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FEEDING POTENTIALITY OF *COCCINELLA SEPTEMPUNCTATA* ON THREE DIFFERENT APHID SPECIES AND ITS MOLECULAR IDENTIFICATION BASED ON MITOCHONDRIAL *COI* GENE SEQUENCE

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ABSTRACT
Predatory efficacy of coccinellid beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae) on three important aphid pests was studied in laboratory. The number of *A. craccivora, A. fabae and A. gossypii* consumed by 1st, 2nd, 3rd and 4th instar *C. septempunctata* larvae were 16.67 ± 0.58, 23.33 ± 1.53, 41.33 ± 0.58, and 151.67 ± 1.15; 13.67 ± 1.15, 22 ± 1.00, 40.67 ± 0.58 and 135.67 ± 1.15; and 12.33 ± 0.58, 21.66 ± 1.15, 39.67 ± 1.53 and 129.33 ± 2.51 respectively. Life cycle studies of *C. septempunctata* revealed that it took 28 ± 2.64, 24.33 ± 0.58 and 23 ± 1.73 days to complete its life cycle while reared on *A. craccivora, A. fabae and A. gossypii* respectively. The mitochondrial cytochrome c oxidase (*COI*) gene of the *C. septempunctata* were sequenced and submitted to the NCBI GenBank (Accession NO.MH 976795.1). The nucleotide composition analysis revealed that the value of A+T (69.3%) was greater than G+C (30.7%). The intraspecific genetic divergence ranged from 0.000 - 0.005. Haplotype analysis showed less genetic diversity (1 mutational step). Phylogenetic analysis by Maximum Likelihood method showed that all the *Coccinella* species originated from a common major clade and *C. septempunctata* originated from different regions of the world. The present research may be useful for attempting any biological control program against the aphid pests.

INTRODUCTION

Aphids (Hemiptera: Aphididae) are one of the most destructive pests of both agricultural and ornamental plants (Difonzo *et al.*, 1997; Raboudi *et al.*, 2002). It is especially detrimental in nurseries and young orchards (Dean and Sterling, 1992). It has been estimated that 37 to 90% production losses are occurred due to aphid infestations (Abate *et al.*, 2000; Ampofo and Massomo, 2009). Approximately 4000 species of aphids have been described to infest over 250 agricultural and horticultural crops throughout the world (Ali and Rizvi, 2007). In many parts of the world including Bangladesh, *Aphis craccivora, Aphis fabae* and *Aphis gossypii* are regarded as detrimental pests of aphid (Hossain *et al.*, 2006; Chowdhury *et al.*, 2008). To protect the plants and environment, biological control of aphids is a good replacement of highly toxic insecticides which is a common practice for its control (Bellows, 2001). In comparison with other biological control agents, the Coccinellid predators are tolerant to many insecticides which are an advantage over other predators (Banks and Stark, 2011). Considering their economic importance, timely and proper identification of coccinellid predator species is important for effective pest management strategies. Taxonomist faces difficulties due to huge morphological variations within the species that lead huge dilemma to identify insects accurately (Ball and Armstrong, 2006; Singh and Singh, 2014).

Coccinellid beetles, especially *Coccinella transversalis and C. septempunctata* are important biological control agents as they are predaceous on several groups of insect pests, including aphids (Karpacheva, 1991; Bajia and Singh, 2014). Predatory performance of C. transversalis on different aphids was studied previously (Shukla and Jadhav, 2014; Sarker *et al.*, 2019). Biology and feeding potential of C. *septempunctata* on different aphid species (*Macrosliphum rosae*, *Liphaphis erysimi*) were previously studied (Varshney *et al.*, 2016; Unal *et al.*, 2017).

But there is lack of data on choice to consume *Aphis craccivora, Aphis fabae* and *Aphis gossypii* aphid species by *C. septempunctata*. Improved understanding of coccinellid activity and predation on aphids in the laboratory could clarify their potential in aphid biological control. Before introducing any biological control agent in the field level, proper taxonomic studies, preferably molecular identification is prime important (Sarker *et al.*, 2019). Unlike other insects, limited molecular studies have been commenced in the members of the subfamily Coccinellidae of the insect order Coleoptera (Lyla and Haseena, 2008). DNA barcoding has the potentiality to mitigate the challenges posed by identification of insect pests (Gariepy *et al.*, 2007; Rugman-Jones *et al.*, 2009; Quicke *et al.*, 2012; Sethusa *et al.*, 2014). DNA barcoding involves the PCR amplification and sequencing of a key genetic marker from a given specimen (Gariepy *et al.*, 2007). A short, standardized region of its genome, specifically the mitochondrial gene, Cytochrome c oxidase subunit 1 (*COI*) is used in most of the cases (Hebert *et al.*, 2004).

Therefore, the goal of the research was to observe biology of *C. septempunctata* and its predatory efficacy on three detrimental aphid species - *Aphis craccivora, A. fabae* and *A. gossypii* under laboratory conditions. Another objective of the present study was to conduct molecular characterization of *C. septempunctata*, which should in turn facilitate quicker and effective implementation of pest management through quicker identification in countries affected by the aphid species.

MATERIALS AND METHODS

Assessment of feeding potentiality

The feeding potentiality of larval stages of *Coccinella septempunctata* was examined on three agriculturally important aphid species *i.e. Aphis craccivora, A. fabae* and *A. gossypii* separately under controlled conditions (26 ± 2ºC and 65 ± 5 % R.H.). The standard mass rearing technique for the coccinellid predators as described by Soni *et al.* (2008). To maintain the supply of aphid and predatory beetle population on a regular basis, a garden of bean, *Lablab purpureus* and brinjal plants, *Solanum melongena* were cultivated and maintained.

To test predatory efficacy, methods of Shukla and Jadav (2014) and Sarker *et al.* (2019) was followed with slight modification. In short, to check feeding potentiality of larvae of the *C. septempunctata*, newly hatched larvae of the predator were placed individually in six petridishes (6.0×1.0 cm). A predetermined number of aphids were provided in each petridish daily. Unconsumed numbers of aphids were checked and noted regularly. After every 24 hours, unconsumed numbers of aphids were counted. The feeding potentiality of larvae of the predator was examined by feeding the larvae on aphids. The number of aphids consumed per day, during the period of study was recorded in each treatment by counting the number of remaining aphids and subtracting them from the total number of aphids provided. The larvae of the predator were also checked daily for their moulting to calculate the duration of each larval instar. This study was continued until pupation.

Molecular identification

DNA isolation

Genomic DNA of *Coccinella septempunctata* was extracted from somatic tissue rich in mitochondria (e.g., leg or elytra) using Wizard® Genomic DNA Purification Kit, USA, following the manufacturer's protocol with slight modification as mentioned in Aslam *et al.* (2019). The remaining parts of insects and respective individuals were kept as voucher specimens. Processed DNA was stored at 4°C or -20°C.

PCR amplification

The extracted DNA was subjected to PCR amplification through Applied Biosystems® Veriti® 96-well thermal cycler of USA following standard protocols. Primers used were forward primer: (LCO 1490 52-GGTCAACAAATCATAAAGATATTG G-32) and reverse primer: (HCO 2198 52-TAAACTTCAGGGTGACCAAAAAATCA-32) (Folmer *et al.*, 1994). The Promega Gotaq® G2 Green Master Mix (Promega Corporation, USA) was used that contained GoTaq® G2 DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of a wide range of DNA templates. Thermocycling consisted of an initial denaturation of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 49°C for 30 sec, extension at 72 °C for 1 min, final extension: 72°C for 10 min and hold: 4°C.

COI gene sequencing

After checking the accuracy of DNA band using gel electrophoresis, the PCR products were purified using Promega Wizard® SV Gel and PCR clean up system manufactured by Promega Corporation, USA following manufacturer's protocol. The quantity and purity of PCR purified products was checked by Nanodrop™ 2000 spectrophotometer (Thermofisher Scientific, USA). DNA sequencing was performed to determine the nucleotide sequence in cytochrome oxidase I region. BigDye® Terminator v3.1 cycle sequencing kit was used in this process. Each species was bi-directionally sequenced to get sequence of both (5′ and 3′) the DNA strands.

Submission of gene to GenBank

BioEdit v.7.0.5 software was used for checking the quality of sequenced data. Homology, insertions - deletions, stop codons, was checked using NCBI BLAST. BankIt, a WWW-based submission tool with wizards to guide the submission process was used. The GenBank database was designed for new sequence data that was determined and annotated by the submitter. Sequence was uploaded to GenBank.

Data analysis

The chromatograms were converted to FASTA format using FinchTV chromatogram viewer software. The DNA sequences in ABI file were manually edited using BioEdit v.7.0.5. Results of sequence editing were analyzed using BLAST (Basic Local Alignment Search Tool) NCBI to indicate the homology from closest species. Nucleotide composition analysis was performed using the MEGA X. Genetic distance analysis was performed using the Kimura 2-parameter model. Phylogenetic tree was constructed using maximum likelihood method, calculation using Bootstrap with 1000 times of repetition in MEGA (Molecular Evolutionary Genetic Analysis) software program v.10.0. (Tamura *et al.,* 2013). For a comparative and vivid bioinformatics analysis more nucleotide sequences of other Coccinellidae species were retrieved from GenBank.

RESULTS AND DISCUSSIONS

Feeding potentiality

The results of the tests of feeding potentiality of the larval stages of *C. septempunctata* on three *Aphis craccivora, Aphis fabae, and Aphis gossypii*, aphid species are presented in Table 1. All the instar of the larvae found to consume highest number of *A. craccivora* aphids (16.67 ± 0.58, 23.33 ± 1.53, 41.33 ± 0.58 and 151.67 ± 1.15) followed by *A. fabae* and *A. gossypii* aphids. According to Varshney *et al.* (2016), the total number of aphids, consumed by each instars of *C. septempunctata*, viz. first, second, third and fourth were (19.3 ± 0.29, 24.38 ± 0.31, 53.16 ± 0.5 and 175.8 ± 0.69) when reared on *Lipaphis erysimi* at 23 ± 2º C, 65 ± 5% RH. Again, Unal *et al.* (2017) reported that all the instars of *C. septempunctata* consumed (20.3, 54.3, 108.2 and 232.7)

numbers of aphids when reared on *Macrosiphum rosae* at 21.3 ± 4º C, 79 ± 9% RH. However, third larval instar seems to be more appropriate for predator release because after fourth larval instar predator will develop to pupal stage in a short time.

Life cycle

Life cycle of *C. septempunctata* was observed on three detrimental aphid pests, viz. *A. craccivora*, *A. fabae*, *A. gossypii* separately under controlled temperature (26 ± 2ºc) and relative humidity (65 ± 3%) in laboratory condition (Table 2). The incubation period varied from 2 to 5 days with an average 2.33 ± 1.52 days and the hatching percentage of eggs was also remarkable (82.33 ± 16.77). According to Vasrhney *et al*. (2016), the incubation period of *C. septempunctata* while reared on *L. erysimi* was 2.6 ± 0.51 and 2.9 ± 0.73 days when reared at 30 ± 2ºc and 70 ± 5% RH, 25 ± 2ºc and 70 ± 5% RH. The duration of fourth instar larvae of *C. septempunctata* varied with an average (4.33 ± 0.58, 4 ± 1 and 4.33 ± 1.53) days when reared on *A. craccivora, A. fabae* and *A. gossypii*, respectively. On the other hand, Vasrhney *et al*., (2016) observed that the duration of fourth instar larvae were 3.1 ± 0.87 and 3.3 ± 0.94 days while reared on *L. erysimi*. However, Unal *et al*. (2017) recorded that fourth instar larvae of *C. septempunctata* lasted for 5.17 ± 0.75 days when reared on rose aphid, *Macrosiphum rosae*. The total life cycle of *C. septempunctata* varied from 25 to 30,

Table 1: Predatory efficacy of the Coccinellid beetle, *Coccinella septempunctata* larval stages on three different aphid species

Larva		Attributes Max	Min	Predatory efficacy
1st instar	*A. craccivora*	17	16	16.67 ± 0.58
	A. fabae	15	13	13.67 ± 1.15
	A. gossypii	13	12	12.33 ± 0.58
2nd instar	*A. craccivora*	25	22	23.33 ± 1.53
	A. fabae	23	21	22 ± 1.00
	A. gossypii	23	21	21.66 ± 1.15
3rd instar	*A. craccivora*	42	41	41.33 ± 0.58
	A. fabae	41	40	40.67 ± 0.58
	A. gossypii	41	38	39.67 ± 1.53
4th instar	*A. craccivora*	153	151	151.67 ± 1.15
	A. fabae	137	135	135.67 ± 1.15
	A. gossypii	132	127	129.33 ± 2.51

Table 2: Life cycle of *Coccinella septempunctata* reared on *A. craccivora*, *A. fabae* and *A. gossypii*

Attributes			Max	Min	Mean ± SD
Incubation period (days)			5	2	2.33 ± 1.52
Hatching percentage			93	63	82.33 ± 16.77
Larva (days)	1st instar	*A. craccivora*	4	3	3.33 ± 0.58
		A. fabae	4	2	3 ± 1
		A. gossypii	3	1	2.33 ± 1.54
	2nd instar	*A. craccivora*	3	2	2.66 ± 0.58
		A. fabae	3	1	2.33 ± 1.15
		A. gossypii	3	3	3 ± 0
	3rd instar	*A. craccivora*	4	3	3.66 ± 0.58
		A. fabae	4	3	3.33 ± 0.58
		A. gossypii	4	2	3.0 ± 1
	4th instar	*A. craccivora*	5	4	4.33 ± 0.58
		A. fabae	5	3	4 ± 1
		A. gossypii	5	2	4.33 ± 1.53
Total larval development period(days)					
Prepupal period (days)		*A. craccivora*	1	1	1 ± 0
		A. fabae	1	1	1 ± 0
		A. gossypii	1	1	1 ± 0
Pupal period (days)		*A. craccivora*	4	3	3.33 ± 0.58
		A. fabae	3	2	2.33 ± 0.58
		A. gossypii	4	2	3 ± 1
Total life cycle (days)		*A. craccivora*	30	25	28 ± 2.64
		A. fabae	25	24	24.33 ± 0.58
		A. gossypii	24	21	23 ± 1.73

Table 3: Nucleotide composition of *Coccinella septempunctata*

Species	T(U)	C	A	G	Total	A+T	G+C
C. septempunctata	38.5	16.1	30.8	14.6	584.0	69.3	30.7

Table 4: Genetic distance among *Coccinella septempunctata* species using Kimura 2 parameter (K2P)

Species name	1	2	3	4	5
C. septempunctata (BD*)	-				
C. septempunctata (Pakistan)	0.002	-			
C. septempunctata (Korea)	0.002	0.003	-		
C. septempunctata (USA)	0.003	0.005	0.002	-	
C. septempunctata (China)	0.000	0.002	0.002	0.003	-

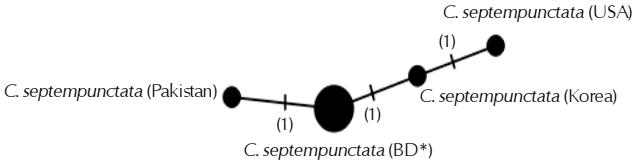

Figure 1: Mitochondrial COI gene haplotype analysis of *Coccinella septempunctata* constructed by Popart1.7 based on TCS network. Big black circles represent the haplotype and small black circle represent the common ancestor. Mutational steps are presented by hatch marks and number

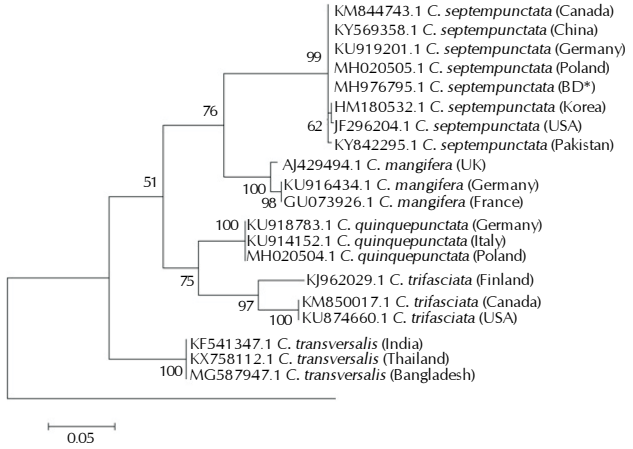

Figure 2: Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together was shown above the branches

24 to 25 and 21 to 24 days when reared on *A. craccivora, A. fabae* and *A. gossypii* with an average 28 ± 2.64, 24.33 ± 0.58 and 23 ± 1.73 days, respectively in laboratory condition. However, Sakurai *et al.* (1991) reported that the quality of food and environmental factors like temperature, humidity also play an important role on different aspects of the biology of Coccinellid beetles. So, this variation may be due to mentioned factor.

Molecular characterization

Sequence result and BLAST analysis

For accurate identification of such important predator, COI gene of preliminary identified further confirmation, *C. septempunctata* was sequenced. The mitochondrial cytochrome oxidase subunit I (COI) gene sequence was 591bp. National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to check homology between the retrieved sequences and GenBank library or database of sequences. This aided to identify sequence similarity across genomes.

BLAST analysis (data not shown) revealed that the observed sequence showed 100% homology with the sequences of *Coccinella septempunctata* in GenBank (MH020505.1, KU919201.1, KU917472.1, KU916544.1 and KU916644.1). It indicated that the observed sample was *Coccinella septempunctata*. The sequence was submitted to the NCBI GenBank and acquired accession number was MH 976795.1. GenBank is a comprehensive database that contains publicly available nucleotide sequences. GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Nucleotide Archive (ENA), and GenBank at NCBI (NCBI; a part of the National Institutes of Health in the United States).

Nucleotide composition of *Coccinella septempunctata*

An important characteristic of nuclecic acid is their nucleotide composition. Retrieved sequence was subjected for analysis of nucleotide composition (Table 3). Codon positions included were 1st + 2nd + 3rd + non-coding. All positions containing gaps and missing data were eliminated from the dataset. From the analysis it was found that the average largest number of nucleotide was thiamine (T) and composed of 38.5% nucleotide. On the other hand, lowest number of nucleotide was guanine (G) which composed of 14.6%. The maximum value of adenine and thiamine (A + T, 69.3) and the minimum value of guanine and cytosine (G + C, 30.7) were found in *C. septempunctata*. As expected, AT content was found significantly higher than the GC content. Similar nucleotide composition was observed in other mitochondrial COI gene sequence analyses (Aslam *et al.*, 2019; Rain *et al.*, 2019; Sarker *et al.*, 2019).

Genetic distance analysis

Genetic distance analysis was performed for reconstructing the history of species and for understanding the origin of species. The numbers of base substitutions per site among sequences are shown in table 4.There were a total of 641 positions in the final dataset. Intraspecific genetic divergence ranged from 0.000-0.005. The lowest genetic distance (0.000) was found in *C. septempunctata* (China) and the highest genetic distance (0.005) was found in *C. septempunctata* (USA).

Haplotype

To know the relationships among the different haploid genotypes among the datasets, haplotype network was performed. Haplotype analysis of mitochondrial COI gene of *C. septempunctata* from different regions of the world showed

very little genetic diversity among them. They were separated from their common ancestor by 1 mutational step. *C. septempunctata* (BD*) showed 1 mutated site from Pakistan, Korea and USA.

Phylogenetic analysis

Maximum likelihood (ML) tree was analyzed to find out the phylogenetic relationship among the *C. septempunctata* from different regions of the world, mentioned beside the species name was used for a proper comparison. According to maximum likelihood with 1000x bootstrap repetition, a phylogeny was constructed (Fig. 2) by the MEGA v.10.0 software using analyzed twenty sequences of *Coccinella* species from different regions of the world. Here, *Thyreus histrionicus*, a solitary bee belongs to the order Hymenoptera used as an out group. All *C. septempunctata* was originated from single clade and showed 99% genetic similarity with the sequenced *C. septempunctata* (marked with BD*). The bar at the bottom provides a scale for the genetic change. In this case, the line segment with the number '0.05' shows the length of branch that signifies an amount genetic change of 0.050.

Now a days, DNA barcoding has gained much popularity as a molecular method for species identification. The goal of DNA barcoding is to create a library of every organism on earth (Kerr *et al.,* 2007). Although the major insect pests in food and their biological control agents are widespread worldwide, only a few studies have been conducted on the DNA barcodes for these species (Seo *et al.*, 2013). This study is the first attempt of construction a DNA reference dataset using the mitochondrial COI gene along with molecular characterization and other related bioinformatic analysis especially of Bangladesh. This dataset may be effectively used to identify this biological control agent that is currently important in commercial field in the perspective of Bangladesh. DNA barcoding can also play an effective role in identifying such agents in any stage of life making easier to effectively this as biological control agent and saving farmers from cost of billion dollars from pest damage (Kaur, 2015; Sarvananda, 2018).

Coccinellid beetle - *C. septempunctata* showed high predation efficiency especially on three detrimental aphid pests, viz. *A. craccivora*, *A. fabae*, *A. gossypii* which could be recommended to use in the field level after more screening. Moreover, DNA barcoding can help in proper identification of the predatory beetle that might facilitate in implementing aphid management program.

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