

E-ISSN: 2320-7078 P-ISSN: 2349-6800 www.entomoljournal.com JEZS 2021; 9(1): 1097-1103 © 2021 JEZS Received: 25-11-2020 Accepted: 27-12-2020

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# Journal of Entomology and Zoology Studies

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### Isolation and characterization of adult gut microflora of *Xenocatantops humilis* (Orthoptera: Catantopidae)

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#### DOI: https://doi.org/10.22271/j.ento.2021.v9.i1o.8713

#### Abstract

Grasshoppers are an integral part of food chain, food web and ecosystem balance. They are consumed in many parts of the world by humans as a protein source. Some species are a serious threat to agriculture and vegetation. Gut microflora plays an essential role in the metabolic activities of insects including reproduction and behavior. Identification and Characterization of gut microbial taxa can lead to better understanding of insect-microbe interactions and development of advanced, more efficient biological methods of pest control. Microbiological and genomic analyses of gut isolates of adult *Xenocatantops* showed the occurrence of *Bacillus paramycoides* of the *Bacillus cereus* group along with other diverse microbial genera.

Keywords: Bacillus, grasshoppers, gut microbiome, 16SrRNA, phylogeny, orthoptera, insects

#### Introduction

Orthopterans which include both short-horned and long-horned grasshoppers, are the key herbivores in grassland ecosystems across the world and are considered as one of the most important insect groups both ecologically and economically. Grasshoppers form an essential part of grassland ecosystems and serve as a food source for birds and also affect the soil enzymatic activity significantly <sup>[1]</sup>. *Xenocatantops humilis* is a grasshopper species belonging to family Acrididae and subfamily Catantopidae <sup>[2]</sup>. It is mainly found in temperate Asian regions of India, China, Malaysia and Papua New Guinea and it is generally found in forests <sup>[3]</sup>. Heavy infestation of *X. humilis* has been found on hill slopes adjoining cultivated fields of paddy, maize, oat cow pea. Adults and nymphs are found in groups under the leaves and also near the edges of the rice fields near aquatic sources <sup>[4, 5]</sup>.

It is mainly consumed as a protein source in the South-East Asian regions like Singapore and are generally bred for the same <sup>[6]</sup>. According to several surveys, *Xenocatantops humilis* was found to be a predominant pest on tea plants and paddy fields <sup>[7][8]</sup>. Out of total 56 species of *Xenocatantops*, *X. humilis* is a pest on pepper leaves thereby reducing its production <sup>[9]</sup>.

Insect microbiomes play an important role in the health and fitness of insect hosts by contributing to nutrient absorption, immune health, and overall ecological fitness <sup>[1]</sup>. They are also involved in multitrophic interactions between the host and other biological factors, including enhancement of the host's immune response. During evolution mutalism between gut microbes and insect host has been established, and the gut microbes developed strategies for adapting to the environment of the insect gut <sup>[10]</sup>. Insects, which generally harbor a lower microbial diversity than vertebrates and have recently emerged as potential model systems to study these interactions <sup>[11]</sup>. Recently microbiomes of economically important insects such as Honeybee, Fruit fly and Beetles are widely studied <sup>[12-16]</sup> but grasshoppers being the pest insects for many crops are still not in forefront with respect to the microbiome studies <sup>[1, 17-19]</sup>. Study of microflora may play a role in the development of new methods of biological pest control and for studying unknown microbes influencing insect physiology and ecology. Insect microbiome studies may contribute in increasing the protein value of edible insects such as *Xenocatantops humilis*. Gene transcriptomics data of *Xenocatantops brachycerus* will provide a useful molecular resource for gene prediction, molecular marker development, studies on

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cellular signaling pathways and will be a reference for the efficient use of other grasshoppers <sup>[21]</sup>. Similar genomics approach can be used for *X*. *humilis* which will lead to better understanding of biology of grasshoppers and their uses as proteinaceous food source and development of next

generation pesticides. In this paper we have isolated and characterized the microflora of *Xenocatantops humilis* which is one of the major pests involved in crop destruction as well as a source of food.

#### **Materials and Methods**

*Xenocatantops. humilis* adults were collected from Vetal Tekdi, Kothrud, Pune (Fig.1). Genus and species were confirmed by using relevant references and expert guidance (S.K. Tandon, 1972).

*X. humilis* were subjected to euthanasia (cold treatment). After euthanasia induction, the organisms were surface sterilized using 70% EtOH. Decapitation was performed, body appendages were removed and the entire abdomen was transferred ice-cold 0.1 M 1X PBS (pH 7.4). Abdomen was homogenized in Teflon-coated Potter Elvehjem homogenizer. Homogenate was serially diluted from 10-1 to 10-4 for obtaining pure colonies. 100  $\mu$ L of sample was plated on Luria Agar (LA) plates by Spread plate method after performing serial dilutions. Colony characterization and CFU/ml estimation was done post inoculation (1 -2 days). Sub-culturing was done on differential and selective media by streak plate method to obtain pure isolates. Gram staining <sup>[22]</sup> and biochemical characterization was performed for identification and characterization of pure isolates.

A single isolate was selected for identification, biochemical characterization, genomic, and phylogenetic analyses using the gold standard 16S rRNA gene sequencing method. 1200 bp sequence length was used for finding out extent of homology with the closest neighbor in the database (EzBioCloud 16S db). Phylogenetic Tree Construction was done using MEGA X 10.2.5 version and ClustalX. Neighbour Joining method (N-J) was used for the same.

#### Results



Fig 1: *Xenocatantops humilis* collection site (Occurrence and habitat)

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Fig 2: Adapted from https://singapore.biodiversity.online/species/A-Arth-Hexa- Orthoptera



Fig 3: Adult Abdomen: Undiluted Homogenate on LA plate (Spread Plate method)



Fig 4: Adult Abdomen: Pure Isolate on LA plate (Streak Plate method)

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Fig 5: Streak plate method : Pure Isolate



Fig 6: EMB Agar plate : No growth



Fig 7: Catalase Test: +Ve



Fig 8: Gram Staining of Isolate Gram +ve rods (Bacilli)

 Table 1: Colony Characterisation

Sr. no.	Colony Characteristics	Isolate 1					
1	Grams Nature	+ve (Fig. 8)					
2	Shape	Rods					
3	Size	Pin point to pin head size 1 mm					
4	Margin	Entire					
5	Colour of Colony	White					
6	Opacity	Opaque					
7	Elevation	Flat					
8	Surface	Smooth					
9	Consistency	Mucoid					
10	Catalase Test	+ <b>ve</b> (Fig. 7)					
11	Motility Test	-ve					

The isolate showed no growth on EMB agar (Fig.6).

#### **Bioinformatics**

Using standard genomic techniques of RNA isolation, purification and PCR, the following 16SrRNA sequence was used for *in silico analysis* involving NCBI BLAST (Fig. 9, 10).

#### >16S rRNA sequence of Xenocatantops isolate B\_FEB\_21\_131

GATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGG ATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGG CTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAG CCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGA TTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACT GTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACT CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTG GGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAA TTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCTCCTTCGGGA GCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGGGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA ACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGG ATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAG ACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGG AATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGCACACACCGCCCGTCA

#### NCBI-BLAST Multiple Sequence Alignment (MSA) for 16SrRNA sequence of Xenocatantops sp. abdominal Isolate

Description	is Graph	nic Summary	Alignments	Taxon	omy	
Reports	Lineage	Organism	Taxonomy			
100 sequence	es selected 🔞					
	Organisr	m	Blast Name	Score	Number of Hits	Description
Bacillaceae			firmicutes		100	
- Bacillus			firmicutes		<u>77</u>	· · · · · · · · · · · · · · · · · · ·
- Bacillus	cereus group		firmicutes		<u>31</u>	
Bacill	us paramycoides		firmicutes	2473	1	Bacillus paramycoides hits
Bacill	us tropicus		firmicutes	2468	1	Bacillus tropicus hits
Bacill	us nitratireducens		firmicutes	2468	1	Bacillus nitratireducens hits
Bacill	us luti		firmicutes	2468	1	Bacillus luti hits
Bacill	us albus		firmicutes	2468	1	Bacillus albus hits
Bacill	us cereus ATCC 1	14579	firmicutes	2462	2	Bacillus cereus ATCC 14579 hits
Bacill	us cereus		firmicutes	2462	4	Bacillus cereus hits
Bacill	us wiedmannii		firmicutes	2457	1	Bacillus wiedmannii hits
Bacill	us proteolyticus		firmicutes	2457	1	Bacillus proteolyticus hits
Bacill	us pacificus		firmicutes	2446	1	Bacillus pacificus hits
Bacill	us paranthracis		firmicutes	2446	1	Bacillus paranthracis hits
Bacill	us toyonensis		firmicutes	2440	1	Bacillus toyonensis hits
Bacill	us pseudomycoid	0.5	firmicutes	2440	2	Bacillus pseudomycoides hits
Bacill	us thuringiensis		firmicutes	2440	3	Bacillus thuringiensis hits
<u>Bacill</u>	us mobilis		firmicutes	2435	1	Bacillus mobilis hits
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Fig 9: NCBI BLAST Significant hits

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Query 9669	(*)1	TATGA	GAACACCA	ataac	GAAGG	CGACTT	CTGGTC	TGTAAC	TGACA	CTGA	aacaca	A 1,346				
NR 157734.1	(+)66	8 x 20 + 1 + 1 + 1 + 1 + 1					NUMBER OF STREET					1.410	Bacillus paramy	coides		
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NR 074540.1	(+)/3						*1.*1.*1.*1.*1					1,417	Bacillus cereus	ATCC 14579	-	
NR 112220.1	(*)40	X										1,390	Bacillus cereus			-
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Fig 10: 16SrRNA sequencing followed by in silico analysis using Ez Bio Cloud DB showed the following results

DDN	Strain No.	Closest Neighbour*		%	
FRIN	Strain NO.	Taxonomic Designation	Accession No.	Similarity	
B_FEB_21_131	1	Bacillus paramycoides NH24A2(T)	MAOI01000012	99.85	



Fig 11: Closest Neighbour\*

Fig 12: Clustal X Multiple Sequence Alignment (MSA) of BLAST hits for 16SrRNA sequence of Xenocatantops humilis abdominal isolate

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Data Edit Search Alignment Web Sequencer Display	Help						
	C ()	+ 🔁	41	₽	Q 9	9	9
DNA Sequences Translated Protein Sequences							
Species/Abbry							
1. Sequence 1							GATTAAGAGC TTGCTCT
2. NR_157734.1 Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA partial sequence	e: C T G C	CTC	GG	TGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
3. NR_157736.1 Bacilius tropicus strain MCCC 1A01406 16S ribosomal RNA partial sequence(2)	CTGO	IC TC	GG	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
4. NR_157732.1 Bacillus nitratireducens strain MCCC 1A00732 16S ribosomal RNA partial sequence	CTGC	ICTC)	GG	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
5. NR_157730.1 Bacillus luti strain MCCC 1A00359 16S ribosomal RNA partial sequence	CTGO	CTC	GG	TGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTE
6. NR_157729.1 Bacilius albus strain MCCC 1A02146 16S ribosomal RNA partial sequence	CTGO	стс	GG	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
7. NR_074540.1 Bacillus cereus ATCC 14579 165 ribosomai RNA (rrnA) partial sequence	TTTC	ATC	TG	G C T C	AGG	ATG	AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGC
8. NR_115526.1 Bacillus cereus strain IAM 12605 16S ribosomal RNA partial sequence	GCGC	CGT	CC	TAAT	ACA	TGC	AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
9. NR_115714.1 Bacillus cereus strain CCM 2010 16S ribosomal RNA partial sequence	CTGO	CTC	AGG.	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
10. NR_112630.1 Bacilius cereus strain NBRC 15305 16S ribosomal RNA partial sequence	1000	GTG	CCT.	AATA	CAT	OCA	A G T C G A G C G A A T G G A T T A A G A G C T T G C T C T T A T G A A G T T A G C G G C G G A C G G
11. NR_113266.1 Bacillus cereus strain JCM 2152 165 ribosomal RNA partial sequence	GCGC	CGT	1 C C	TAAT	ACA	TGC	AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
12. NR_114582.1 Bacillus cereus ATCC 14579 16S ribosomal RNA partial sequence	ATGA	ACG	TG	G C G C	CGT	GCC	TAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTA
13. NR_152692.1 Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA partial sequence	CTGG	CTC	GG	ATGA	ACGO	CTG	GCGGCGTGCCTAATACATGCAAGTCGAAGCGAATGGATTAAGAGCTTGCTCT
14. NR_157735 1 Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA partial sequence	e: C T G C	CCC	AGG.	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCT
15. NR_170494.1 Bacillus fungorum strain 17-SMS-01 16S ribosomal RNA partial sequence	AGAO	AAA	TA.	C II	TAT	TGG	AGAGITIGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGC
16. NR_157733.1 Bacillus pacificus strain MCCC 1A06182 16S ribosomal RNA partial sequence	CTGO	CTC	GG	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCT
17. NR_157728.1 Bacillus paranthracis strain MCCC 1A00395 16S ribosomal RNA partial sequence	CTGC	CTC	GG	ATGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCT
18. NR_121761.1 Bacillus toyonensis strain BCT-7112 16S ribosomal RNA partial sequence	IGCT0	AGG	TG	AACO	CTG	o c o	GCGTGCCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAA
19. NR_113991.1 Bacillus pseudomycoides strain NBRC 101232 16S ribosomal RNA partial sequer	A G C G C	CGT	C C	TAAT	ACA	TGC	AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
20. NR_043403.1 Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA partial sequence	GCGG	CGT	CC	TAAT	ACA	TGC	AAGTCGAGCGAATGGATTGAGAGCTTGCTCTCAAGAAGTTAGCGGCGGACG
21. NR_114581.1 Bacillus thuringlensis strain ATCC 10792 16S ribosomal RNA partial sequence	ATGA	ACGO	TG	GCGG	CGT	GCC	TAATACATGCAAGTCGAGCGAATGGATTGAGAGCTIGCTCTCAAGAAGTTA
22. NR_112780.1 Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA partial sequence	GCCC	CGT	CC	TAAT	ACA	TGC	AAGIC DAGC GAAIGGAIIGAGAGC IIGC ICICAAGAAGIIAGC GGC GGAC G
23. NR_157731.1 Bacilius mobilis strain MCCC 1A05942 16S ribosomal RNA partial sequence	CTGO	CTC,	GG	TGA	ACG	CTG	GCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTGAGAGCTTGCTCT
24. NR_113996.1 Bacillus mycoides strain NBRC 101238 16S ribosomal RNA partial sequence	GCGG	CGT	CC	TAAT	ACA	TQC	AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
25. NR_113990.1 Bacilius mycoides strain NBRC 101228 165 ribosomal RNA partial sequence	10000	CGT	CC	AAT	ACA	TGC	AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACG
26. NR_115993.1 Bacillus mycoides strain ATCC 6462 16S ribosomal RNA partial sequence	CGAO	CGA	TG	GATT	AAG	AGC	TIGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTA
27. NR_036880.1 Bacillus mycoides strain 273 16S ribosomal RNA partial sequence	CGTO	CCT	AAT	ACAT	GCA	AGT	C G A G C G A A T G G A T T A A G A G C T T G C T C T T A T G A A G T T A G C G G C G G A C G G G T G
28. NR_024697.1 Bacilius mycoides strain DSM 11821 16S ribosomal RNA partial sequence	CAGO	ATG	AC	GCTG	GCG	GCG	I G C C T A A T A C A T G C A A G T C G A G C G A A T G G A T T A A G A G C T T G C T C T T A T G A A
29. NR_148248.1 Bacillus bingmayongensis strain FJAT-13831.16S ribosomal RNA partial sequence	TGGA	TIA	GA	GCTT	GCT	CTT	ATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAG
30. NR_041248.1 Bacillus anthracis strain ATCC 14578 16S ribosomal RNA partial sequence	TIAT	GAA	TT	AGCG	GCG	GAC	GGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGA
31. NR_114422.1 Bacillus pseudomycoides 16S ribosomal RNA partial sequence	CCTC	GCT	AG	GATO	AAC	GCT	OGCOGCOTOCCTAATACTTOCAAOTCGAGCGAATGGATTAAGAGCTTGCTC
Site # 82 🕢 with 🕐 w/o gaps Editing	enabled						Selected genetic code: Standard

Fig 13: MSA of 16SrRNA sequence of the isolate yielded significant hits for Genus Bacillus with highest similarity to Bacillus paramycoides



Fig 14: Phylogenetic tree construction in MEGA X using N-J method

Based on colony characterization and 16S rRNA sequencing the isolate was found to belong to *Bacillus cereus* group further it with 99.85 % confirmed similarity to *Bacillus paramycoides* (Fig.13,14).

#### Discussion

Most herbivorous insect intestines contain various cellulose and other biopolymer degrading symbiotic microorganisms (Mannesmann 1972). Insect diet profoundly affects gut microflora and gut microbes can adapt remarkably to changes in insect diet (Kaufman and Klug 1991; Santo Domingo *et al.*) 1998). Occurrence of *Bacillus cereus* group members in the gut of insects like grasshoppers may be co-related to their herbivorous diet, since *Bacillus cereus* group is commonly found in soil, on vegetables and even processed foods <sup>[23]</sup>. *Bacillus paramycoides* hydrolyses starch, skimmed milk and casein <sup>[20]</sup>. This indicates the possible role of *B. paramycoides* in metabolism of starch and cellulose by secretion of extracellular amylases in *X. humilis* gut <sup>[20]</sup>.

#### Conclusion

Genomic analysis of entire gut microbiome of Xenocatantops

*humilis* will definitely lead to development of novel biocontrol strategies (bio-pesticides) and pest control.

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\_vegetables\_and\_weeds\_be\_used\_to\_farm\_crickets\_and\_ grasshoppers\_in\_Singap

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