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Research Article

Biochemical and molecular diversity analysis of culturable bacteria in *Cotesia plutellae* (Kurdjumov)[1] (Hymenoptera: Braconidae), a parasitoid of diamondback moth, *Plutella xylostella* (Linnaeus)

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ABSTRACT: Geographical populations of *Cotesia plutellae*, a predominant endolarval parasitoid of the diamondback moth, *Plutella xylostella* (Linnaeus) were screened and analyzed for bacterial diversity. The culturable bacterial species were isolated and identified by sequence analysis of 16S rRNA gene. Eleven bacterial isolates were identified *viz., Pseudomonas* sp., *Enterobacter cancerogenus, Bacillus* spp., *Pseudomonas putida, Pantoea agglomerans, Bacillus thuringiensis, Pantoea* sp. and *Bacillus cereus.* The molecular characterization and phylogenetic analysis placed these phylotypes into two major classes *i.e.* Bacilli and Gamma proteobacteria. The evolutionary distance matrix (Pairwise distance) showed similarity between the sequences. The bacterial diversity observed was low in the different geographic populations. The nucleotide sequences were blasted and submitted to GenBank.

[1]Synomously referrred to as *Cotesia vestalis* (Haliday).

KEY WORDS: Bacteria, *Cotesia plutellae,* 16S rRNA, symbiont

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INTRODUCTION

The insects system represents a large source of microbial diversity. Parasitoids have developed a natural arsenal and also a number of physiological mechanisms so as to enable them to successfully colonize the host and regulate host development to their own benefit. One of these is through symbiotic association. The symbiotic associations of these microbes inflict various types of metabolic, physiological and reproductive alterations. Their role in pesticide degradation, vitamin synthesis, pheromone production and pathogen prevention has been documented (Wigglesworth, 1929; Breznak, 1982; Campbell, 1990; Dillon and Dillon, 2004).

The diamondback moth (DBM), *Plutella xylostella* (Linn.) is a considered as the major pest of brassicas (cabbage, cauliflower, radish, khol-rabi, turnip, beetroot, mustard and amaranthus) worldwide. In India, *P. xylostella* is of national significance and yield losses to the tune of 80-95% have been reported (Dhumale *et al*., 2009). Cost of managing *P. xylostella* was estimated globally at US$ 1 billion in 1992 (Talekar and Shelton, 1993). Resistance of this noxious pest to all groups of insecticides had warranted an alternate strategy for its management and biological control methods have gained importance (Kranthi *et al.,* 2001).

Among them, use of *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae), a solitary larval endoparasitoid of DMB parasitizing up to 72% of the larvae is the most important. The rates of parasitism are highly variable geographically. Several biotic stresses influence the efficiency of the parasitoids. These include the pesticide applications on the host and their toxicity, host induced plant odours, competitive interaction between the different parasitoids / hyper parasitism, reproductive isolation (in compatibilities) and associated endosymbionts. Endo-symbionts are known to play role in the metabolism, physiology and reproduction of their host (Consoli *et al*., 2006, Iturbe and O'Neill, 2007**,** Rattan *et al.,* 2011). Geographic populations of the parasitoid may differ in their infection status of these symbionts and contribute to genetic divergence (David Bickford *et al*., 2006). Parasitoid strains differ in their propensity to search host species and parasitism depends on location specific strain and its adaptation to environment. The genetic divergence

influences the population dynamics of the parasitoid and the tritrophic interactions (Potting *et al.,* 1997; Christopher *et al.,* 2010). Therefore, characterization of the associated microflora in the parasitoids is necessary to assess the divergence or homology between the geographic populations, which influences their dynamics. In the present study molecular evidence for the presence of culturable bacterial gut flora through 16S rRNA gene markers and biochemical techniques were carried out to assess the diversity and discuss the rationality of their use in pest management programs.

MATERIALS AND METHODS

Collection and isolation of microflora from *Cotesia plutellae*

Population of the parasitoid were collected from different geographical locations of India, *viz.*, Delhi (29.61° N, 77.23°E), Anand (Gujarat) (22.55° N, 72.95° E), Rajahmundry (Andhra Pradesh) (16.98° N, 81.78VE), Nawashahar (Punjab) (31.21° N, 76.11° E), Shillong (Meghalaya) (25.56° N, 91.88° E), Solan (Himachal Pradesh) (30.90° N, 77.09° E), Palani (Tamil Nadu) (10.45° N, 77.51° E), Jorhat (Assam) (26.75°N, 94.22° E), Tirupati (Andhra Pradesh) (13.65° N, 79.42° E), Hoskote (Karnataka) (13.07° N, 77.80° E) and Bhubaneshwar (Orissa) (20.27° N, 85.84° E). The populations of *C. plutellae* were brought to NBAII laboratory in Bangalore and maintained on *P. xylostella* larvae reared on mustard seedlings raised in ice cream cups (55.2 x 8.9 x 45.1mm) containing vermiculite. The individual cups were placed in cages (24 x 24 x 24") to enable maintenance of populations.

Isolation of bacteria from *Cotesia plutellae*

Culture dependent method

The head, wing and thorax of adult *C. plutellae* was dissected out carefully, surface sterilized with 75% ethanol transferred to distilled water and homogenised for isolation of bacterial fauna. The homogenates were plated in Luria broth agar (LB) media. The colonies were selected based on colony characters (involving colony size, shape, colour and margin) and morphology of isolate based on Gram's staining technique. The isolates were sub-cultured by streaking in LB agar media**.**

Identification of bacterial isolate

Bacterial genomic DNA was isolated using Qiagen kit. The 16S rRNA gene was amplified using 16S universal primers (16S F 5'-ACTCCTACGGGAGGCAGCAG-3'and 16S R 5'-ATTACCGCGGCTGCTGG-3'). PCR protocol consisted of an initial denaturation at 95°C for 1 min. followed by 30 cycles at 95°C (1 min), Annealing at 55°C (1 min) and 72°C (2 min) and a final extension step at 72°C for 2 min. The PCR product was verified by running samples on a 1.5% agarose gel.

Screening of isolates on the basis of antibiotic sensitivity assay

Bacterial isolates were grown individually in Luria broth (LB) at 37°C, 200 rpm for 24h and hundred micro liter of culture was spread on Nutrient agar plates. Each isolate was tested against 6 different antibiotic discs of known concentrations. Ampicillin 25 mcg, penicillin G 50 mcg, rifampicin 15 mcg, wtreptomycin10 mcg, tetracyclin 10 mcg and chlorempenicol 10 mcg were placed on Nutrient agar (NA) plates at the rate of 2 discs per plate. Plates were incubated overnight at 35°C. Zone of inhibition was measured (diameter in mm) to segregate the isolates.

Identification based on metabolic characteristics

Different isolates were inoculated individually onto media such as LB agar (as control), casein hydrolysate (1%), and starch (1%), to identify their abilities to produce the enzymes amylase and protease. All the plates were incubated at 37°C for 24h. The production of these enzymes was checked by observing for a clear zone around each bacterial isolate. Coomassie staining was done on plates containing casein hydrolysate for the detection of protease activity. Similarly, for starch the clear zone was observed by staining the plates with iodine solution**.** Voges–Proskauer (VP) test and methyl red (MR) test were done for all the isolates and the development of red color indicated positive and yellow or brown color confirmed the negative.

Phylogenetic analysis of 16S rRNA gene library

All reference sequences were obtained from the GenBank and similarity between query sequence and the GenBank data base was assessed using online Blast search (Madden *et al.,* 1996). Phylogenetic tree was constructed using Maximum-Likelihood tree MEGA-5 bioinformatics tool and the genetic relatedness between the isolates was analyzed. The accuracy of the phylogeny was tested by bootstrap analysis using 500 iterations and the constructed tree was visualized with Tree viewer program. All the sequences were submitted to the Gene bank data base (Table 3). The gamma distribution shape parameters and substitution rates were used in phylogenetic analysis. The evolutionary distance matrix was analyzed by nucleotide pair wise distance calculation using Maximum Composite Likelihood Model of MEGA-5 bioinformatics software.

RESULTS AND DISCUSSION

Biochemical and molecular characterization for identification of bacterial isolates

The bacterial cultures obtained from the different field collected populations of *C. plutellae,* upon plating were subjected for Gram's staining technique. The isolates contained both Gram positive bacteria: *Bacillus* sp., *Bacillus cereus, Bacillus thuringieinsis,* and Gram negative bacteria: *Enterobacter cancerogenus, Pseudomonas* sp., *Pantoea* sp., *Pseudomonas putida, Pantoea agglomerans* (Table 1). Based on their differential sensitivity to the various antibiotics screened, the microbial isolates were further isolated (Table 2). *E. cancerogenus, Pantoea agglomerans, Pseudomonas* sp. and *Pseudomonas putida* gave positive result for VP test and negative for MR test and all others were negative for both MR-VP tests.

The ability of these bacterial isolates to solublize the various substrates such as starch, and casein were also variable; *Bacillus* strains were among the high amylase and protease producers, whereas *P. agglomerans* and *Pantoea sp*. showed amylase activity. In few of the bacterial isolates, based on the zone of hydrolysis, no activity was observed (Table 1). Hence, for further confirmation of physiological, biochemical and molecular characterization were performed. Eleven bacterial species were isolated and identified, *viz.*, *Enterobacter cancerogenus, Bacillus* sp., *Pseudomonas* sp., *Pantoea agglomerans, Bacillus* spp., *Bacillus thuringiensis, Bacillus cerus, Pantoea* sp. and *Pseudomonas putida*. The list of isolated microflora from the different regions of India is given in the Table 3.

Phylogenetic analysis of 16S rRNA sequences

Eleven bacterial isolates by culture dependent methods were identified by 16S rRNA sequencing, from the different geographical populations of the *C. plutellae* (Fig. 1). Phylogenetic tree placed the 11 different bacterial isolates, with their closest matches based on 16S rRNA gene. The bacterial strains obtained were *Enterobacter* sp., *Bacillus* spp., *Pseudomonas* sp., *Bacillus thuringiensis, Bacillus cereus, Pantoea* sp., *Pseudomonas putida,* and *Pantoea agglomerans* (Fig. 2). Sequences with more than 97% similarity were considered to be of the same OTUs (operational taxonomic unit). Bacterial phylotypes are

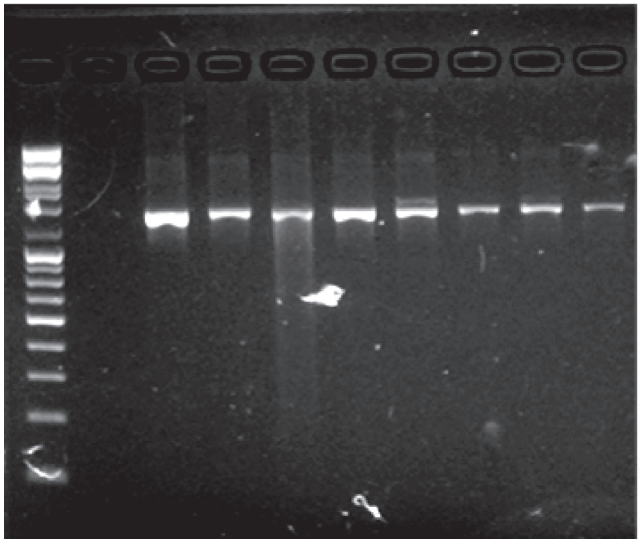

Fig. 1: Agarose gel electrophoresis of 16S rRNA gene of eight bacterial isolates from *Cotesia plutellae* Lane M –Marker (100-3000bp), Lane 1 – 8 (1000bp) PCR amplicons from 8 bacterial isolate

Table 1: Biochemical characteristics of the bacterial isolates from *Cotesia plutellae*

Strain	Gram staining	Starch hydrolysis test	Hydrolysis of casein	Methyl red test	Voges-Proskauer test
CpG-13	-ve, rods	–	–	–	+
CpR-12	+ve, rods	+	+	–	–
Cp-B.sp2	+ve, rods	+	+	–	–
Cp-B.sp1	+ve, rods	+	+	–	–
Cp-Bt	+ve, rods	+	–	–	–
Cp-Pt.a	-ve, rods	+	–	–	+
Cp-Bs.sp3	+ve, rods	+	+	–	–
Cp-pt.a1	-ve, rods	+	–	–	–
Cp-Ps.sp	-ve, rods	–	–	–	+
Cp-Ps.pt1	-ve, rods	–	–	–	+
Cp-BC	+ve, rods	+	–	–	–

Table 2: Antibiotic sensitivity assay of microbial strains isolated from *Cotesia plutellae*

Sl. No.	Strain ID.	Antibiotics					
		Ampicillin	Pencillin	Chlorempenicol	Rifampicin	Streptomycin	Tetracyclin
		25 mcg	50 mcg	10 mcg	15 mcg	10 mcg	10 mcg
	Cotesia plutellae	Zone of inhibition in (mm)					
1	Cp-Ps.sp	10	–	32	–	–	–
2	CpG-13	16	–	–	–	–	–
3	CpR-12	–	–	–	–	–	–
4	Cp-B.sp2	–	–	–	–	–	–
5	Cp-Pt.a	32	10	30	20	4	32
6	Cp-B.sp1	–	14	–	–	18	30
7	Cp-Bt	12	–	24	–	10	–
8	Cp-Bs.sp3	30	10	–	20	10	–
9	Cp-Pt.a1	16	10	34	20	10	30
10	Cp-Ps.pt1	10		24	–	–	–
11	Cp-BC	–	–	–	–	14	–

Table 3. Culturable bacteria isolated and identified in the various population of *Cotesia plutellae* using 16S rRNA gene

Strain ID	Bacteria	Place of collection	Genbank Acc. No
Cp-Ps.sp	*Pseudomonas* sp.	Shillong	KC441059
CpG-13	*Enterobacter cancerogenus*	Anand (Gujarat)	KC139361
CpR-12	*Bacillus* sp.	Rajahmundry (Andhra Pradesh)	KC139360
Cp-B.sp1	*Bacillus* sp.	Nawanshahr (Punjab)	KC512245
Cp-Pt.a	*Pantoea agglomerans*	Palani (Tamil Nadu)	KC512244
Cp-b.sp2	*Bacillus* sp.	Solan (Himachal Pradesh)	KC512246
Cp-Bt	*Bacillus thuringiensis*	Jorhat (Assam)	KC512243
Cp-Bc	*Bacillus cereus*	Delhi	KC582828
Cp-Pt.a1	*Pantoea* sp.	Bhubaneshwar (Orissa)	KC582827
Cp-Ps.pt1	*Pseudomonas putida*	Hoskote (Karnataka)	KC589741
Cp-Bs.sp3	*Bacillus* sp.	Tirupati (Andhra Pradesh)	KC582829

placed into two major classes *i.e.* Bacilli (54.54%) and Gammaproteobacteria (45.45%). Genus *Bacilli* was most abundant amongst all (54.54%) followed by genus *Pantoea, Pseudomonas* (18.18%) and genus *Enterobacter* with 9% (Fig. 3). The analysis involved 11 nucleotide sequences. The evolutionary distance was calculated using pairwise distance matrix and the evolutionary distance between the sequences was calculated by computing the proportion of nucleotide differences between each pair of sequences. There was a total of 270 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

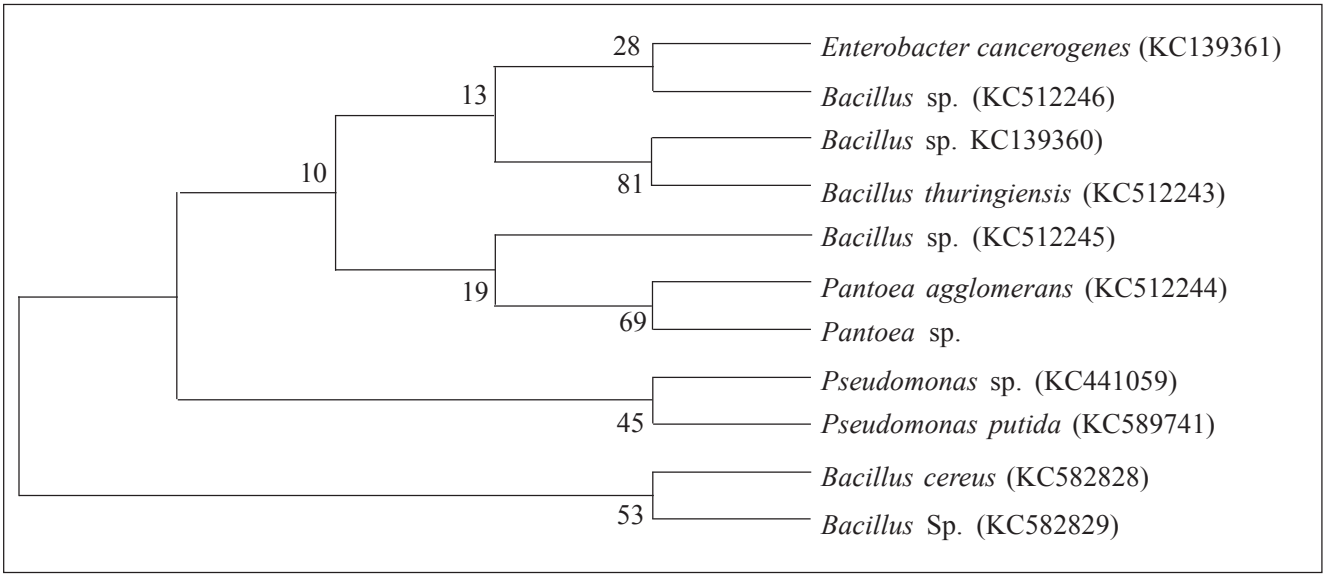

Fig. 2. Phylogenetic tree constructed for partial 16S rRNA gene of bacterial isolates cultured from field-collected *Cotesia plutellae*

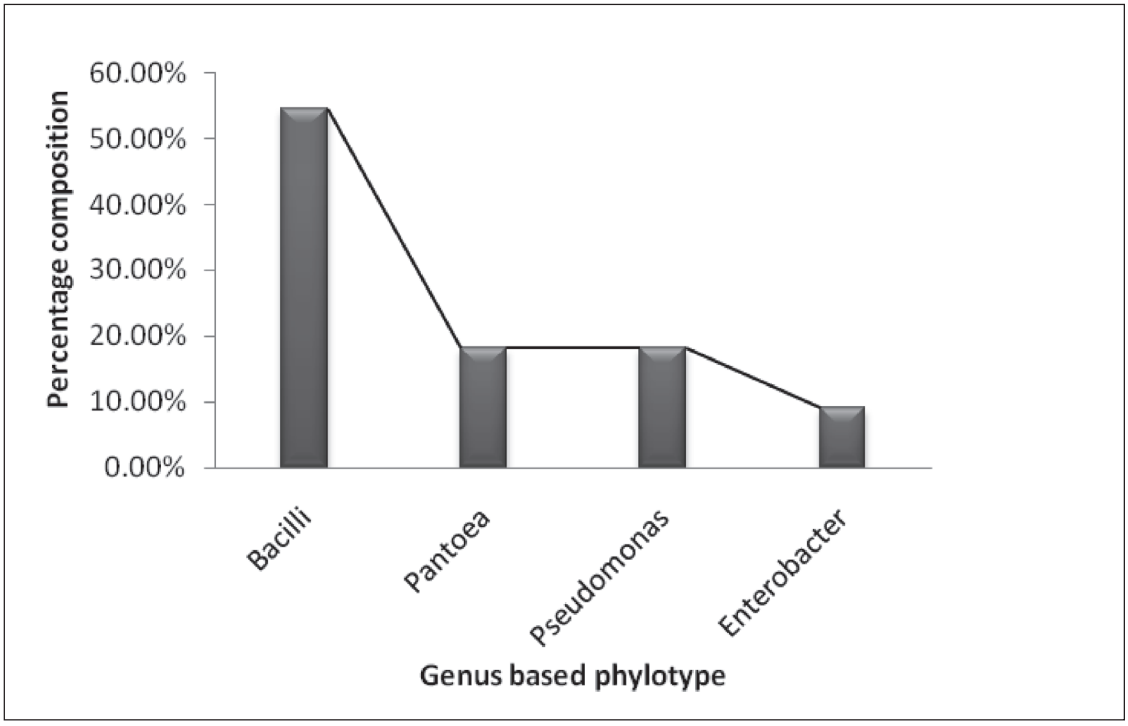

Fig. 3: Percentage composition of bacteria in *Cotesia plutellae*

In the present study, bacterial diversity in the different geographical populations of *C. plutellae* was observed. However, the variations were not significant as remarked by the constructed phylogenetic tree and distance matrix. *C. plutellae* from Rajahmundry, Solan, Nawanshahar, Jorhat, Tirupati and Delhi were diversified with *Bacillus* sp., *Bacillus cereus* and *Bacillus thuringiensis* where as the populations from Anand, Bhubaneshwar and Palani harbored *Pantoea* sp., *Enterobacter* sp. and *Pantoea agglomerans*. Shillong and Hoskote (Karnataka) populations diversified with *Pseudomonas* sp. and *Pseudomonas putida*. The phylogenic tree showed that genera *Enterobacter* and *Bacillus* were closely related. Genera *Bacillus* and *Pantoea* were closely related. Similarly genera *Bacillus* and *Pseudomonas* were related to each. The evolutionary distance matrix of the bacterial flora analyzed from *C. plutellae* is shown in (Table 4).

The bacterial isolates identified in the present study were commonly found in most of the insect species. Thakur *et al.,* (2005) have isolated *Bacillus* sp., *Pseudomonas* sp. from the gut of the *Discladispa armigera* (Olivier). *Bacillus* sp. and *Pseudomonas* sp. also occurred in the gut of larvae of *H. armigera* (Madhusudan, 2011). Our results on the gut microbials corroborate with the reports of these workers.

Bacteria are known to be ubiquitous, living in nearly all environments (Martin and Kukor 1984). Chapman (1990) has reported that the most commonly occurring microorganisms in insects are bacteria and bacterium-like organisms. The occurrence of gut bacteria in the different populations may prove to be useful for biological manipulations of the parasitoid, since they are known to play role in the metabolism and physiology of the host (Martin and Kukhr 1984, Kehinde 2011). These bacteria can drive allelic genes through inheritance among the populations. They can be used as vectors for spreading desirable genetic modifications in pest populations or to enhance productivity of the parasitoid (Consoli and Elliot, 2006). Rapid advances in DNA based technologies have expanded the range of possibilities for utilization of these bacterial symbionts for such long term goal. Sequencing the bacterial genome facilitates understanding the relationship between the parasitoids and their symbionts, to comprehend the genome interdependence that occurs between host and the bacteria (Doughlas, 1994; Girin and Bouletreau, 1995).

Symbiosis results in a reduction in bacterial lineages that loose preferentially genes involved in catabolic pathways. Therefore, infections can be manipulated by elimination, transfection or genetic modification. Our studies indicated the occurrence of bacteria reported to play role in the fitness benefits of the parasitoid corroborating with the reports of earlier researchers (Consoli *et al*., 2006; Mochiah *et al*., 2002; Mandrioli, 2009). The information would add to the existing knowledge on the parasitoid for its effective exploitation in the management of diamondback moth.

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Table 4. Evolutionary distance matrix for bacterial isolates of *Cotesia plutellae*

Organisms	1	2	3	4	5	6	7	8	9	10
*Pseudomonas*_sp										
Enterobacter_cancerogenus	0.730									
*Bacillus*_sp1	0.756	0.715								
*Bacillus*_sp2	0.741	0.700	0.737							
Pantoea_agglomerans	0.763	0.756	0.733	0.778						
*Bacillus*_sp	0.781	0.704	0.774	0.696	0.719					
Bacillus_thuringiensis	0.756	0.741	0.767	0.756	0.756	0.559				
*Pantoea*_sp	0.763	0.726	0.741	0.744	0.681	0.730	0.752			
Pseudomonas_putida	0.707	0.789	0.730	0.752	0.759	0.719	0.730	0.756		
Bacillus_cereus	0.744	0.733	0.763	0.756	0.770	0.770	0.770	0.778	0.778	
*Bacillus*_sp3	0.752	0.756	0.770	0.756	0.763	0.770	0.804	0.744	0.770	0.730

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