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Development of DNA barcodes for Natural enemies of cole crops insect pests in mid-hills of Meghalaya

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Abstract
Significant morphological similarities within or in between species of insects has made reliable taxonomic identification difficult. DNA barcoding has appeared to be a useful tool in resolving the issues related to the identification of taxonomically difficult insect species. The North eastern region of India is one of the mega biodiversity hotspots in the world and the climatic conditions of the region are highly conducive for reproduction and multiplication of insects. The efforts were undertaken to study the biodiversity of insects and to develop molecular database by developing DNA barcodes of natural enemies of cole crops insect pests in mid hills of Meghalaya. Limited information is available on natural enemies in cole crops ecosystem in mid –hills of Meghalaya. A total of 17 natural enemies belonging to three insect orders *viz*., Coleoptera (4) Predator, Hymenoptera (5) (1 nymphal/pupal; 3 Larval parasitoid; 1 Pupal parasitoid), Diptera (8) (5 Predator, 3 pupal parasitoid) Hemiptera (2) were documented during the year 2014-2015. Natural enemies especially Predators of aphids *viz.*, *Coccinellid* beetles, Syrphid flies were recorded, Parasitoids observed were; nymphal/adult Parasitoid of aphids, *Diaeretiella rapae*, larval parasitoid of cabbage butterfly, *Hyposoter* sp and *Cotesia glomerata* and Pupal parasitoid including *Brachymeria femorata* and *Tachinidae* sp. The collected species were identified by established taxonomic keys, by taxonomists and/or molecular basis. DNA was successfully extracted from multiple specimens of 17 insect species and molecular assays were also undertaken for presence of *Wolbachia* infection. The *Wolbachia* infected specimens were discarded and not used for further analysis. The DNA barcodes were successfully developed for 17 species by sequencing partial Cytochrome oxidase I (COI) gene of mitochondrial DNA. The molecular identity of the insect species was established through BLAST-n at NCBI. The total nucleotide length of barcodes varied from species to species (501bp to 682bp). All the analysed sequences were submitted to National Centre for Biotechnology Information (NCBI) and Accession numbers were obtained (KT175578 to KT 175606). The comprehensive taxonomical and molecular database developed in this study for a total of 17 species observed in cole crop ecosystem could be used as diagnostic guide at both morphological and molecular level.

Keywords: COI, DNA barcoding, biodiversity, parasitoid, predator and mtDNA

Introduction
Cole crops, the most abundantly consumed vegetables in the world belonging to the family Brassicaceae comprises about 380 genera and over 3000 species of cultivated and wild plants that have almost similar insect pest complex (Heywood, 1993) [15]. Throughout the world, a total of 51 insect pests species (Maison, 1965) [19] and a total of 37 insect pest species from India have been reported to feed on cruciferous crops (Lal, 1975) [16]. The enormous yield and economic losses in *Brassica* crop production every year caused by insects is a threat to global agriculture. Sometimes the yield loss by insects reaches as high as 60-70% and a report has been made that Indian agriculture is currently suffering an annual loss of about ₹ 86.39 million due to insect pests (Dhaliwal *et al*. 2007) [7]. On an average 25-30% yield loss in vegetables worldwide is caused by insect pests (Reddy and Zehr, 2004) [23]. Besides insect pests, several natural enemies also harbored in cole crops ecosystems. They play a key role in reducing the pest populations below those causing economic injury level. Most of the natural enemies (predators, parasitoid and pathogens) available in the agricultural and urban environments are naturally occuring, which provide excellent regulation of many pests with little or no assistance from human.

Significant morphological similarities within or in between species of insects had made reliable taxonomic identification difficult. DNA barcoding has appeared to be a useful tool in resolving the issues related to the identification of taxonomically difficult insect species. The efforts were undertaken to study the biodiversity of natural enemies and to develop molecular

database by developing DNA barcodes of natural enemies of insect pests of cole crops in mid hills of Meghalaya.

North east India, considered as one of the mega biodiversity hot spots has a predominantly humid sub-tropical climate with hot, humid summers, severe monsoons and mild winters. Meghalaya is a part of North Eastern Himalayas and it is a land-locked territory with a geographical area of 22 429 km^2, lying between 25° 47' and 26° 10' N latitude, and 89° 45' and 92° 47' E longitude, exceptionally rich in biodiversity of insect pests and their natural enemies. Vegetable cultivation is an important part of economy in north eastern states of India including Meghalaya where the geo-climatic condition offers an excellent scope for growing different types of horticultural crops including Cole crops which is a major component of diet in this region. Among the many challenges in sustaining crop productivity and nutritional security, direct and indirect damages by insect pests is of paramount importance. Firake *et al*. (2012) [8] reported about 21 natural enemies in cruciferous ecosystems of Meghalaya and *Hyposoter ebeninus* (Grav.) (Hymenoptera: Ichneumonidae) and *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) are the two most important larval parasitoid of *P. brassicae* naturally controlling more than 70% larvae of *P. brassicae* during peak season.

Till date no comprehensive information is available on molecular characterization and/or DNA barcoding, especially of natural enemies of cole crop ecosystem of India or Meghalaya although some studies have been done. Many more unidentified insect species might have been harboring under cole crop ecosystem in this region. Accurate identification of already identified species is also is an issue as evidenced in different species of cabbage white butterfly (*Pieris brassicae*, *P. napae*, *P. rapae* and *P. canidia*) (Pachuau *et al.,* 2012) [22]. Despite of this fact, the comprehensive information on morphological and molecular data on cole crops pest complex is lacking. Therefore, major aim of this study is to develop molecular data by developing DNA barcodes of natural enemies of cole crops ecosystem in mid hills of Meghalaya. The details DNA data base on natural enemies of cole crop ecosystem would be very useful and could be share with other research community and quarantine agencies across the globe.

Materials and Methods

Location and Site

Studies on "Development of DNA barcodes for natural enemies of major insect pests of cole crops ecosystem in mid-altitude of Meghalaya" was carried out during 2014-15 in the IPM and Insect Molecular Biology laboratories of Entomology section of Crop Protection Division, ICAR Research Complex for North Eastern Hill (NEH) Region, Umiam, Meghalaya. The institute is situated at Umiam (Barapani), 25º41'-21" North latitude and 91º55'-25" East longitude having an elevation of 1010 m above the msl. The climatic condition in this area is of mid tropical zone, with an average annual rainfall of 2810 mm with maximum temperature range of 20.9^{0}C to 27.4^{0}C and minimum temperature from 6.7^{0}C to 18.1^{0}C. The biodiversity of natural enemies of cole crops in this area was observed during the experimental period.

Sample collection

Specimens (Maximum 10 each) were collected from two multiple experimental plots of cole crops at two different locations *viz.,* Entomology experimental farms of Entomology Section and Horticulture Division of ICAR Research Complex for North East Hill Region, Umiam (Barapani), Meghalaya during October 2014 to March 2015 (Table 1). The collected samples were cleaned and placed in > 95% ethanol in individual 1.5mL Eppendorf tubes until genomic (gDNA) extraction.

Species identification

Preliminary identification was done based on established taxonomic key or by matching the characters with identified species in Insect Museum of Entomology section of Crop Protection Division, ICAR research complex for NEH Region, Umiam, Meghalaya. Some samples which were not identified by established taxonomic keys were also sent to IARI (Indian Agricultural Research Institute) and NBAIR (National Bureau of Agricultural Insect Resources), Bengaluru. All the Syrphid species reported in this studies were identified personally by Dr. Kumar Ghorpade, an eminent taxonomist (Diptera) during his visit to entomology section.

Isolation of genomic DNA

Total genomic DNA was extracted from all the specimens used in this study by modified phenol: Chloroform method developed by Behere *et al*., 2007. [3]

The specimens were removed from the individual ethanol vials and allowed to dry in on sterilized blotting paper for an hour at ambient temperature. DNA was extracted from either a single leg or antennae (in case of large insect) and whole insect (in case of small insects) in two separate batches for each species along with a negative control; thereby any chance of cross contamination during Gdna extraction could be detected through the inclusion of blank extraction. The DNA and additional two insect voucher specimens of each identified species have been deposited and preserved at insect museum of Entomology section, ICAR Research Complex for NEH, Region, Umiam, India.

Determination of *Wolbachia* (Bacterial symbiont) infection

All specimens of identified species were checked for the presence of *Wolbachia* infection by using *Wolbachia* gene specific primers: WOL 16S & WOL 16R (O'Neill *et al*., 1992) [21] and WSP 81F& WSP 96R (Zhou *et al*., 1998) [30] synthesized by Chromous Biotech Pvt. Ltd. Bengaluru, India were used for detection of *Wolbachia* infection in test insects in this study. For the determination of *Wolbachia*, PCR composition and profile as described in Murthy *et al*., (2011) was used.

PCR and sequencing

A partial 709bp cytochrome oxidase I (COI) gene of mtDNA was PCR amplified by using universal primers; LCO-1490 (forward) and HCO-2198 (Reverse) of (Folmer *et al.* 1994) [10]. The PCR (Polymerase chain reaction) amplifications were carried out in thermal cycler (Eppendorf, India) to test the amplifications of all the samples with two standard DNA barcoding primers. The PCR reaction was carried out in a total of 10µl volume. PCR profile consist of an initial denaturation at 94°C for 2 min, followed by 5 cycles of denaturation at 94°C for 30seconds, annealing at 45°C for 40 seconds and extension for 1 min at 72°C, again followed by 35 cycles of denaturation at 94°C for 30seconds, annealing at 51°C for 40 seconds and extension for 1 min at 72°C. A final extension was allowed for 10 min at 72°C. After the completion of the PCR reactions the amplified products were allowed to hold at 10^{0}C for 1 hrs and then stored in -20°C.

The PCR products were electrophoresed in agarose gel electrophoresis stained with 2µl ethidium bromide and visualized on a UV trans-illuminator. For sequencing, separate PCR reactions were carried out in a total volume of 50µl with LepF1/LepR1 primer and for some samples which were failed to amplify with LepF1 and LepR1 were amplified with LCO and HCO primers. The PCR profile was similar as described above. After completion of PCR amplification only 10µl of each PCR product was used for gel electrophoresis and documentation. A remaining 40µl Post-PCR product of each species was transferred into 1.5ml sterilized Eppendorf tubes and the tubes were packed properly and sent for sequencing in frozen conditions to M/S Xcelris Pvt. Ltd, Ahmedabad. The nucleotide composition especially AT% and GC% for all the species was determined in Clustal W. The AT and GC% was also determined for first, second and third positions of codon in partial COI gene. For each species a total 2 to 4 samples were sequenced. Each species was bidirectionally sequenced from both the ends (5' and 3').

Sequence analyses

All the sequenced were analyzed in sequence analysis software Staden Package (Staden *et al*., 2000) [27]. The sequencing analysis was carried of multiple samples of individual species. During sequencing analyses all the sequences were also checked manually within the software for accuracy. The messy 5' and 3' ends of sequences were trimmed for all the sequences. All sequences were also checked for Open Frame Reading (OFR), and protein translation was carried out by using invertebrates genetic code. Multiple amino acid sequence alignment of all identified species was performed in Clustal X.

Phylogenetic analysis

The evolutionary relationships/Phylogenetic analysis between different identified species of natural enemies in cole crop ecosystem were also carried out. The evolutionary history was inferred using Maximum Parsimony (MP) method by employing 1000 bootstraps replicates. Out of three trees generated the most parsimonious consensus tree was selected. The Maximum Parsimony tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the final tress were obtained by the random addition of sequences (10 replicates). The tree was generated based on amino acid sequences of a total of 17 species. Evolutionary analyses were conducted in MEGA6.

Results and Discussion

Biodiversity of natural enemies in cole crops ecosystem of Meghalaya

The North-Eastern Himalayan region of India is exceptionally rich in terms of flora and fauna and is also considered to be one of the mega biodiversity hotspot in the World (Mayer *et al*., 2000) [20]. In present investigation, a total of 17 insect Pest species were collected, identified and documented from cole crops ecosystem during the year 2014-2015 in mid altitude of Meghalaya. (Table 2). Out of the 17 species collected, nine species were predators consisting of four coccinellids and five hover/syrphid fly. Coccinellids were *Coccinella septempunctata*, *C. transversalis*, *Micraspis* sp and *Oenopia* sp (Table 2: Plate 1-4). Predatory hover fly Syrphid fly were; *Episyrphus viridaureus*, *Melanostoma orientale*, *Sphaerophoria macrogaster*, *Eristalis cerealis* and *Macro syrphus confrator* (Table 2: Plate 5-9). Among 17 natural enemies, eight were parasitoids and out of eight parasitoids, three were larval parasitoid (*Hyposoter* sp., *C. glomerata* and *Cotesia* sp) (Table 2: Plate 10-12) and five were pupal parasitoids, (*D. rapae*, *B. femorata*, *Tachinidae* sp1, *Tachinidae* sp2 and *Tachina* sp) (Table 2: Plate 13-17). The population of natural enemies was very high in cole crops ecosystem in this region. Lal and Chandra (1976) [17] recorded five larval and three pupal parasites attacking P. brassicae in cole crops ecosystem in India. Bhat and Bhagat (2009) [4] studied the biodiversity of natural enemies in cole crops and reported three potential hymenopteran parasitoids *viz*., *H. ebeninus*, *C. glomerata* and *B. femorata* OD cabbage butterflies from Kashmir valley of India. The natural enemies reported and identified in present investigation on cole crops were further supported by findings of Firake *et al*. (2012) [9] where authors observed similar kind of natural enemies in Brassica crops of Meghalaya. Firake *et al*. (2012) [9] reported several natural enemies especially, Predators of aphids, Diaeretiella rapae, larval parasitoids of cabbage butterfly, Hyposoter ebeninus and Cotesia glomerata, Exorista larvarum, Exorista bombycis, Pteromalus puparum, etc in cole crops ecosystems of Meghalaya. Among the parasitoids attacking this pests, *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) and Hyposoter ebeninus (Gravenhorst) (Hymenoptera: Ichneumonidae) are the two most important endo-larval parasitoids, with both being widely distributed across the world (Lozan *et al*., 2008; Harvey *et al*., 2010) [18]. As observed in present investigation, predators like Coccinellids beetle and syrphid fly preying upon aphids and other soft bodies insects were abundant throughtout the season. Sathe and Bhosale (2001) [24] reported 21 species of coccinelids beetles feeding on aphids and several soft bodies' homopterous pests of agricultural and forest plants from Maharashtra States. Brunetti (1907, 1917) [5, 6] recorded a total of about 76 species (in 36 genera) of syrphidae from Himachal Pradesh, Ghorpade (1973, 1974) [12, 13] studied the fauna of Bangalore and reported 20 species in 25 genera. Ghorpade *et al.* (2011) [11] also confirmed 16 species of 10 genera which were sampled from the Coromandel Coast of Andhra Pradesh, India. All the above mentioned studies, strongly supports findings reported in present investigation that coccinellids beetle and syrphid fly are most common predators of aphids which is indeed a major insect pests of cole crops.

The preliminary identification of the collected insect pests were established based on known taxonomic keys. The species/specimens which were difficult to identify based on known taxonomic keys were sent to ICAR-NBAIR, Bengaluru or IARI, New Delhi, India. Based on their taxonomic classification, the collected 17 specimens were classified under three major insect orders, Diptera (8), Coleoptera (4) and Hymenoptera (5). The images of the insect whenever possible were also documented and all these information are presented in Table 2.

Detection of *Wolbachia* (Bacterial symbiont) infection on natural enemies of insect pests.

Wolbachia are also transmitted horizontally between arthropod species (Avtzis *et al.*, 2014) [2]. As the determination of presence of *Wolbachia* infection is a pre requisite for any insect DNA barcoding research work. All species of insects analyzed in this study were found to be free from endosymbiont *Wolbachia* infection except for three species of predatory syrphids, belonging to family order Diptera in which all the specimens were tested with two different pairs of primers specific to Wolbachia genes**.** The

samples which were positive for presence of *Wolbachia* infection were discarded and were not used for further DNA barcoding work. It was interesting to note that *Wolbachia* infection was more especially in Hover Fly Syrphid flies *viz.*, *S. macrogaster*, *M. orientale* and *E. viridaureus*. As having *Wolbachia* infection in DNA was not a good indication, all the infected DNA samples were discarded. *Wolbachia* infection also manipulates the sequencing reactions and masks the extent of genetic variations in mitochondrial genes. The *Wolbachia* infection in study insects were detected by absence of band in PCR which used the *Wolbachia* gene specific primers (Table 3 and Fig 1).

Table 3: List of insect species found to be positive for *Wolbachia* infection.

Sl. no	DNA Code	Name of sample	Wol16SF/ Wol 16SR	Wsp1/ Wsp2
1	618	Sphaerophoria macrogaster	+ve	+ve
2	607	Melanostoma orientale	+ve	+ve
3	650	Episyrphus viridaureus	+ve	+ve

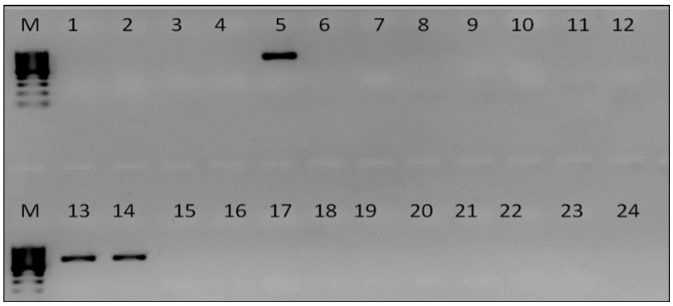

Fig 1: PCR amplification of Wolbachia gene specific primer WOL 16SF and WOL 16SR

Werren and Windsor, (2000) [28] reported that 15 to 75% of insect species harbor *Wolbachia*, it was detected in each of the major insect orders, including Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, and Orthoptera. The finding reported on *Wolbachia* in this study was further supported by Whitworth *et al.* (2007) [29], who observed that the patterns of mitochondrial variability was related to the spread of maternally transmitted bacteria that co-segregate with mitochondria. During the DNA barcoding of different species of bird blowfly *Protocalliphora*, a total of 12 species of *Protocalliphora* were found positive for *Wolbachia* infection (Whitworth *et al*., 2007) [29,] hence the barcoding was not possible for almost 60% species of *Protocalliphora*. Due to the *Wolbachia* infection DNA barcoding work not possible as this technique might underestimate the observed genetic variations in target species. Smith *et al.* (2005) [25, 26] analyzed more than 2 million insect COI trace files on the BOLD and reported that *Wolbachia* COI was present in 0.16% of the cases and concluded that the presence of the *Wolbachia.* DNA in total genomic extracts made from insects was unlikely to compromise the accuracy of the DNA barcode library and suggested that regular assays for *Wolbachia* presence should be undertaken for any DNA barcoding project. Considering and keeping in mind the above mentioned points, the samples which were positive for presence of *Wolbachia* infection were discarded and were not used for further DNA barcoding work as infection could manipulate the COI DNA barcoding results.

Sequencing Analysis and Submission of sequence to NCBI

The target region of COI gene was successfully amplified in all specimens, and the good quality sequences obtained. No INDELs (Insertion and deletions) were detected among the sequences, hence the sequence analysis and alignment was easily carried out. The molecular identity of all the specimens was successfully established through BLASTn search at National Centre for Biotechnology (NCBI), where all the 17 species shows 95-100% identity with reported sequences in the NCBI GenBank

DNA was successfully extracted from all the 17 species using modified Phenol: Chloroform method. The DNA barcodes based on partial COI gene for all the 17 species have been developed. The total length of DNA barcodes varied from species to species and it was ranged from 501 to 682 bp. The shortest good quality sequenced was obtained from *M. confrator* and the longest good quality final sequenced was obtained from *Hyposoter* sp. For others 15 species the final analyzed sequence length was always more than 600bp. All the 17 DNA barcodes have been deposited to NCBI and accession numbers were obtained for all of them. All analyzed sequences have been submitted to NCBI vide accession numbers KT175578 - KT175606. The nucleotide composition of all the 17 sequence was AT rich and no significant variations were observed in terms AT and GC% at first, second and third position of codon.

The evolutionary relationships/Phylogenetic analysis between different identified species of insect pests in cole crops ecosystem was inferred using Maximum Parsimony (MP) method. After 1000 bootstrap replications all the insect species group together as per their insect order

Table 1: Collection details of identified natural enemies during experimental season (2014-15)

Sl.no	Name of insect species	Order	Family	Date collection	Host	Location	No of specimens
1	Brachymeria femorata	Hymenoptera	Chalcididae	Nov. 2014	Pieris pupa	Ento. Field	10
2	Hyposoter sp	Hymenoptera	Ichneumonidae	Nov. 2014	*Pieris* sp	Ento. Field	10
3	Oenopia sp	Coleoptera	Coccinellidae	Nov. 2014	Aphids	Ento. Field	10
4	Micraspis sp	Coleoptera	Coccinellidae	Nov. 2014	Aphids	Ento. Field	10
5	Coccinella.transversalis	Coleoptera	Coccinellidae	Dec. 2014	Aphids	Ento. Field	10
6	Cocinella.septempunctata	Coleoptera	Coccinellidae	Dec. 2014	Aphids	Ento. Field	10
7	Tachinidae sp1	Diptera	Tachinidae	Jan. 2015	*Spodoptera* pupa	Ento. Field	10
8	Tachinidae sp2	Diptera	Tachinidae	Jan. 2015	*Spodoptera* pupa	Ento. Field	10
9	Tachina sp	Diptera	Tachinidae	Jan. 2015	Pupa	Ento. Field	10
10	Sphaerophoria macrogaster	Diptera	Syrphidae	Jan. 2015	Aphids	Ento. Field	10
11	Melanostoma orientale	Diptera	Syrphidae	Jan. 2015	Aphids	Ento Field	10
12	Diaeretiella rapae	Hymenoptera	Braconidae	Jan. 2015	Aphids	Ento. Field	10
13	Episyrphus viridaureus	Diptera	Syrphidae	Jan. 2015	Aphids	Ento. Field	10
14	Macrosyrphus confrator	Diptera	Syrphidae	Jan. 2015	Aphids	Ento. Field	10

15	Cotesia glomerata	Hymenoptera	Braconidae	Jan. 2015	*Pieris* sp	Ento. Field	10
16	Cotesia sp	Hymenoptera	Braconidae	Jan. 2015	*Pieris* sp	Ento. Field	10
17	Eristalis cerealis	Diptera	Syrphidae	Jan. 2015	Aphids	Ento. Field	10

Table 2: Image and Biodiversity of Natural enemies of cole crops insect pests in mid hills of Meghalaya

Sl.no	Scientific name	Status	Plate no.
1	Cocinella. septempunctata	Predator	1
2	Coccinella. transversalis	Predator	2
3	Micraspis sp	Predator	3
4	Oenopia sp	Predator	4
5	Episyrphus viridaureus	Predator	5
6	Melanostoma orientale	Predator	6
7	Sphaerophoria macrogaster	Predator	7
8	Eristalis cerealis	Predator	8
9	Macrosyrphus confrator	Predator	9
10	Hyposoter sp	Larval parasitoid	10
11	Cotesia glomerata	Larval parasitoid	11
12	Cotesia sp	Larval parasitoid	12
13	*Diaeretiella rapae*	Pupal parasitoid	13
14	Brachymeria femorata	Pupal parasitoid	14
15	Tachinidae sp1	Pupal parasitoid	15
16	Tachinidae sp2	Pupal parasitoid	16
17	Tachina sp	Pupal parasitoid	17

Plate 1: *Cocinella. septempunctata*

Plate 2: *Coccinella. transversalis*

Plate 3: *Micraspis* sp

Plate 4: *Oenopia* sp

Plate 5: *Episyrphus viridaureus*

Plate 6: *Melanostoma orientale*

Plate 7: *Sphaerophoria macrogaster*

Plate 8: *Eristalis cerealis*

Plate 9: *Macrosyrphus confrator*

Plate 10: *Hyposoter* sp

Plate 11: *Cotesia glomerata*

Plate 12: *Cotesia* sp

Plate 13: *Diaeretiella rapae*

Plate 14: *Brachymeria femorata*

Plate 15: *Tachinidae* sp1

Plate 16: *Tachinidae* sp2

Plate 17: *Tachina* sp

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Conclusions
In this study, we have analysed and documented only 17 natural enemies of major insect pests of cole crops, but there may be more species harboured in this region of India. Therefore, additional studies have to be undertaken to get a clear picture of insect pests diversity and the pest status in the region. The comprehensive data generated from present study would be useful in further understanding of the biodiversity of arthropod fauna associated with natural enemies of cole crops insect pest in other regions of the country and this study would certainly have implications for pest management, taxonomy, quarantine and trade and for development of diagnostic guide both at molecular and morphological level.

Disclosure statement
No potential conflict of interest was reported by the authors

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