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Characterization and identification of gut associated endosymbiotic bacteria in *Paramecops farinosa* (Wied.) (Coleoptera: Curculionidae)

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Abstract
The *Paramecops farinosa* is a monophagous pest on *Calotropis procera.* We examined the diversity of the gut microbiota and characterized using both culture-dependent and culture-independent methods. In total, 25 bacterial colonies were characterized based on their morphological, biochemical and molecular characteristics. Out of twenty-five isolates, fifteen isolates were gram-negative, nine isolates were amylase producers, six isolates were Lipase producers and three isolates were cellulase producers. All the 25 isolates were able to produce catalase and ammonia. In total of 25 isolates, 16S rRNA gene sequence of 2 isolates revealed the presence of *Pseudomonas* sp. (PFI03) and *Enterobacter* bacterium (PFI07).

Keywords: *Paramecops farinosa*, *Calotropis procera*, gut associated bacteria, enterobacteriaceae

Introduction
Insects occupy every niche on the earth, belong to a largest taxon and all of them have endosymbiotic association with many microbes including bacteria. These bacteria inhabit all the parts of their insect partners and some they are unique to some insect parts [4]. The symbiotic relation of bacteria has inevitable role in the physiology of insects such as metamorphosis, production of enzymes and vitamins, tolerance for environmental factors, neutralization of pestilent chemicals consumed from their plant diet [5, 8, 9] etc. The endosymbionts of insects also facilitate the digestion of lignin [13], generation of methane, fixation of nitrogen [16], protection against pathogen invasion [6], supply nutrients such as vitamins and amino acids, detoxify the toxic dietary secondary metabolites [7]. The bacterial taxa in the guts of insects vary with their phylogeny, diet, habitat and geography; the bacteria were categorized using 16s rRNA gene [22]. In the recent days, 16S rRNA gene sequence of bacteria is believed as most reliable approach to classify bacteria and search for novel bacteria [20].

Paramecops farinosa is a monophagous pest on *Calotropis sp.;* both adult beetle and grub feed on the growing buds and tender leaves [18]. The pest consumes many toxic compounds and bacteriolytic enzymes produced in the latex by the host plant [15]. It is a well-established fact that the gut of animals proved to be a microbiome and harbors a variety of microbes. In the present study, it is looked for bacterial fauna in such a hostile environment, the gut of *P. farinosa.*

Material and Methods
Collection of insect and dissection of gut
The adult *P. farinosa* was collected from Puducherry (11° 57' 10.85" N and 79° 48' 34.80" E) in February, 2017. The insect was surface sterilized with 70% ethanol for one minute and rinsed in sterile water before dissection and the gut was dissected under sterile conditions. The head, wings and last abdominal segment of insect were severed, and pressure was applied anterior to the crop to release the gut. The gut was homogenized in 0.86% NaCl solution [2].

Isolation of dominant bacteria
The gut was macerated in a sterile tissue homogenizer to release the gut contents, the volume was made to 10 ml with sterile water and the supernatant was serially diluted 10^{-2} to 10^{-5} times. 1 ml of sample from each concentration was added to nutrient agar petri plate and triplicates were maintained. The petri plates were incubated for 24 hours at 37 ˚C under sterile condition.

The colonies thus grown were grouped according to colony morphology and cell characteristics [1].

Screening and identification of bacteria

Each colony, grown on the petri plate, was recorded its shape, size and colour. Further, the colonies were subjected to Gram's staining and biochemical tests like Amylolytic test [11], Cellulolytic test [19], Lipolysis test [3] and Catalytic test [14] and the results were recorded. Bacterial growth was studied for all the isolates for 72 hours and the means of OD values at 24 hours interval were compared by one way ANOVA. The ratio between the zone of degradation and the size of the colony was calculated as enzymatic index [10].

16S rRNA gene sequencing

16S rRNA gene sequencing was carried out for two select colonies (PF103 and PF107) which showed distinct features from other colonies. For PCR amplification of 16S rRNA gene, primers 518F 5' CCAGCAGCCGCGGTAATACG 3' and 800R 5' TACCAGGGTATCTAATCC 3' were used. The purified PCR product of approximately 1, 400 bp was sequenced.

Phylogenetic tree construction

The two sequences thus obtained were subjected to BLAST analysis, the phylogenetically related sequences were selected from the Gene Bank and they were subjected to multiple sequence alignment and then align sequence were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6.

Results and Discussions

In this study, we isolated 25 dominant isolates, the colonies were denoted with specific identification number through PF101 to PF125 and the morphology of each colony is given in the Table 1. Punctiform colonies represented 56 percent, remaining were either round or filamentous and there was a single filamentous cream coloured colony. The growth of the isolates in nutrient broth was ensured by their OD values at 24 hours interval. The select isolates were tested for their biochemical activities such as amylolytic; lipolytic; cellulolytic; catalytic; ammonia production and are presented in Fig 1. Among 25 isolates, 60 percent of colonies represented gram negative; 100 percent of colonies tested positive for ammonia and catalytic test; 12 percent was cellulolytic positive; 24 percent of isolates tested positive for lipolytic test; 36 percent isolates were amylolytic positive. The isolates PF103 and PF107 were pure cultured and 16S rRNA gene was cloned and sequenced, a phylogenetic tree was constructed (Fig 2 and 3). The sequence obtained for the isolate PF107 was submitted to Gene bank.

Gene bank accession number of bacterial isolate

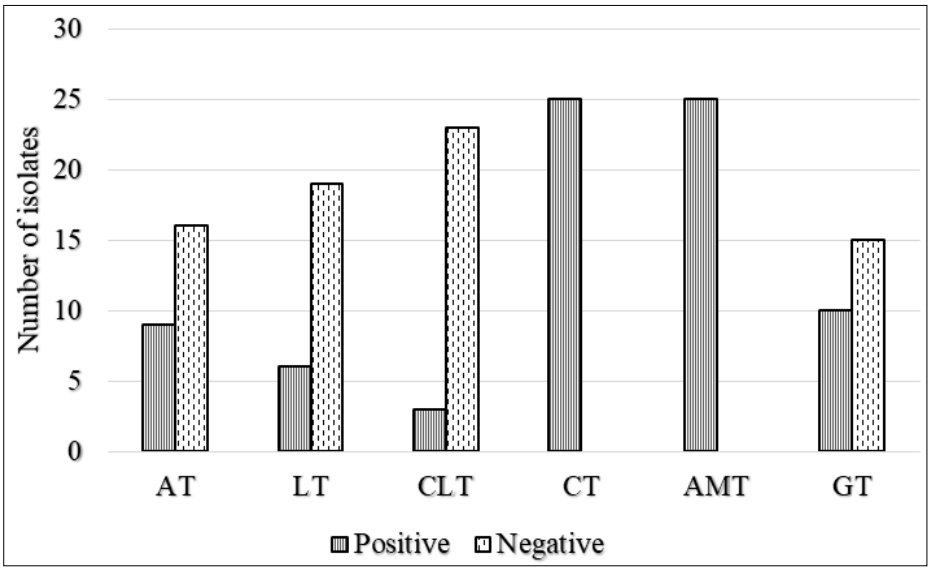
The Gene Bank Accession Number for the partial sequence of the 16S rRNA gene sequences for the isolate PFI07 (Enterobacteriaceae bacterium) is MF769775.

Like a finger print, the composition of gut microbiota of an organism is unique and support that organism in innumerable ways like food digestion, immunity, nutrient supply, behaviour etc. The gut microbiota of *P. farinosa* showed a variety of species composition and it was witnessed based on the morphological and biochemical characteristics. Ramachandiran *et al.*, (2018) [17] isolated twenty types of bacteria, reported three species *Bacillus subtilis*, *Bacillus vietnamensis* and *Bacillus anthracis* from the gut of brinjal spotted beetle, *Epilachna vigintioctopunctata*. *Bacillus subtilis* is supposed to be a pathogen to insects and *Bacillus anthracis* is deadly pathogen on man and cattle. The studies reveal that the gut of animals is major reservoir of bacterial communities. Many colonies isolated from the gut of *P. farinosa* were punctiform and it may be due to the environment where they live that suits the adaptive benefits [21].

The association of bacteria with animals is a highly complicated, poorly understood and a thorough study on interdependencies is required. The amylolytic bacteria found in the gut of *P. farinosa* might involve in the digestion of the starch received from the leaves of *Calotropis sp.;* the presence of ammonia producing bacteria and catalase producing bacteria indicates that these bacteria might involve in removal of secondary metabolites and removal of hydrogen peroxide respectively [12]. The colonies which were less than 1mm diameter were considered as pin point type and the reason for such colonies could not be studied due to challenge in the subculture of these colonies. Also, the domination of pinpoint colonies in the present stud might be due to nutritional deficiency in the culture plates. The phylogenetic tree was constructed using partial sequence of 16S rRNA gene of the randomly selected isolates PF103 and PF107. The gene sequence of colony PF103 could not be interpreted due to its failure to form cluster with sequences on nucleotide data bank. The bootstrap value of the sequence of the isolate PF107 was 85 percent with that of *Enterobactor mori*. This species of bacterium is a plant opportunistic gram-negative pathogen also found in the soil of oil fields [23]. *Enterobacter mori* might involve in the degradation of toxic compounds received through its food in the gut of *P. farinosa*.

Table 1: Characterization of bacterial colonies

Colony	Shape of the colony	Size of the colony	Colour of the colony
PFI01	Rhizoid	Medium	White
PFI02	Round	Punctiform	White
PFI03	Round	Punctiform	White
PFI04	Rhizoid	Medium	Cream
PFI05	Rhizoid	Medium	Cream
PFI06	Rhizoid	Medium	Cream
PFI07	Rhizoid	Medium	Cream
PFI08	Round	Punctiform	White
PFI09	Round	Punctiform	White
PFI10	Round	Punctiform	White
PFI11	Round	Punctiform	White
PFI12	Round	Punctiform	White
PFI13	Rhizoid	Medium	Cream
PFI14	Filamentous	Large	Cream
PFI15	Round	Punctiform	White
PFI16	Rhizoid	Medium	Cream
PFI17	Round	Punctiform	White
PFI18	Round	Punctiform	White
PFI19	Round	Punctiform	White
PFI20	Round	Punctiform	White
PFI21	Rhizoid	Small	Cream
PFI22	Round	Punctiform	White
PFI23	Round	Punctiform	White
PFI24	Rhizoid	Small	Cream
PFI25	Rhizoid	small	Cream



AT - Amyleolytic Test; LT - Lipolytic Test; CLT - Cellulolytic Test; CT - Catalytic Test; AMT- Ammonia Test; GT - Gram's Test

Fig 1: Biochemical activities of bacterial isolates

Table 2: Enzyme activity of bacterial isolates

S. No.	Test	Colonies	Enzymatic index
1	Amylolytic	PFI01	2.2
		PFI02	3.0
		PFI04	1.6
		PFI18	1.5
		PFI19	2.0
		PFI20	1.3
		PFI21	1.5
		PFI23	1.4
		PFI24	2.2
2	Cellulolytic	PFI06	1.2
		PFI21	1.5
		PFI23	1.5

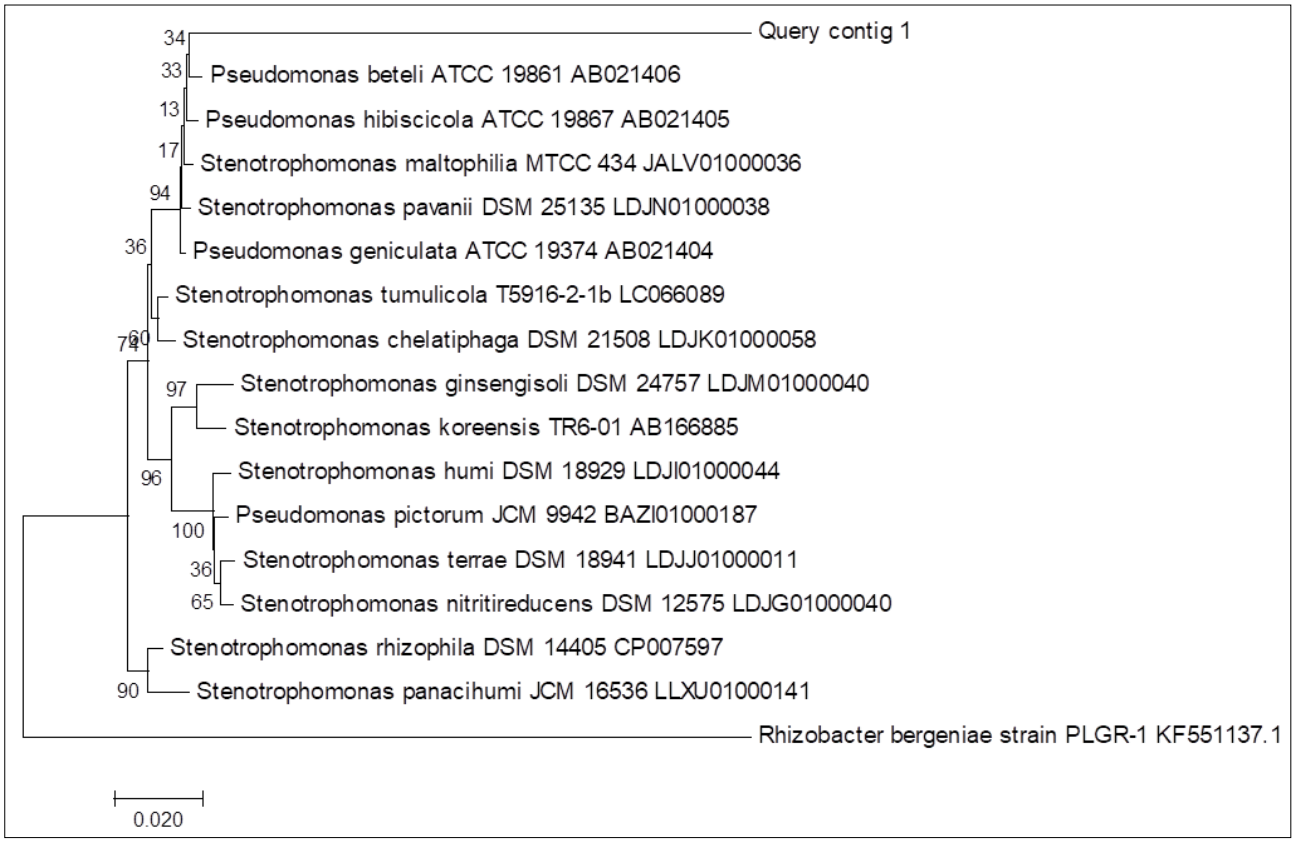


Plate 1: Phylogenetic tree of PFI03

Plate 1: Phylogenetic tree based on sequences of 16S rRNA gene obtained in the present study; the scientific names indicate the sequences obtained from GenBank. The numbers associated with the inner nodes are bootstrap values (%) obtained after 1000 replicates. The scale bar, 0.020 substitutions per nucleotide position.

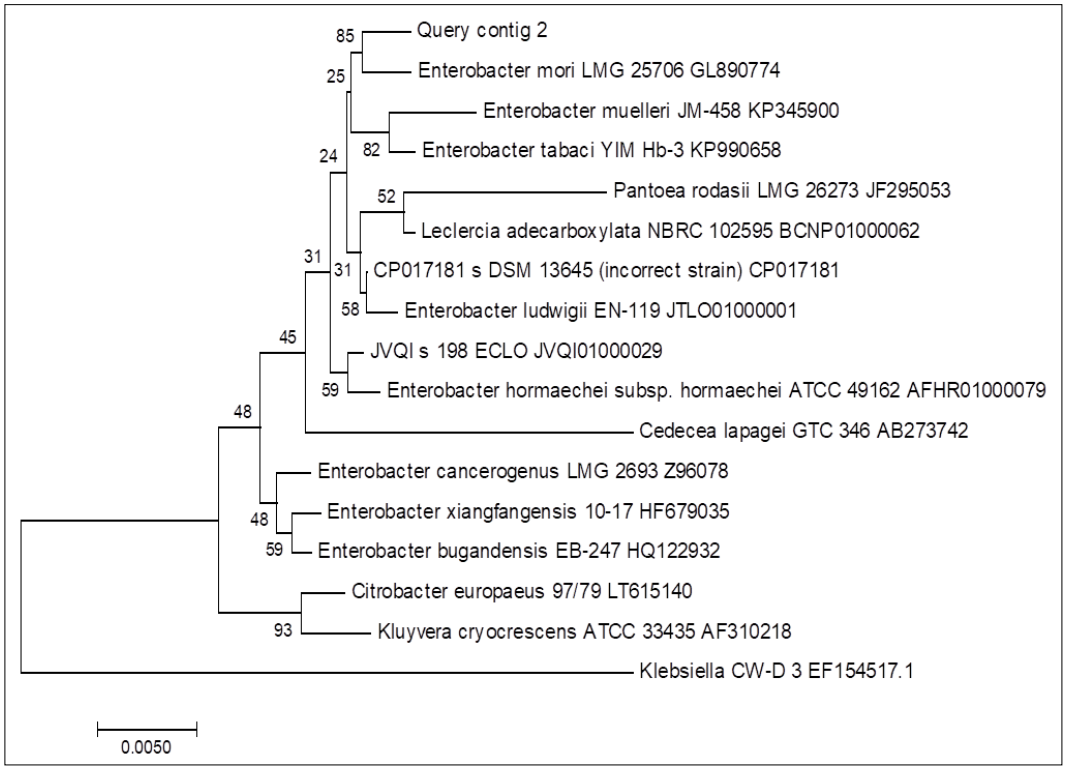


Plate 2: Phylogenetic tree of PFI07

Plate 2: Phylogenetic tree based on sequences of 16S rRNA gene obtained in the present study; the scientific names indicate the sequences obtained from GenBank. The numbers associated with the inner nodes are bootstrap values (%) obtained after 1000 replicates. The scale bar, 0.005 substitutions per nucleotide position.

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