

Probing Gut Microflora Community of the Bihar Hairy Caterpillar, *Spilosoma obliqua* Walker: By Next Generation Sequencing

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As part of a metagenomic analysis of gut microflora, V3 region of the 16s rRNA gene of the bihar hairy caterpillar, *Spilosoma obliqua* collected from the sunflower field was sequenced using a MiSeq desktop sequencer (Illumina, San Diego, California). The sequence comprised of 381 074 paired-end reads with 50.76 % GC content and 35.95 average base quality (Phred score), after the reads were passed through a conserved region filter, a spacer sequence filter, a read quality filter, and mismatch filter. Singleton and chimeric sequences were removed as part of pre-processing, and the 255 904 paired-end reads that remained were considered for the analysis. After removing singleton operational taxonomic units (OTUs) and chimeric sequences, 206 OTUs were identified, representing 13 phyla, 22 classes, 32 orders, 48 families, 38 genera, and 16 species. To our knowledge, this is the first successful application of Illumina-based *de novo* sequencing for the analysing the microbial community of the gut of a bihar hairy caterpillar.

Keywords: Gut, metagenomic, microflora, operational taxonomic units.

Insects are one of the most successful and diverse groups on earth; they are adapted to a wide variety of diets and live in practically any habitat. The microorganisms associated with the insect gut could be particularly important because their function in the gut may help in food digestion and supply nutrients and growth factors (Six, 2003). Bacterial communities associated with the gut of bihar hairy caterpillar have not been well investigated in as much as detail as those found in the guts of other insect groups has been studied widely (Moran *et al.*, 2005).

The Bihar hairy caterpillar, *Spilosoma* (= *Diacrisia*) *obliqua* (Walker) (Arctiidae: Lepidoptera), is an intermittent pest widely distributed in India, China, Bangladesh, Myanmar,

Nepal and Pakistan (Crop Protection Compendium, 2004). It is a serious pest in Bihar, Madhya Pradesh, Uttar Pradesh, Punjab, Manipur and other states. It is a polyphagous pest having a very wide range of host plants damaging oilseeds, fiber crops, pulses, vegetables and some medicinal plants (Atwal and Dhaliwal, 1997) and in case of severe infestation, the entire crop is damaged badly causing up to 40 per cent defoliation of leaf area (Thippaiah, 1997). The studies on *S. obliqua* in southern part of India are relatively scanty (Kotikal and Devaiah, 1988; Peerajade *et al.*, 1999; Muthusamy *et al.*, 2011), however in the central and north eastern parts, it is an insect pest of high significance and its status is well documented. At Pantnagar, *S. obliqua* is an established insect pest of sporadic nature damaging different pulse crops, particularly soybean (Chand, 2012). The pest makes its first appearance from its winter hibernation in March. The caterpillars feed gregariously and

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voraciously on a variety of food plants and having ability to destroyed one field, and move in swarms to another field. There are six generations of this pest per annum and, passes the first generation mostly on weeds, it should be destroyed in the weed itself before the pest multiplies and migrates to the cultivated crops (Yadav *et al.*, 2001). An outbreak of this pest was noticed on finger millet (*Eleusine coracana* L.), castor (*Ricinus communis* L.) and field bean (*Lablab purpureus* L.) in 1917, in the Ilawala village near Mysore, wherein people burnt about half a cartload of collected caterpillars (Puttarudraiah, 1975).

In the present scenario, where insects have become resistant to most insecticides including organophosphates, carbamates, and pyrethroids (Elbert and Nauen 2000), it becomes necessary to understand their microbiome and further isolate bacterial strains with functional aspects. Earlier studies based on the traditional approach of plating the insect homogenate on different nutrient agar media failed to analyze the majority of gut bacterial communities because they could not grow outside the host body on artificial media (Vaughan *et al.*, 2000). However, with the advent in technology, 16S rDNA sequencing (Ohkuma and Kudo 1996) and denaturing gradient gel electrophoresis (Reeson *et al.*, 2003) were developed as major tools for revealing bacterial diversity in several insects. Thus, exploiting both traditional and modern day techniques could present a better picture of insect gut flora.

Metagenomics is a culture-independent strategy involving direct extraction and cloning of DNA from an assemblage of microorganisms, thereby capturing their genetic potential in a surrogate host (Handelsman, 2004). Metagenomic analysis of insect-associated microorganisms has contributed to better understanding of the genome of *Buchnera*, an obligate intracellular symbiont of aphids (Perez-Brocail *et al.*, 2005), and of biosynthetic pathways of secondary metabolites in the bacteria associated with beetles (Piel *et al.*, 2004). Xylanases with unusual primary sequences and novel domains of unknown function were discovered in metagenomic libraries of microbiota associated with members of the orders *Isoptera* (termites) and *Lepidoptera* (moths) (Brennan *et al.*, 2004). The present investigation attempts to analyse the richness and composition of the gut

micro flora of bihar hairy caterpillar, *Spilosoma obliqua* using next generation sequencing (NGS).

MATERIALS AND METHODS

Currently, sequencing the diversity of the 16S rRNA gene in Bihar hairy caterpillar guts using NGS technology is one of the easiest and more cost effective tools available for characterizing the microbial communities associated with Bihar hairy caterpillar gut. For conducting present experiment following procedure was followed

Insect collection

Larvae (4/5 instar) of the Bihar hairy caterpillar were collected from a field of sunflower at the of Main Agricultural Research Station Dharwad, Karnataka, India. The species is neither endangered nor protected. Larvae after collection from field were brought to the laboratory.

Extraction, PCR and quantification

Steps for extraction

1. Larvae before dissection are disinfected and cleaned to ensure that only microbes of gut are present in the gut extract. The collected larvae were killed by asphyxiation and dissected inside a laminar airflow chamber to remove their entire gut. The whole intestine was removed from the larvae with sterile forceps.
2. Extraction of DNA was done using bead-beating method because that is one of the best methods for cell disruption and it was method of choice (Mattila *et al.*, 2012; McFrederick *et al.*, 2012)
3. After obtaining gut sample, it was placed in 2 mL microcentrifuge tube with a sterile 5 mm stainless steel bead (QIAGEN, Valencia, CA), 500 µL of 0.1 mm glass beads (Scientific Industries, Inc.; Bohemia, NY, USA), and 500 µL RLT buffer (QIAGEN; Valencia, CA, USA) with 5 µL of β- mercaptoethanol.
3. The gut sample was run in tissue lyser at 30 Hz for 5 minutes.
4. The gut sample was centrifuged at 5000rpm at 5 °C for 2 minutes to separate the beads and the buffer.
5. In new 1 mL centrifuge tube 200 µL of 100 percent ethanol and 200 µL of supernatant were taken and gently mixed the contents using vortex mixture.

6. DNA was recovered from gut sample by applying the gut sample from step 5 to a QIAamp minispin column and followed the tissue protocol of QIAamp DNA minikit (QIAGEN; Valencia, CA, USA).
7. Eluted DNA stored in nanopure water at -20 °C.
8. DNA obtained from above step was quantified by using gel-electrophoresis, nano drop concentration, spectrophotometer reading and qubit concentration.

Primer choice and 16S rRNA region

Primary concern for primer choice is universality. We have considered and amplified V3 region of the 16S rRNA gene. Primer pair used for amplification of V3 region is

Forward primer, 28F: 5'–GAGTTTGATCNTG GCTCAG–3'

Reverse primer, 519R: 5'–GTNTTACNGCGGC KGCTG–3'

PCR condition

We have used HotStarTaq Plus Master Mix Kit (QIAGEN; Valencia, CA, USA) for 50 µL reaction. For this 25 µL HotStarTaq plus master mix was taken in to PCR tubes to which 2 µL each of 10 µM forward and reverse primer, 5 µL DNA template (100 ng), 16 µL sterilized nanopure water were added. Using the PCR reaction conditions as described above, the PCR cycle involved in initial denaturation at 95°C for 5 minutes followed by 25 cycles of denaturation 94°C for 30 seconds, annealing at 60°C for 40 seconds and elongation at 72°C for 1 minute and final elongation at 72°C for 10 minutes.

Bioinformatics analysis

The analysis involved the following steps.

- 1) Fastq quality checking: base quality, base composition, GC content
- 2) Filtering and identification of V3 region from paired end data includes three steps
 - (a) Reading, trimming, and identification of V3 sequences (b) Constructing consensus sequence from paired-end reads (c) Filtering of sequences

A proprietary wet-lab approach was followed to sequence the 16S rRNA in the V3 region of the *S. obliqua* gut bacteria. The region was extracted using Illumina paired-end sequences, as

follows.

- (a) Trimming of the spacer and the conserved region
- (b) Building a consensus V3 region from the trimmed paired-end reads
- (c) Filtering through the following filters to identify high-quality V3 region sequences viz., conserved region filter, spacer sequence filter, read quality filter and mismatch filter.

Usually, a paired-end sequence from the V3 metagenomics region contains some portions of the conserved region, spacer, and V3 region. As the first step, the spacer and the conserved region portions were removed from the paired-end reads. After trimming the unwanted sequences from the original paired-end data, a consensus V3 region sequence was constructed using Clustal Omega. The four filters mentioned above were used to ensure that only high-quality V3 region sequences were chosen for the various downstream analyses.

The following pre-processing steps were carried out before the analysis. The singletons that were probably included because of sequencing errors and can result in spurious OTUs were removed by removing the reads that did not cluster with other sequences. Chimeras were also removed using the *de-novo* chimera removal method UCHIME implemented using the software USEARCH.

- 3) Operational taxonomy units (OTUs) and taxonomic classification
 - (a) Identification of OTUs (b) Assignment of taxonomy to each OTU (c) Identification of read abundance

The above steps were performed using pre-processed consensus V3 sequences. Pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using UCLUST (similarity cutoff = 0.97) [Lozupone *et al.*, 2013; D'Argenio *et al.*, 2014].

For the entire downstream analysis, QIIME was used [Caporaso *et al.* 2010]. A representative sequence was identified for each OTU and aligned against the Greengenes core set of sequences using PyNAST [DeSantis *et al.*, 2006a; DeSantis *et al.*, 2006b]. These representative sequences were aligned against reference chimeric data sets. Taxonomic classification was performed using an RDP classifier and Greengenes OTUs database. Both rare and abundant microbiota were

identified from the gut samples [Galand *et al.*, 2009; Aravindraja *et al.*, 2013].

RESULTS

The result showed that primer pair provided clear band in the gel picture (Fig. 1) and by nanodrop and qubit techniques, concentration of the DNA in the gut of bihar hairy caterpillar was estimated as 11.6 ng/μL, and 0.682 ng/μL, respectively. Spectrophotometer reading, 260/280 ratio is 1.34. This analysis showed V3 region of the gut microflora had amplified.

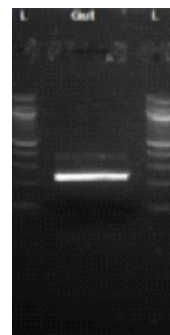


Fig. 1. Gel picture showing DNA of gut sample: Gut – *Spilosoma obliqua* larval gut sample, L – Ladder

Table 1. Raw read summary

Sample	Total Reads (Paired-End)	Sequence Length (bp)	Total Data (Mb)	%GC	Average base quality (Phred score)
GUT	381,074	151	114.32	50.76	35.95

Table 2. Phred score distribution of the paired-end reads for the samples

Sample	Q0-Q10	Q10-Q20	Q20-Q30	>= Q30
GUT	0.00	5.84	1.71	92.44

Table 3. Base composition distribution of the samples

Sample	Base Composition (%)			
	A	C	G	T
GUT	24.34	23.86	26.89	24.89

Clostridia, in orders – Clostridiales, in families – Ruminococcaceae, in genus – Oscillospira and in species – Saprophyticus were top most respective taxa in the gut of the bihar hairy caterpillar.

DISCUSSION

Bihar hairy caterpillar possesses a simple

Table 4. Read Summary Table

Sample Name	Total Reads	Passed Conserved Region Filter	Passed Spacer	Passed Read Quality Filter	Passed Mismatch Filter
GUT	381,074	356,283	355,947	355,926	284,081

Table 5. Pre-processing reads statistics

Sample	Consensus Reads	After Singletons Removal	Chimeric Sequences	Pre-processed Reads
GUT	284,081	279,080 (98.24 %)	23176 (8.16%)	255,904 (90.08 %)
Total Reads	255,904			
Total OTUs	206			

Sequence quality check

Raw read summary

Data from the raw Fastq files obtained from the sequencer are summarized in Table 1.

Fastq quality check

Base quality score distribution

The base quality and Phred quality is shown in Table 2. Nearly 92 per cent of the total reads have a Phred quality score greater than 30 ($p = 0.001$). The Phred score distribution of the sample is given in following table.

Base composition distribution

The base composition of left and right end of the paired-end read sequences was calculated. Since the target sequence was that of V3 region, a sequence composition bias was observed in the gut sample. Overall base compositions of gut sample was provided in Table 3.

GC distribution

The average GC distribution of the sequenced read of the gut sample was 40-50%.

Identification of V3 region from paired-end reads

In making a consensus V3 sequence, more

than 80 per cent of the paired-end reads aligned to each other with zero mismatches and an average contig length of ~160 to ~165bp. A summary of reads that passed each filter can be found in Table 4.

Pre-processing of reads: Singletons removal and chimera filter

Results of singleton removal and chimera filter based on gut sample was given in Table 5.

A total of 206 OTUs were identified from 255,904 reads and taxonomical annotations of 206 OTUs based on Greengenes database. The sequences do not have any alignment against taxonomic database are categorized as "Unknown". Based on OTUs data, 198 phyla, 189 classes, 133 orders, 32 families, 11 genus and 2 species of microbes were identified remaining 8 phyla, 17 classes, 74 orders, 175 families, 196 genus and 204 species were unidentified or unknown. A total taxa of 13 phyla, 22 classes, 32 orders, 48 families, 38 genera, and 16 species of microbes were ascertained from the gut of the bihar hairy caterpillar. In phyla – Firmicutes, in classes –

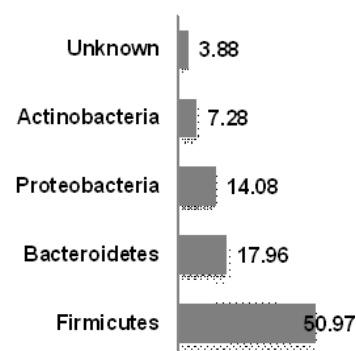


Fig. 2. Top five phyla percentage

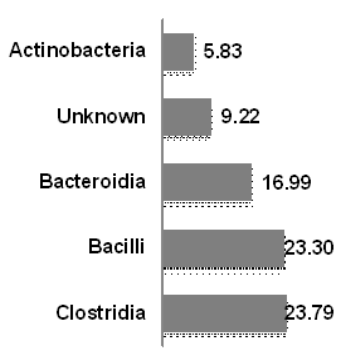


Fig. 3. Top five classes' percentage

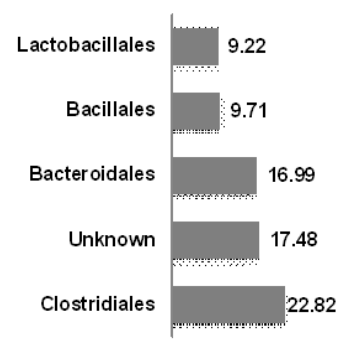


Fig. 4. Top five orders percentage

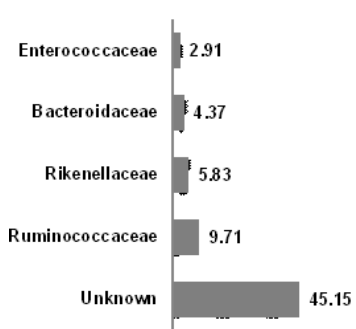


Fig. 5. Top five families' percentage

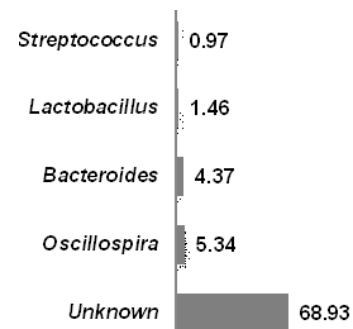


Fig. 6. Top five genus percentage

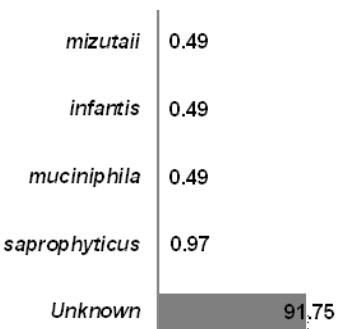


Fig. 7. Top five species percentage

tube-like alimentary tract, which is the largest part of the whole body and lacks any specialized substructures. A very few researches have applied NGS technique to study gut microbiota. In spite of its easiness, a huge extent of microbiota has been found occupying the gut making the gut an ideal environment for diverse microbial activities. In general, NGS reads were dominated by taxa from Firmicutes and Bacteroides. The complexity of this community, comprised of 206 OTUs, is clearly compared to the gut microbiota of insects from orders such as termites of Isoptera, or vertebrates, which often harbor hundreds of phylotypes (Warnecke *et al.*, 2000; Perkins *et al.*, 2012). The microbes, which were more predominant, were taken for explanation and discussed. Apart these most of the microbes were unknown. Percent distribution of top five phyla, classes, orders, families, genus and species were depicted in the form of bar graph from Fig.2 to Fig.7. However, three families, including Ruminococcaceae and Rikenellaceae are particularly more, and these are likely the core functional populations living inside the gut. The colonization of *Clostridia* has commonly been linked to its highly efficient cellulose digestion and its ability to ferment a variety of sugars (Watanabe and Tokuda, 2010). It can be presumed that the order Clostridiales bacteria associated with bihar hairy caterpillar play a similar role. In addition, *Clostridia* may enhance host immunity (Atarashi *et al.*, 2011).

As larvae grow bigger, less oxygen can penetrate into the gut lumen over the thicker gut wall and elongated alimentary tract, which cause consistently low oxygen tension in the gut compartment (Tang *et al.*, 2012). This largely anoxic condition probably promotes the development of anaerobic, anoxygenic, genus *Oscillospira*. Although *Oscillospira* bacterium was first described almost a century ago (Chatton and Pe´rard, 1913), growth in pure culture has not been reported (Gibson, 1974 and Gibson, 1986) hence, little is known of its ecological role and physiological properties in the intestinal tract. *Oscillospira* and other large bacteria attached rapidly to the cuticular surface of clover and grass leaves in the cattle and sheep rumen (Clarke, 1979) suggesting that the cuticle of green leaves constitutes a specific niche for these bacteria. Metagenomic analysis of the microbial DNA from

this study will increase our knowledge of other functions supplied by the active microbiota. During coevolution, indigenous gut bacteria have adapted to work together in this distinct ecological niche and supply their metabolic benefits to the host. On the other hand, different physicochemical conditions with respect to host development, such as gut alkalinity, oxygen tension, might impact the community's activity, which in turn influences its composition and consequently metabolic functions (Shao, 2014). Our data also setup the preliminary catalogue of the gut microbiota of a representative organism from poly-phytophagous Lepidoptera, bihar hairy caterpillar. Knowledge of the gut bacteria in such a major herbivore insect may also provide new targets for agricultural pest management.

CONCLUSION

We have explored key method (Non-culture-based approach) for studying symbiotic microorganisms in bihar hairy caterpillar guts. NGS sequencing improved our capabilities, when it comes to identifying the microbial communities associated with bihar hairy caterpillar. This study is a basic type of research, which gives us an idea about existing microbes inside the gut of bihar hairy caterpillar. This information helps for further strategic types of works like knowing the role of microbes inside the gut of the bihar hairy caterpillar and helps in management of it on field crops.

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