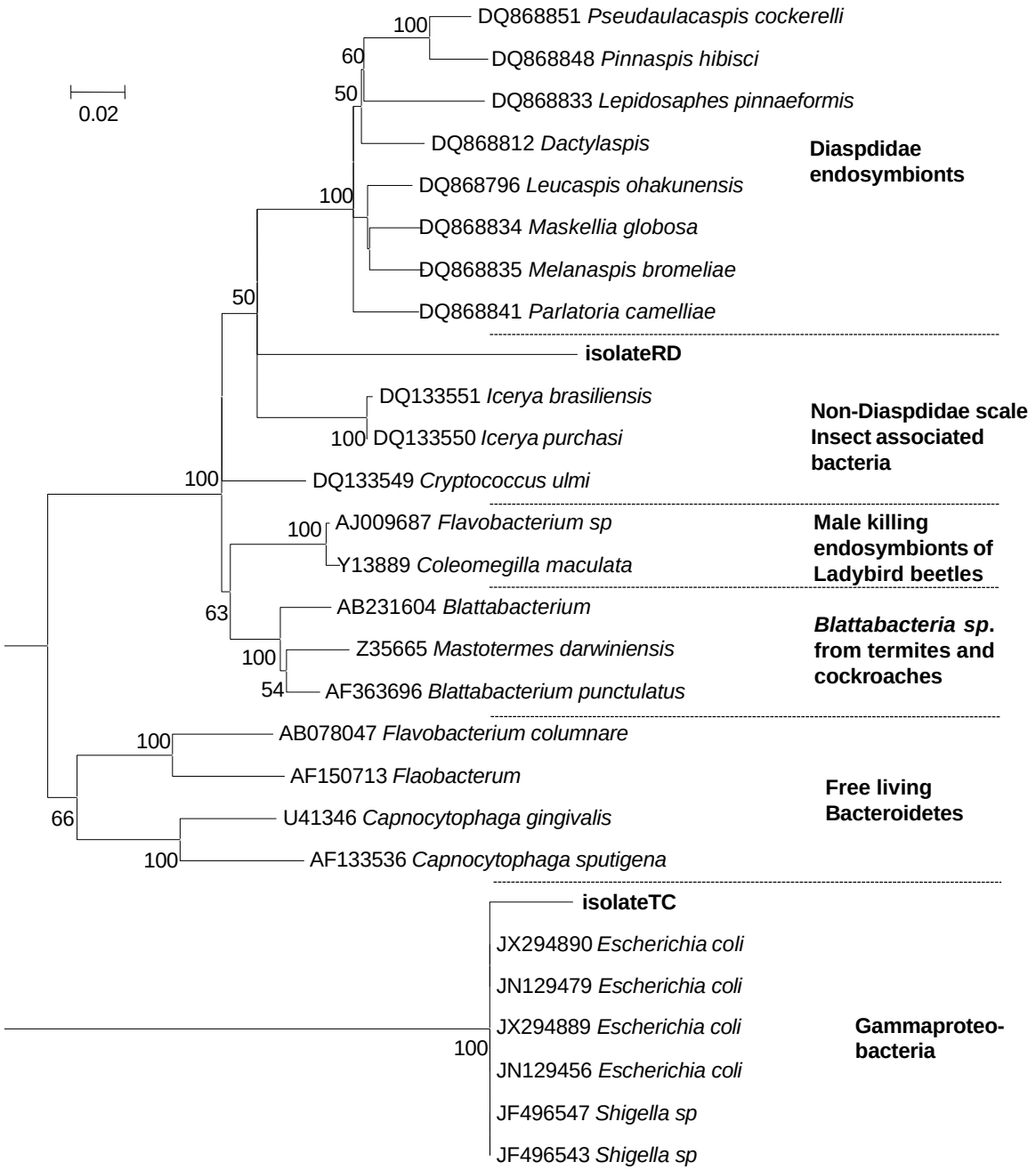


Fig.1. Phylogenetic tree of the eubacterial isolates from *R. dominica* and *T. castaneum* using partial 16S rRNA gene sequence by Neighbor-joining method. TC – Clone from *T. castaneum*; RD – Clone from *R. dominica*. The percentage of 1000 bootstrap replicates are shown at the left nodes when at least 50%. The scale bar indicates two changes per 100 bp nucleotides.

communities present in gut of two major grain pests. However, further studies can be undertaken to characterize specific endosymbiont and their role in insecticide degradation, more particularly phosphine degradation and also the mechanism to overcome the problem of high resistance in stored grain insects.

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Received after revision : December 26, 2016; Accepted : December 30, 2016