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Exploration of gut bacteria in white grub *Anomala* sp. (Coleoptera: Scarabaeidae) , a major pest of vegetables and fruit trees in India

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Abstract:

White grubs are among the most destructive pests living in the soils causing severe economic loss to most of the agriculturally important crops. An attempt was made in the present study to isolate the gut bacterial communities of white grub, *Anomala* sp. (Coleoptera: Scarabaeidae) collected from citrus trees soil. A culture based approach was deployed for exploring the diversity of gut bacterial isolates in this pest. About 24 bacterial species were isolated from the gut of this insect by using diverse growth media and generic identification of the same was done based on 16s ribosomal RNA gene identification method. The proportional distribution of the gut bacteria revealed that bacterium *Ochrobactrum* sp. (25 %) was the most dominant one followed by *Bacillus* (21 %), *Citrobacter* (21 %), *Pseudomonas* (17 %), *Enterobacter* (8%) and *Paenibacillus* (8%). Phylogenetic association between these gut bacterial isolates, their possible functional role and scope and utility of these gut bacterial isolates in pest management have been discussed in this work.

Introduction

The grubs of scarabaeids are among the most destructive root feeders and of soil insect pests thus cause serious economic losses to diverse agricultural and horticultural crops across the world (Huang *et al*., 2010).

Insects signify as one of the largest reservoirs of bacterial diversity on earth and about 15 per cent of all insects harbour diverse communities of bacteria (Brooks, 1963 and Moran *et al*., 2008). The insect bacterial association has co-evolved for more than 250 million years and have resulted in manifold interactions between insects and bacteria, ranging from pathogenicity to highly complex symbiotic relationships (Douglas and Beard, 1996 and Oliver *et al*., 2005). The number of bacteria within an insect outnumbers the total number of cells within the insect body (Ann and Fergus, 2006). These gut bacteria may play a role in the nutrition, physiology, reproduction, overall, growth and development of the insect host (Dale *et al*., 2006).

Recent studies have shown that the native gut inhabitants drive the

intestinal immunity in order to regulate the colonization of the gut by other non-indigenous microbes including pathogens. The collective genome of indigenous microbiota is described by the term 'microbiome' (Lederberg and McCray, 2001). It has been recognized that some bacteria can incorporate their whole genome into the host DNA for successful transmission (Dunning-Hotopp *et al*., 2007). The composition of the gut flora reflects natural selection at both the microbial and host levels that forms a mutualistic relationship with each other. Petri (1910) described one of the first bacterial symbiotic associations in an insect species, the olive fly, *Bactrocera oleae* (Rossi) (Diptera : Tephritidae) .

The hindgut of scarabaeid grubs have a unique physiological structure called fermentation chamber, which is nothing but an enlarged and modified ileum housing a complex and dense aerobic and anaerobic microbial communities (Cazemier *et al*., 1997). Previous studies had shown that 25–65 per cent of the ingested pure cellulose were degraded by Scarabaeid grubs and the intestinal bacteria present in the hindgut were found to be associated with the cellulose degradation (Cazemier *et al*., 1997). Furthermore, several cellulolytic bacterial species have been successfully isolated from the gut contents of some Scarabaeids (Cazemier *et al*., 2003). These studies demonstrated that the hindgut of scarab larvae represent an ideal resource for identifying microorganisms and enzymes that can be used for biofuel production and to improve biofuel production technology (Huang *et al.*, 2010).

Usage of broad range 16SRNA gene as a tool for identification of bacteria is possible because the 16S ribosomal RNA (16s rRNA) gene is present in all bacteria (Woese, 1987). The 16S rRNA gene has a highly conserved nucleotide sequences, scattered with variable regions that are genus or species-specific. Bacteria can be identified by nucleotide sequencing of the PCR product followed by comparison of this sequence with the known sequences stored in a database (Clarridge, 2004). The previous works for bacterial isolation from Scarabaeid larval gut concerned about many species, while, little is known about the gut bacterial diversity of *Anomala* sp. a major pest of several fruits and vegetable crops.

The present work explores the gut bacterial diversity of this important pest of agricultural crops and the information generated may be of practical utility in exploiting the gut microbes for management of this pest. Studies were carried out on isolation and identification of bacteria culture by using the 16srRNA gene sequence from the gut of white grub, *Anomala* sp. Besides, phylogenetic analysis was done to understand the relationship between the gut bacterial isolates. The possible functional role and scope and utility of these gut bacterial isolates in pest management have been discussed.

Materials and methods

1. Larval collection:

Fully grown healthy third instar grub of *Anomala* sp. were collected from citrus trees from the experimental fields of Indian Agricultural Research Institute, New Delhi, (28° 38′ 5.430″ N; 77° 09′ 8.410″ E), India . Grubs were maintained individually in rearing jars containing sprouted potatoes in a soil medium at 25±2˚ C and 60 % RH inside the insect growth chambers.

2.Dissection and extraction of guts:

The grubs of *Anomala* sp. selected for this study were pre starved for 24h. and subsequently anesthetised at -20˚ C before extraction of gut compartments. The grubs were surface sterilized with 70 % ethanol for 60 sec. followed by immersion in 5 % sodium hypochlorite (NaOCl) solution followed

by thorough rinsing with sterilized water to remove the disinfectant. The surface sterilized larvae were then dissected under aseptic conditions to extract the intestinal tract. The gut was dissected to get the mid-gut and hind gut sections including the fermentation chamber. The extracted gut compartments of each grub were homogenised in 0.85% NaCl using a sterile motorised homogenizer. Care was taken to extract the gut contents under aseptic conditions to avoid contamination of gut sections.

3. Isolation and enumeration of gut bacteria:

The isolation of gut bacteria from *Anomala sp*. was done by using three different media: Nutrient Agar, Brain Heart Infusion Agar and *Pseudomonas* Isolation Agar. The media were autoclaved at 121˚ C for 20 min. The gut homogenate samples were serially diluted in NaCl solution and spread on agar plates. The inoculated plates were incubated at 37˚ C for 24h. The colonies were differentiated on the basis of their size, colour and morphology and a single isolate was transferred to an agar slant. Enumeration of gut bacterial isolates was performed by counting the colony forming units (CFU). The mean values of CFU were used to calculate the viable count of bacteria. After incubation of 24 h, the colonies were picked up from the spread plate and purified by streaking on respective agar plates. Streaking of gut bacteria was repeated for four to five times to ensure the purity of each bacterial culture. Gram Staining was differentiated as Gram positive or Gram negative bacteria. The purified strains were maintained in glycerol stock at -80 ˚C. For the experimental purposes, the bacteria were revived in nutrient broth containing 3.0 g/l beef extract and 5.0 g/l peptone.

4. DNA Extraction and PCR Amplification of 16S rRNA:

Isolated gut bacterial cultures were generically identified by using 16S rRNA gene sequencing technique. The bacterial isolates were grown in nutrient broth for 24h. at 37˚ C. The inoculated broth was then centrifuged at 10621g. to separate the pellet and the supernatant. The pellet of the broth was then used for DNA extraction using a modified cetyltrimethylammoniumbromide (CTAB) method. The quality of bacterial nucleic acid was checked on an agarose gel. Bacterial DNA was amplified by using universal primers 27F (5`-AGAGTTTGATCCTGGCTCAG-3`)1492R-(5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR was carried out in an AB-Applied Biosystems thermal Cycler as follows: one cycle at 94.0 ˚ C for 5 min, 30 cycles at 94 ˚ C for 1 min, 58 ˚ C for 1 min and 72 ˚ C for 1 min 40 s, followed by 72 ˚ C for 10 min and 4 ˚ C forever. The PCR products were then examined on horizontal gel electrophoresis on a 0.8% agarose gel, and the bands were visualized by staining with ethidium bromide. Gels were visualized under UV Gel Documentation system of Alpha Imager™ gel imaging system. The PCR products were sequenced by Sanger's sequencing technique with M/s. Sci Genome Pvt Ltd, India. The high-quality curated sequences of bacterial isolates were compared with the 16S rRNA sequences retrieved from Gen Bank data base by using the Basic Logic Alignment Search Tool (BLAST) algorithm. Bacterial isolates were generically identified based on their similarity with existing sequences.

5. Phylogenetic tree analysis:

The high quality sequences were chosen and thus assigned to phylogenetic tree analysis. The phylogenetic tree for the gut bacteria isolated from *Anomala* sp. was constructed. The sequences were assembled and aligned BIOEDIT V. 7.0 (Hall, 1999) and tree was constructed using MEGA V. 6.0 software program (Tamura *et al*., 2011) by using the Maximum parsimony method. To

calculate the support for each clade, bootstrap analysis was performed with 1000 replications (Felsenstein, 1985). *Geothrix fermentans* (U41563.1) was chosen as an out-group and the sequence of which was obtained from National Centre for Biotechnology Information (NCBI) database.

6.Statistical analysis:

The data of colony forming unit was analyzed using one way analysis of variance and Tukey's honest significant difference post hoc test by using SPSS 16.0 program.

Results and discussin

1. Isolation of gut bacteria:

The isolation of bacterial flora from the intestinal tract of the *Anomala* sp. was carried out by deploying a culture-dependent approach coupled with 16S rRNA gene sequencing for generic identification of bacteria. A total of 35 pure colonies of gut bacteria were isolated from the larvae of *Anomala* sp. on different media. The gut bacterial counts were enumerated on different media and were expressed as colony forming units (CFU). The average CFU of gut bacteria was calculated from all three media and it was found to be 2.21± 0.15 on Nutrient agar media, 2.15±0.10 on Brain Heart media and 1.15±0.11 on *Pseudomonas* isolation agar media. The results revealed that the mean CFU of the gut bacteria was found to be significantly higher on Nutrient agar media as compared to other two media. However it was found that the maximum CFU was observed on the Brain Heart Infusion agar. The mean CFU of these gut bacterial isolates are given in the Table (1) .

Table (1): Colony forming unit (CFU) means of isolated bacteria from three different media of *Anamola* sp. gut.

No.	Media used for the isolation	Colony Forming Unit CFUml^{-1} (× 10^4)
1	Nutrient agar	1.15±0.11^c
2	*Pseudomonas* agar	2.21±0.10^a
3	Brain Heart Infusion Agar	2.15±0.15^b
	CV%	28.10
	SE(M)	0.13

CV is the Coefficient of Variation; SE (M) is the Standard Error of the mean; the values after ± indicates the standard deviation. Means of the Colony Forming Units (CFU) were calculated for the gut bacteria of 3rd instars of *Anamola* Sp. The analysis was done by using the SPSS v.16.0.

2.16srRNA gene sequencing:

The sequences of gut bacterial isolates obtained were checked for the quality and 24 unique, high quality, non-repetitive and no redundant sequences were shortlisted for further analysis. Comparative BLAST analysis revealed that most of the bacterial isolates had shown 99 per cent similarity and a few showed 88 % similarity to their closest relatives retrieved from the GenBank database. The 16S rRNA sequences of gut bacterial isolates generated from this study were submitted to GenBank (accession No. MK235187 to MK235210) (Table, 2).

The results showed that the gut of *Anomala* sp. consisted of diverse gut bacteria with *α-proteobacteria* being the most dominant group represented by the genus *Ochrobactrum* sp. constituting 25 per cent of the total gut bacterial isolates followed by the *γ-proteobacteria* group represented by the genus *Citrobacter* (20.8%) consisting of single species *viz., C. Koseri; Pseudomonas* (16.6%): *P. aeruginosa and Enterobacter* (8.33%) were the other bacteria belonging to the *γ-Proteobacteria. Firmicutes* were represented by genera such as *Bacillus* spp. (20.8%) and *Paenibacillus* (8.33%) represented by *P. Jamilae* (Figure, 1).

Table (2): Gen Bank accession details of gut bacterial isolates from the white grub *Anamola* sp.

Isolate No.	Organism of closest match identified from Genbank	Gene Bank Accession no.	Similarity %	Family	Class
1	*Pseudomonas aeruginosa*	MK235187	99%	*Pseudomonadaceae*	*γ-proteobacteria*
2	*Pseudomonas aeruginosa*	MK235188	99%	*Pseudomonadaceae*	*γ-proteobacteria*
3	*Bacillus pseudomycoides*	MK235189	99%	*Bacillaceae*	*Bacilli*
4	*Ochrobactrum* sp.	MK235190	99%	*Brucellaceae*	*α-proteobacteria*
5	*Ochrobactrum* sp.	MK235191	99%	*Brucellaceae*	*α-proteobacteria*
6	*Paenibacillus jamilae*	MK235192	99%	*Paenibacillaceae*	*Bacilli*
7	*Ochrobactrum anthropi*	MK235193	99%	*Brucellaceae*	*α-proteobacteria*
8	*Bacillus aryabhattai*	MK235194	99%	*Bacillaceae*	*Bacilli*
9	*Citrobacter koseri*	MK235195	99%	*Enterobacteriaceae*	*γ-proteobacteria*
10	*Citrobacter koseri*	MK235196	99%	*Enterobacteriaceae*	*γ-proteobacteria*
11	*Citrobacter koseri*	MK235197	88%	*Enterobacteriaceae*	*γ-proteobacteria*
12	*Bacillus aryabhattai*	MK235198	100%	*Bacillaceae*	*Bacilli*
13	*Citrobacter koseri*	MK235199	99%	*Enterobacteriaceae*	*γ-proteobacteria*
14	*Bacillus* sp.	MK235200	99%	*Bacillaceae*	*Bacilli*
15	*Ochrobactrum* sp.	MK235201	99%	*Brucellaceae*	*α-proteobacteria*
16	*Enterobacter* sp.	MK235202	99%	*Enterobacteriaceae*	*γ-proteobacteria*
17	*Pseudomonas aeruginosa*	MK235203	99%	*Pseudomonadaceae*	*γ-proteobacteria*
18	*Paenibacillus jamilae*	MK235204	99%	*Paenibacillaceae*	*Bacilli*
19	*Citrobacter koseri*	MK235205	99%	*Enterobacteriaceae*	*γ-proteobacteria*
20	*Bacillus* sp.	MK235206	100%	*Bacillaceae*	*Bacilli*
21	*Pseudomonas aeruginosa*	MK235207	99%	*Pseudomonadaceae*	*γ-proteobacteria*
22	*Ochrobactrum anthropi*	MK235208	99%	*Brucellaceae*	*α-proteobacteria*
23	*Enterobacter* sp.	MK235209	99%	*Enterobacteriaceae*	*γ-proteobacteria*
24	*Ochrobactrum* sp.	MK235210	99%	*Brucellaceae*	*α-proteobacteria*

3.Phylogenetic tree analysis:

A total of 24 non reductant sequences were curated and aligned with the outgroup sequence of *Geothrix fermentans* (Accession no. U41563.1). Phylogenetic tree was analysed using Maximum Parsimony algorithm and is shown in (Figure, 2). The phylogenetic tree of the gut bacterial isolates of *Anomala* sp. showed seven different clades. Four clades of the phylogenetic group belong to the group *γ-proteobacteria* with the genus *Enterobacter* (with 2 isolates) and *Pseudomonas* (with 4 isolates) and two clades represented by the genus *Citrobacter* (with five bacterial isolates). Another major clade belongs to the group *α-proteobacteria* with 6 bacterial isolates of the genus *Ochrobactrum* which is found to be the most predominant gut inhabitant of *Anomala* Sp. Other two clades represented by the group *Bacilli* with two isolates of genus *Paenibacillus* and *Firmicutes* with five isolates of *Bacillus*.

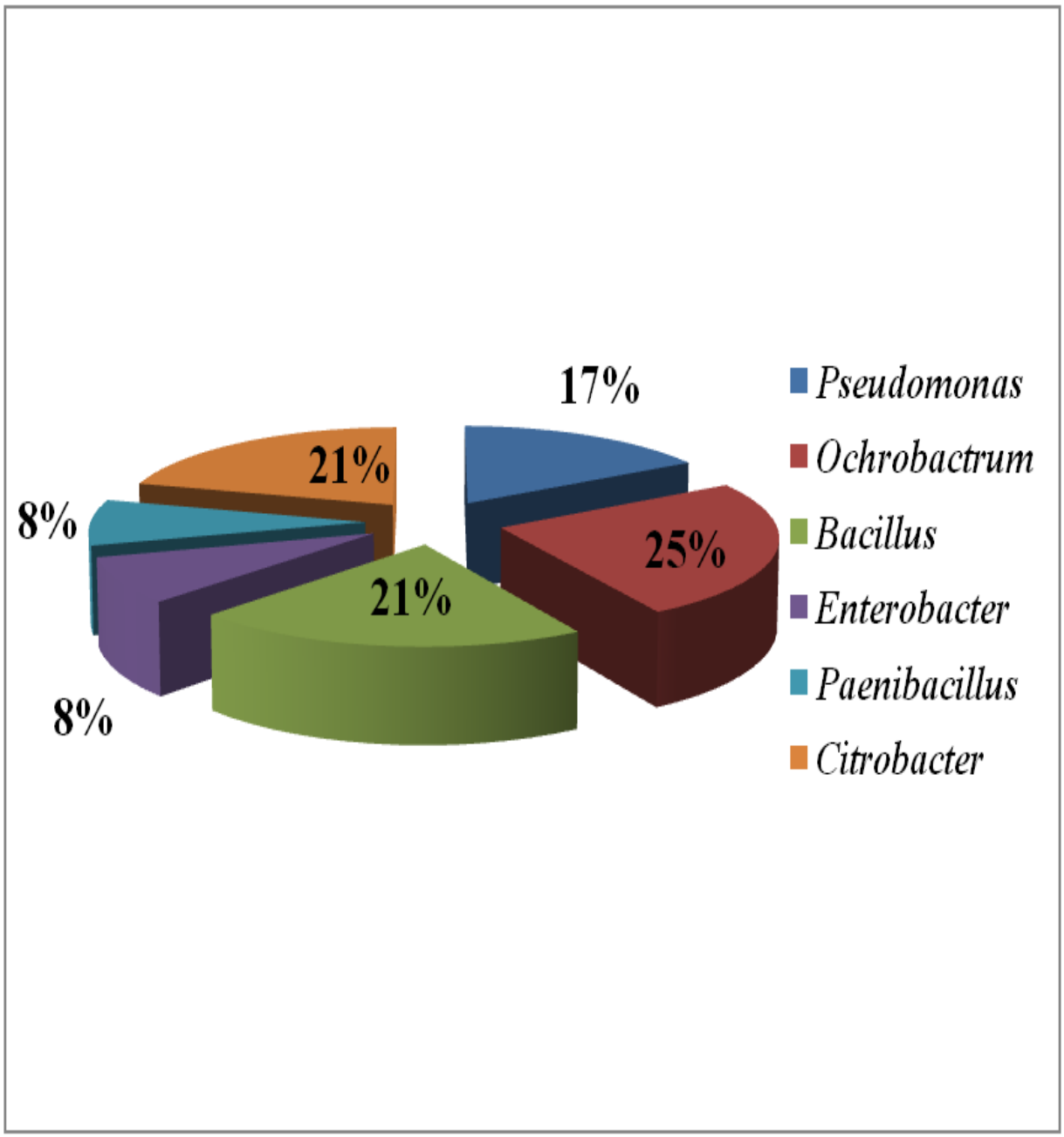

Figure 1: Proportionate distribution of 24 gut bacteria isolated and identified from *Anamola* sp. at genus level.Distribution of different bacteria among the gut of *Anamola* sp. Pseudomonas is 17 percent, Orchrobactrum is 25 percent, Bacillus is 21 percent, Enterobacter is 8 percent, Paenibacillus is 8 percent and Citrobacter is 21 percent. Among all the presence of Orchrobactrum was found more than other bacteria. The presence of Bacillus , Citrobacter, Paenibacillus and Enterobacter was the same.

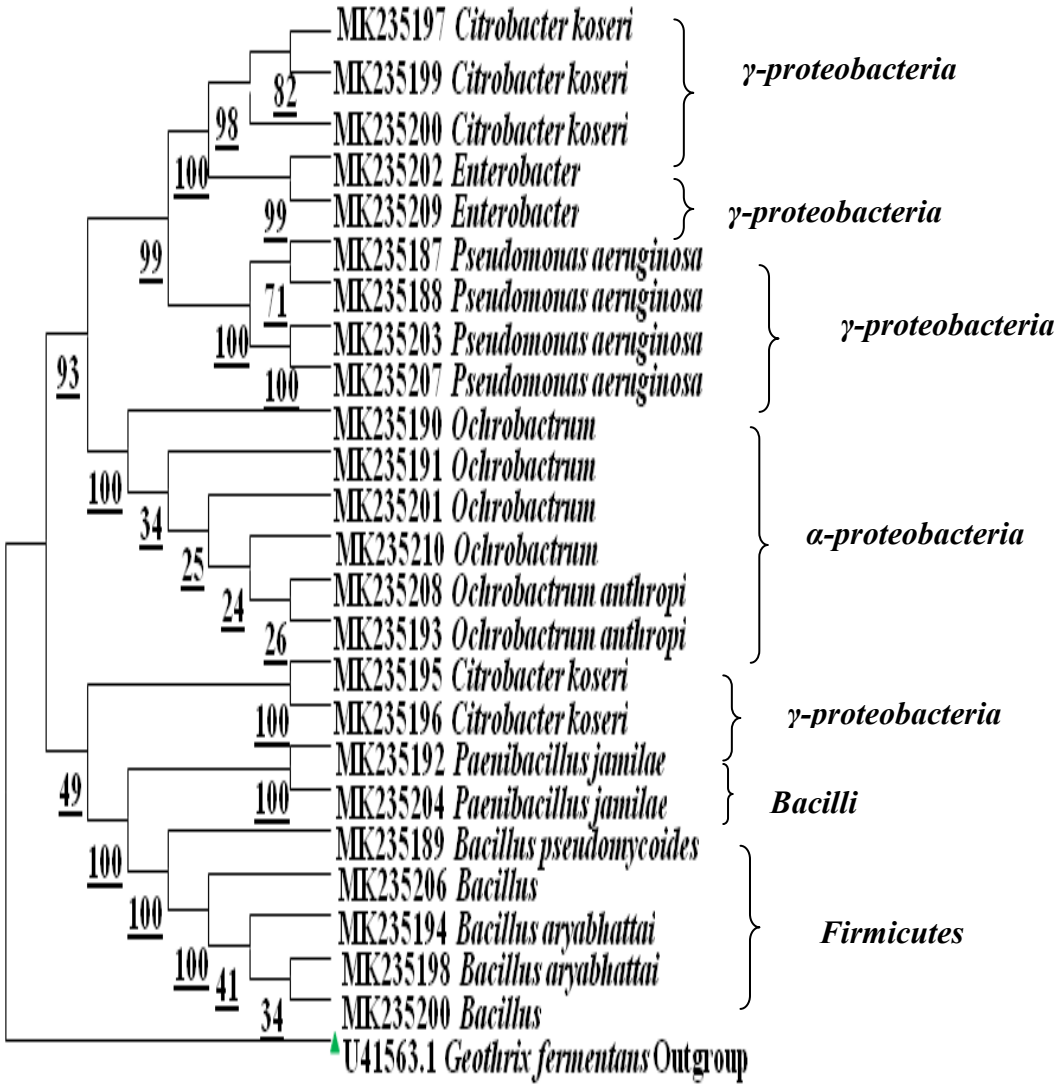

Figure (2): Phylogenetic tree of 24 bacteria from the gut of *Anamola* sp. 16S rRNA gene sequences aligned using Bioedit 7.0, with the ClustalW program, and a phylogenetic tree was constructed based on the Maximum parsimony algorithm supportrd by bootstrap values with 1000. symbol indicates the outgroup used in the phylogenetic tree.

Very little is known about the gut bacterial diversity of the *Anomala* sp. However, there are similar results were obtained by (Lehman *et al*., 2008), they reported the presence of *Citrobacter freundii* and *Pseudomonas sp.* inhabiting the digestive tract of ground beetle, *Poecilus chalcites*. We used culture-dependent 16S rRNA gene sequence-based approaches to identify six major genera of bacteria *Pseudomonas*, *Ochrobactrum*, *Paenibacillus*, *Citrobacter*, *Bacillus* and *Enterobacter.* Results of the present study revealed that a variety of gut bacteria belonging to different groups such as *γ-proteobacteria, Firmicutes, α-*proteobacteria inhabit the gut of *Anomala* sp. Studies with Lepidopteran insects showed that *Proteobacteria* and

Firmicutes were the dominant gut microflora (Broderick *et al*., 2004). The presence of *Pseudomonas* and *Bacillus* species has been documented as the dominant bacterial communities in the gut of the gypsy moth, *Lymantria dispar* (Lepidoptera), (Broderick *et al*., 2004). Similarly, Gayatri Priya *et al*. (2012) isolated and identified the members of *Bacillus firmus* and *Bacillus niabense*, *Paenibacillus jamilae*, *Cellulomonas variformis, Acinetobacter schindleri*, *Micrococcus yunnanesis*, *Enterobacter sp.* and *Enterococcus cassiliflavus* from the midguts of fifth-instar larvae of the lepidopteran moth *Helicoverpa armigera* by using cultural techniques. Cheng *et al*. (2017) reported that the gut symbiont, *Citrobacter* sp. isolated from the peach fruit fly *Bactrocera dorsalis* played a key role in the degradation of Organophosphate insecticides. Similarly, gut bacterial species isolated from the silkworm *Bombyx mori* were including *Pseudomonas vulgaris*, *Klebsiella pneumoniae* and *Citrobacter freundii* and they were known to have high cellulolytic activity, while, *Pseudomonas fluorescens* and *Erwinia sp*. showed good pectinolytic activity, moreover, *Aeromonas* sp. and *Serratia liquefaciens* were found to be cellulolytic and pectinolytic (Anand *et al*. , 2009). A similar study conducted by Desiely *et al*. (2010) reported the presence of 39 bacterial genera including *Serratia*, *Enterobacter*, *Klebsiella*, *Pantoea* and *Citrobacter* from the gut of the mosquito *Aedes aegypti*. Also, Park *et al.* (2009) isolated bacteria belonging to the genus *Paenibacillus* from the subterranean termite gut *Diestrammena apicalis* a novel bacterium capable of degrading pectin which was identified by 16srRNA gene sequence analysis. Results concerning *Ochrobactrum sp*. are in agreement with those obtained by Huang *et al.* (2010), it was identified as a potential cellulolytic bacteria associated with the fermentation chamber of a Scarabaeid beetle, *Holotrichia parallela.* Detailed functional characterization of the gut bacterial isolates and identified in this study offers scope for utilization some of these isolates as cellulose degraders in bioremediation, preparation of microbial consortia for decomposing of plant wastes, bio fuel potential and development of bacterial volatile based novel lures for monitoring and mass trapping of this pest. Further studies are needed for characterization and utilization of the gut bacterial isolates from *Anomala* sp.

Symbiotic microorganisms present one way to convene the expected demand for novel insect pest management strategies created by growing human populations and global climate change. Generic characterization of gut bacterial isolates is an essential step towards profiling the cultivable gut bacterial diversity in an organism. The present study outlines a detailed investigation of the composition of common gut symbionts of the white grub *Anomala* sp. and these gut bacteria may help in developing enhanced methods of biological control of this pest.

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