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Gut Microflora Associated with Adult Dung Beetles *Oniticellus cinctus* and *Onthophagus dama* (Coleoptera: Scarabaeidae)

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A B S T R A C T

Dung beetles gut harbour various microorganisms providing benefits to the host as well as to the environment. Experiments were conducted to identify the bacterial diversity associated with gut of the adult dung beetles *Onthophagus dama* and *Oniticellus cinctus* through culture dependent as well as culture independent method. Eight culturable bacterial isolates were isolated from the gut of *O. cinctus* and ten isolates from *O. dama*. Metagenomics research has been developed over the past decade to elucidate the genomes of the uncultured microorganisms with an aim of understanding microbial ecology. Hence metagenomics studies were also done by traditional 16s rDNA cloning resulted in identification of uncultured *Enterobacter* sp., uncultured *Aeromonas* sp., uncultured *Ralstonia* sp., uncultured *Dysgonomonas* sp., uncultured *beta proteobacterium* form *O. dama* and uncultured *Enterococcus* sp., uncultured *Firmicutes* bacterium, uncultured *Gilliamella* sp., uncultured *Peptoniphilaceae*, uncultured *Dysgonomonas* sp., uncultured *Burkholderiales* bacterium, uncultured *Porphyromonas* sp., uncultured *Gilliamella* sp. from gut of *O. cinctus* which were reported for the first time.

Introduction

Dung beetles (Coleoptera; Scarabaeidae) play very important role in the wellbeing of our planet. They improve nutrient recycling and soil structure (Brown *et al.,* 2010). The habitats of dung beetles are diverse which includes, farmland, grasslands, desert and forest (Losey and Vaughan, 2006; Nichols *et al.,* 2008). The gut microbiota of insects plays crucial roles in the growth, development and environmental adaptation to the host insects. The gut of insects contain 10 times more microbes than total cells of the insect and 100 folds more microbial genes than animal genes (Rajagopal, 2009) which will have important role in digestion as well as metabolism and mostly advantageous to the host. As they feed on dung and dung is the undigested residue of plant matter it is possible that dung beetles will be having the microflora present in the gut which helps in digestion of complex carbohydrates. Hence it is very important to know the total microflora present in the gut of these beetles. Metagenomic, the study of metagenome will help us identification the genomes of all microorganisms from different environmental samples. As culture dependant

technique will not reveal the entire bacterial community present in the gut (Gilliam, 1997). Hence, Metagenomics can make it possible to relate potential function of the specific microorganisms within the gut communities. As research on gut microflora of adult dung beetles *Onthophagus dama* (Fab.) and *Oniticellus cinctus* (Fab.) has not been reported yet. In this study, the total microbial diversity was studies from adult dung beetles *O. dama* and *O. cinctus*. It is possible that these gut microbes associated with the beetle may degrade complex organic matter, which is the future aspect of the work.

Materials and Methods

Dung beetles collection and dissection of insect gut

Dung beetles (*Oniticellus cinctus* and *Onthophagus dama*) were collected from a dairy farm located in the district of Bengaluru, Karnataka, India and kept in aerated container. Identity was confirmed by Dr. K. Veenakumari, Principal Scientist and taxonomist, ICAR-NBAIR, Bengaluru. The adult beetles were maintained live using cow dung as medium. The collected beetles were starved overnight, so that gut remains clear of any food particle. The insect was surface sterilized twice with 70% ethanol for 1 min and washed with distilled water. Entire gut was aseptically removed in a UV laminar flow hood. The isolated gut was kept in 10mM phosphate buffer and minced with the help of sterile micro pestle (Vasanthakumar *et al.*, 2006).

Culture dependant method

Isolation of gut microflora

The gut extracts collected were serially diluted and spread plated on Nutrient agar (NA), yeast peptone dextrose adenine agar (YPDA), potato dextrose agar (PDA). Plates were incubated at 37ºC for 48hrs. All chemicals used for media preparation were acquired from HiMedia Laboratories Pvt. Ltd. The bacterial isolates were differentiated based on their gram's nature.

16S rDNA analysis

The bacterial DNA was extracted using Hipura Himedia genomic DNA extraction kit. Universal primer fd1 and rp2 were used for 16S rDNA PCR. The PCR reaction conditions consisted of an initial denaturation step at 94°C for 3 mins, final denaturation at 94°C for 1mins, annealing at 45°C for 1 min, extension at 72°C for 2 min, and a final extension cycle at 72°C for 10 mins, 35 cycles. The generated sequences were compared with sequences available in GenBank by using the BLASTn program (http://www.ncbi.nih.gov) (Shayne *et al.,* 2003). The sequences were aligned using BioEdit alignment editor and sequences were submitted to Genbank to get the accession numbers.

Culture independent method

Isolation of metagenomic DNA

Isolation of genomic DNA from gut of dung beetles, *O. cinctus* and *O. dama* was done using cell and tissue DNA kit (magspin 35, APS Lifetech, India) method as described by manufacturer in kit instruction manual. Isolated DNA was checked using 1% agarose gel electrophoresis and it was documented (GelDoc-XR, Bio-Rad Laboratories, Inc.). The concentration of DNA was estimated using 260/280 nm absorbance using UV-Vis spectrophotometer (HITACHI U-2910).

PCR amplification of 16S rDNA and gene cloning

PCR of the bacterial 16S rDNA was performed using universal eubacterial primers 27F/806R. This pair of PCR primers generated

around 790 bp PCR products upon successful amplification. Every PCR reaction mixture contained 5.0 uL of genomic DNA; 10 pM of each primer; 200 pM (each) dATP, dCTP, dGTP and dTTP; and 1.0 U of Taq DNA polymerase in the PCR buffer provided in the kit. Reaction products were analyzed by electrophoresis through 2% (W/V) agarose (Promega Corporation, Madison, WI 53711 USA) gel slabs (10 cm by 16 cm by 6 mm) with 0.5x Tris-borate- EDTA buffer. Gels were stained with ethidium bromide, placed over a source of UV light, and then photographed. The molecular sizes of PCR amplicons, relative to molecular size standards scale 100bp (APS LABS, Pune) were determined. The PCR product was purified using APS LABS PCR purification kit to remove unused dNTPs and primers. Cloning of 16S rDNA amplicons was performed using Promega TA cloning kit consisting of pGEMT vector and *E. coli* XL1 blue Chemical competent cells. The TA Cloning reaction (10.0 µL) for eventual transformation into chemically competent *E.coli* XL1 blue cells was performed as: Fresh PCR product 3.0 µL, pGEMT Vector 1.0 µL, T4 DNA ligase 1.0 µL, 10x Ligation buffer 1.0 µL and Final Volume 10.0 µL. The reaction was mixed gently and incubated for Overnight at 4-8°C temperature. After Screening of transformants colony, each colony was further subjected to boiling lysis followed by colony PCR using T7 and SP6 Primers.

Bioinformatics analysis and phylogenetic tree construction

The sequences were subjected to search analysis Basic Local Alignment Search Tool (http://www.ncbi.nih.gov) to identify sequences of the highest similarity and submitted to Genbank to obtain accession numbers for each sequences. Respective Bioproject and Biosamples were created for both the dung beetles. The phylogenetic tree was constructed using maximum likelihood method using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) program based on Kimura-2 parameters with 1000 replicates of bootstrap values.

Results and Discussion

Isolation and identification of isolates

Upon plating the gut contents of *O. cinctus* and *O. dama* on different nutrient media, 8 bacteria were isolated from *O. cinctus* and 10 isolates from *O. dama* as unique isolates and assigned strain numbers. Initial characterization showed both were Gram positive as well as negative isolates. The purified cultures from both the beetles were identified by 16S rDNA sequence analysis. The analysis identified the eight isolates from *Oniticellus cinctus* as *Acinetobacter baumannii* (OS5.1), *Staphylococcus saprophyticus* (OS5.2), *Enterobacter ludwigii* (OS5.4), *Enterobacter cloacae* (OS5.6), *Bacillus cereus* (OS8.2), *Ralstonia mannitolilytica* (OS8.6), *Stenotrophomonas maltophilia* (OS10.8)and nine isolates from *Onthophagus dama* as *Bordetella avium* (DHN10.2), *Achromobacter marplatensis* (DHN10.3), *Bacillus aerophilus* (DHN10.4), *Achromobacter xylosoxidans* (DMN8.4), *Bacillus tequilensis* (DFY8.16), *Achromobacter piechaudii* (DMN8.7), *Bacillus safensis* (DMN8.1), *Bacillus licheniformis* (DFN8.3), *Bacillus subtilis* (DMY8.15).The identified bacteria showed 96–100% nucleotide homology with other bacterial strains available in the NCBI database and the sequences were submitted to NCBI (Table 1).

Though studies on the type of microbes inhabiting the digestive tract of scarab beetles have been well documented (Cazemier *et al.*, 2003; Egert *et al.*, 2005; Zhang and Jackson 2008); the type of bacteria inhabiting the digestive tract of adult beetles is limited or not available.

Fig.1 Molecular phylogenetic analysis of unculturable microflora from *Onthophagus dama* and *Oniticellus cinctus* gut by maximum likelihood method using 16S rRNA gene sequences of NCBI GenBank. The numbers at branch points of the tree designate boot strap values

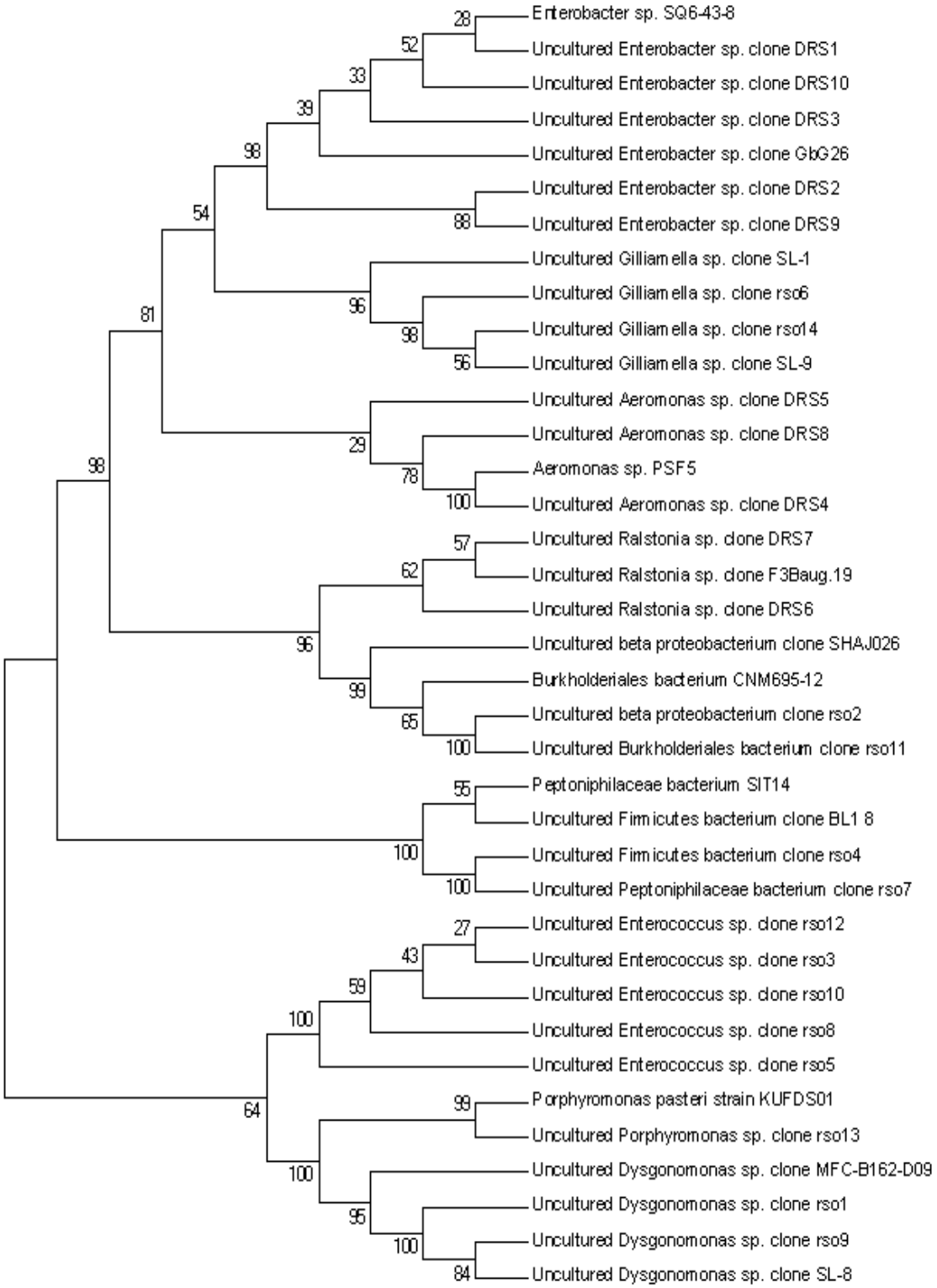

Table.1 Closest BLASTn matches for the 16S rDNA bacterial sequences in GenBank and accession number of each strain

Host	Culture type	Strain	Most similar species	Accession No.
Oniticellus cinctus	Culturable	OS5.1	*Acinetobacter baumannii*	KX242271
	Culturable	OS5.2	*Staphylococcus saprophyticus*	KX242270
	Culturable	OS5.4	*Enterobacter ludwigii*	KX242269
	Culturable	OS5.6	*Enterobacter cloacae*	KX242268
	Culturable	OS8.2	*Bacillus cereus*	KX242264
	Culturable	OS8.6	*Ralstonia mannitolilytica*	KX242263
	Culturable	OS10.6	*Kocuria koreensis*	KX242261
	Culturable	OS10.8	*Stenotrophomonas maltophilia*	KX242259
Onthophagus dama	Culturable	DHN10.2	*Bordetella avium*	KP984765
	Culturable	DHN10.3	*Achromobacter marplatensis*	KP984766
	Culturable	DHM10.4	*Bacillus aerophilus*	KX809598
	Culturable	DMN8.4	*Achromobacterxylosoxidans*	KT853099
	Culturable	DFY8.16	*Bacillus tequilensis*	KT853100
	Culturable	DMN8.7	*Achromobacter piechaudii*	KT853101
	Culturable	DMN8.1	*Bacillus safensis*	KX809601
	Culturable	DFN8.3	*Bacillus licheniformis*	KX809603
	Culturable	DMY8.15	*Bacillus subtilis*	KT853098
	Culturable	DHN10.1	*Bacillus pumilus*	KX809597
Onthophagus dama	Unculturable	DRS1	Uncultured *Enterobacter* sp.	KX585900
	Unculturable	DRS2	Uncultured *Enterobacter* sp.	KX585902
	Unculturable	DRS3	Uncultured *Enterobacter* sp.	KX585903
	Unculturable	DRS4	Uncultured *Aeromonas* sp.	KX585904
	Unculturable	DRS5	Uncultured *Aeromonas* sp.	KX585905
	Unculturable	DRS6	Uncultured *Ralstonia* sp.	KX585906
	Unculturable	DRS7	Uncultured *Ralstonia* sp.	KX585907
	Unculturable	DRS8	Uncultured *Aeromonas* sp.	KX585908
	Unculturable	DRS9	Uncultured *Enterobacillus* sp.	KX585909
	Unculturable	DRS10	Uncultured *Enterobacter* sp.	KX585910
Oniticellus cinctus	Unculturable	RSO1	Uncultured *Dysgonomonas* sp.	KX523835
	Unculturable	RSO2	Uncultured beta *proteobacterium*	KX523836
	Unculturable	RSO3	Uncultured *Enterococcus* sp.	KX523837
	Unculturable	RSO4	Uncultured *Firmicutes* bacterium	KX523838
	Unculturable	RSO5	Uncultured *Enterococcus* sp.	KX523839
	Unculturable	RSO6	Uncultured *Gilliamella* sp.	KX523840
	Unculturable	RSO7	Uncultured *Peptoniphilaceae* bacterium	KX523841
	Unculturable	RSO8	Uncultured *Enterococcus* sp.	KX523842
	Unculturable	RSO9	Uncultured *Dysgonomonas* sp.	KX523843
	Unculturable	RSO10	Uncultured *Enterococcus* sp.	KX523847
	Unculturable	RSO11	Uncultured *Burkholderiales* bacterium	KX523844
	Unculturable	RSO12	Uncultured *Enterococcus* sp.	KX523845
	Unculturable	RSO13	Uncultured *Porphyromonas* sp.	KX523846
	Unculturable	RSO14	Uncultured *Gilliamella* sp.	KX523848

It is assumed that adults will pass on some of these bacteria to its progeny and some could be acquired from the environment.

Identification of culture independent bacteria

The 16S rRNA profiling studies have provided indispensable understanding into the taxonomic composition of the gut microflora, which in turn has facilitated the inference of broad evolutionary patterns. Amplification specific to 16S rDNA gene reveals size of the PCR amplicons near around 790 bp. The pGEMT cloning vector DNA with cloned insert shows an amplicon of 175+800 = 975 bp. The empty vectors that do not contain insert show amplicons of size 175 bp. Total ten clones were selected from *O. dama* and fourteen clones were selected from *O. Cinctus*.

To the total microbes present in the environment more than 90% microflora cannot be cultured and hence yet unrevealed. From this study pertaining to unculturable microflora, we reported bacterial isolates *Gilliamella* sp. belonging to Gammaproteobacteria from gut of *O. cinctus*. This isolate has been also reported from honey bee (*Aphis mellifera*) as well as bumble bees (*Bombus bimaculatus*) gut (Kwong and Moran 2013), which has role in detoxification of toxic carbohydrates present in honeybee diet (Zheng *et al.,* 2016). *Enterococcus* sp. is dominant bacterial group in insects and was seen in both the beetles (*O. dama* and *O. cinctus*). *Enterococcus* sp. was also reported to be associated with guts of Gypsy moth (Allen *et al.,* 2009), *S. litura* (Tang *et al.,* 2012). We have also identified *Enterobacter* sp., *Aeromonas* sp., *Ralstonia* sp., *Dysgonomonas* sp., *beta proteobacterium* form *O. dama* and *Firmicutes* bacterium, *Peptoniphilaceae, Dysgonomonas* sp., *Burkholderiales* bacterium, *Porphyromonas* sp., from gut of *O. cinctus,* which is also the first report on these dung beetles.

Phylogenetic analysis

Phylogenetic analysis methods like maximum likelihood (ML), maximum parsimony (MP), neighbour-joining (NJ), minimum evolution (ME), UPGMA and Bayesian approach are used by different workers (Sriram *et al.,* 2013). ME gives more importance to the recent process of evolutionary variations. The MEGA 6 programme was used for phylogenetic tree construction with 1000 bootstrap value (Fig. 1). The maximum likelihood tree was constructed to see the phylogenetic relationship of the sequences obtained in this study with its closest strain from Genbank database (GenBank, NCBI) (Table 1). The phylogenetic analysis of all bacterial isolates was performed after editing the sequences using multiple alignments. The analysis involved 37 nucleotide including test and reference sequences in culture independent approach. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Two genetic groups were formed from 24 bacterial strains from *O. cinctus* and *O. dama* gut. Group I represented 16 bacterial isolates with >99% similarity (*Enterobacter* sp. DRS1, *Enterobacter* sp. DRS2, *Enterobacter* sp. DRS3, *Enterobacter* sp. DRS9, *Enterobacter* sp. DRS10, *Gilliamella* sp. RSO6, *Gilliamella* sp. RSO*14*, *Aeromonas* sp. DRS5, *Aeromonas* sp. DRS8, *Aeromonas* sp. DRS4, *Ralstonia* sp. DRS6, *Ralstonia* sp. DRS7, *beta proteobacterium* RSO2, *Burkholderiales* bacterium RSO11, *Firmicutes* bacterium RSO4, *Peptoniphilaceae* bacterium RSO7). Group II consisted of 8 isolates with >97% similarity (*Enterococcus* sp. RSO12, *Enterococcus* sp. RSO3, *Enterococcus* sp. RSO10, *Enterococcus* sp. RSO8, *Enterococcus* sp.

RSO5, *Porphyromonas* sp. RSO13 *Dysgonomonas* sp. RSO1, *Dysgonomonas* sp. RSO9) (Table 1).

Nucleotide Accession numbers

The sequence obtained were deposited to Genbank under the accession numbers KX523835-KX523848, Bioproject PRJNA321463, Biosample SAMN05001291 in case of *O. cinctus* and accession numbers KX585900, KX585902-KX585910, Bioproject PRJNA329583, Biosample SAMN05415091 in case of *O. Dama*.

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