Aphid Predation Efficacy of *Coccinella Septempunctata* and Its Molecular Characterization Based on *COI* Gene Sequence

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Abstract

Predatory efficacy of *Coccinella septempunctata* (Coleoptera: Coccinellidae) larvae on three agriculturally important aphids (*Aphis craccivora, A. fabae and A. gossypii*) was studied. The predation efficacy was increased with the progress of the larval instars up to the fourth which was the most voracious. The 4th instar larvae of *C. septempunctata* consumed highest 151.67 ± 1.15 number of *A. craccivora* aphids followed by *A. fabae* (135.67 ± 1.15) and *A. gossypii* (129.33 ± 2.51). Life cycle studies of *C. septempunctata* revealed that it took maximum (28 ± 2.64) days to complete life cycle while reared on *A. craccivora* followed by 24.33 ± 0.58 and 23 ± 1.73 days while reared on *A. fabae* and *A. gossypii* respectively. For molecular identification, mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene of *C. septempunctata* was sequenced and submitted to NCBI GenBank (Accession No. MH976795.1). The partial region of *COI* gene contained 550 bp, where A+T content (69.3%) was greater than G+C content (30.7%). The intraspecific genetic divergence ranged from 0.000-0.005. Haplotype analysis showed less genetic diversity (1 mutational step). Phylogenetic analysis exhibited that *C. septempunctata* species from different regions were placed in a single major clade. Such study of *C. septempunctata* would be helpful in biological control program of aphid pest.

Keywords: *Coccinella septempunctata,* Aphid, predatory efficacy, *COI* gene, molecular characterization

1. Introduction

Aphids (Hemiptera: Aphididae) are distributed worldwide and broadly recognized as one of the most destructive pests of both agricultural and ornamental plants [1-2]. It is especially detrimental to nurseries and young orchards [3]. It has been estimated that 37 to 90% production losses are occurred due to aphid infestations [4-5]. Approximately 4000 species of aphids have been described to infest over 250 agricultural and horticultural crops throughout the world [6]. In many parts of the world including Bangladesh, *Aphis craccivora, A. fabae* and *A. gossypii* are regarded as detrimental pests of aphid [7-8]. 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 [9]. In comparison with other biological control agents, the Coccinellid predators are tolerant to many insecticides which is an advantage over other predators [10]. Both the adult and larval stages of the coccinellid beetle, *Coccinella septempunctata* feed primarily on diverse aphids [11]. But there is lack of data on choice to consume aphid species by this predator. Improved understanding of coccinellid activity and predation against aphids in the laboratory could clarify their potential in aphid biological control. The present study was conducted to observe the biology of *C. septempunctata* and its predatory efficacy on above mentioned three detrimental aphid species of agricultural crops, i.e. *Aphis craccivora, A. fabae* and *A. gossypii* under laboratory conditions.

Timely and proper identification of coccinellid predator species is important for effective pest management strategies. Taxonomist faces difficulties due to enormous morphological variations within the species that lead huge dilemma to identify insects accurately [12-13]. Unlike other insects, limited molecular studies have been commenced in the members of the subfamily Coccinellidae of the insect order Coleoptera [14]. DNA barcoding has the potentiality to mitigate the challenges posed by identification of insect pests [15-18]. DNA barcoding involves the PCR amplification and sequencing of a key genetic marker from a given specimen [16]. A short, standardized region of its genome, specifically the mitochondrial gene, Cytochrome c oxidase subunit 1 (*COI*) is used in most of the cases [19].

Therefore, the goal of the research was to observe biology of *C. septempunctata* and its predatory efficacy on three detrimental aphid species. Another objective of the present study was to avail tools that contribute to accurate molecular identification and characterization of *C. septempunctata* based on *COI* gene sequence. Finally this result should in turn facilitate quicker and effective implementation of pest management and strengthening of the IPM system in countries affected by the aphid species.

2. Materials and Methods

2.1 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.). To maintain the supply of aphid and predatory beetle on a regular basis, a garden of bean, *Lablab purpureus* and brinjal plants, *Solanum melongena* were cultivated and maintained.

To assess feeding potentiality of larvae of the *C. septempunctata* against aphids, newly hatched six larvae

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were placed individually in six petridishes (6.0×1.5 cm). A predetermined number of aphids (50 nos. for 1st and 2nd instar; 100 for 3rd instar and 300 for 4th instar) were provided in each petridish daily. 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.

2.2 Molecular identification

2.2.1 DNA isolation

Genomic DNA of *Coccinella septempunctata* was extracted from somatic tissue rich in mitochondria (e.g., leg or elytra of adult) using Wizard® Genomic DNA Purification Kit, USA, following the manufacturer's protocol with slight modification as mentioned in Aslam *et al*., 2019 [20]. The remaining parts of insects and respective individuals were kept as voucher specimens. The extracted genomic DNA was stored at 4^0C or -20^0C.

2.2.2 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 5′-GGTCAACAAAT CATAAAGATATTG G-3′) and reverse primer: (HCO 21985′-TAAACTTCAGGGTGACCAAAAAATCA-3′) according to Folmer *et al*., 2019, which amplify a 710 bp [21]. 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: 72^0C for 10 min. and hold: 4^0C.

2.2.3 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 partial nucleotide sequence in cytochrome oxidase I region. BigDye® Terminator v3.1 cycle sequencing kit was used in this process. The species was bi-directionally sequenced to get sequence of both (5' and 3') the DNA strands.

2.2.4 Gene submission to genbank and data analysis

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.

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. Haplotype network was performed using Popart1.7 software. 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. [22]. For a comparative and vivid bioinformatics analysis more nucleotide sequences of other Coccinellidae species were retrieved from GenBank.

3. Results and Discussion

3.1 Feeding potentiality

The results of the tests of feeding potentiality of the larval stages of *C. septempunctata* on three *Aphis craccivora, A. fabae, and A. gossypii*, aphid species are presented in Table 1. All the four instars 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 [23], 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 [24], 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 within a short time.

3.2 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 and relative humidity 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 Varshney [23], 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, Varshney [23] 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 [23] 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, 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. Nevertheless, Sakurai [25] 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.

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

Attributes			Max	Min	Predatory efficacy (Mean ± SD)
Larva					
	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

3.3 Molecular Characterization

3.3.1 Sequence result and BLAST analysis

For accurate identification, *COI* gene of morphologically identified *C. septempunctata* was sequenced. The mitochondrial cytochrome oxidase subunit I (*COI*) gene sequence was determined and contained in total 550bp. 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.

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
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

BLAST analysis (data not shown) revealed that the observed sequence showed 100% homology with the sequences of *Coccinella septempunctata* in GenBank with the accession numbers: 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 MH976795.1. GenBank is a comprehensive database that contains publicly available nucleotide sequences.

3.3.2 Nucleotide composition of Coccinella septempunctata

An important characteristic of nucleic 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 base was thiamine (T) and composed of 38.5% nucleotide. On the other hand, lowest number of nucleotide base was guanine (G) which composed of 14.6%. The percentage of A+T content was 69.3 and G+C was 30.7. A-T content was found significantly higher than the G-C content. Similar result was observed in mitochondrial *COI* gene sequence analysis of stored grain pest [20].

Table 3: Percentage of nucleotide composition of *COI* gene of *Coccinella septempunctata*

Species	T (%)	C (%)	A (%)	G (%)	Total	A+T (%)	G+C (%)
C. septempunctata	38.5	16.1	30.8	14.6	550.0	69.3	30.7

3.3.3 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).

Table 4: Genetic distance of *COI* gene 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	-

3.3.4 Haplotype

To know the relationships among the different haploid genotypes among the datasets, haplotype network was performed (Fig. 1). 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.

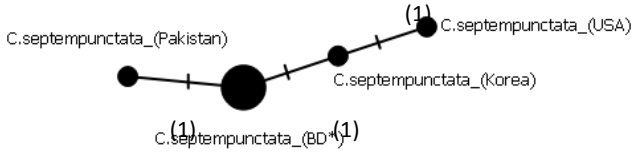

Fig. 1: Mitochondrial *COI* gene haplotype analysis of *Coccinella septempunctata* constructed by Popart1.7 based on TCS network. Big black circles represent the haplotype and small black circle represent the common ancestor. Mutational steps are presented by hatch marks and number

3.3.5 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 phylogenetic tree 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.

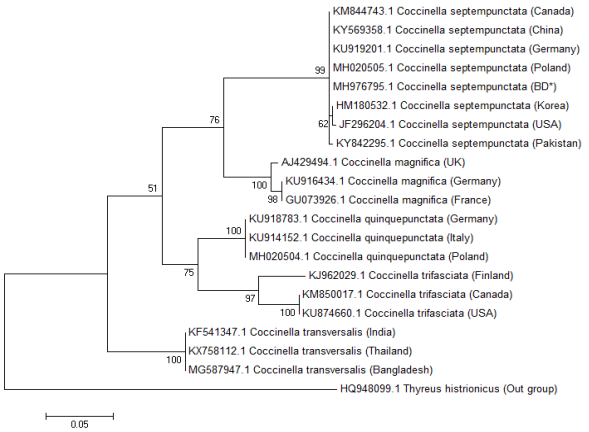

Fig. 2: Molecular Phylogenetic analysis by Maximum Likelihood method based on the nucleotide sequenc of *COI* gene. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model. The tree was drawn to scale, with branch lengths measured in the number of substitutions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

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 [26]. 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 [27].

4. Conclusion

C. septempunctata showed high predation efficiency on three detrimental aphid pests, which could be recommended to use in the field level after more screening. Moreover, DNA barcoding can play an effective role in identifying such important biological control agent even in immature stage of life. This study is the first attempt of construction a DNA reference dataset using the mitochondrial *COI* gene along with molecular characterization especially in Bangladesh. These information might facilitate in implementation of aphid management program and save farmers from cost of billion dollars due to pest damage.

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References

1. C. D. Difonzo, D. W. Ragsdale, E. B. Radcliffe, N.C. Gumestad and G. A. Secor, Seasonal abundance of aphid vectors of potato virus Y in the Red River Valley of Minnesota and North Dakota, J. Econ. Entomol., **90**, 824-831 (1997).
2. F. Raboudi, A. Benmoussa, H. Makni and M. Marraakachi, Serological detection of plant viruses and host plant in Tunisia, EPP. Bulle., **32,** 495-498 (2002).
3. D. A. Dean and W. L. Sterling, A comparison of sampling methods to predict phonology of predaceous arthropods in a cotton agro-ecosystems, J. Agri. Res., **46(8),** 257-282 (1992).
4. T. Abate, H. A. Van and J. K. Ampofo, Pest Management Strategies in Traditional Agriculture: An African Perspective, Ann. Rev. Entomol., **45**, 631-659 (2000).
5. J. K. Ampofo and S. M. Massomo, Some Cultural Strategies for Management of Bean Stem Maggots (Diptera: Agromyzidae) on Beans in Tanzania, Afr. Crop Sci. J., **6(4),** 351-356 (2009).
6. A. Ali and P. Q. Rizvi, Development and predatory performance of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) on different aphid species, J. Biol. Sci., **7(8),** 1478-1483 (2007).
7. A. F. Hossain, J. Ferdous and M. M. R. Salim, Relative abundance and yield loss assessment of Lentil aphid *Aphis craccivora* Koch in relation to different sowing dates, J. Agric. Rural. Dev., **4(1&2),** 101-106 (2006).
8. S. P. Chowdhury, M. A. Ahad, M. R. Amin and N. A. Rasel, Bean aphid predation efficiency of lady bird beetle *Micraspis discolor* F. (Coleoptera: Coccinellidae), J. Soil Nature., **2(3),** 40-45 (2008).
9. T. S. Bellows, Restoring population balance through natural enemy introductions, J. Biocon., **21**, 199-205 (2001).
10. J. E. Banks and J. D. Stark, Effects of a nicotinic insecticide, insidacloprid and vegetarian diversity on movement of a common predator, *Coccinella septemounctata*, Bioseptic, Int.,**7(2),** 113-122 (2011).
11. N. S. Karpacheva, Ladybird beetle, *Zaschchita rastenii*, J. Econ. Entomol., **10**, 34-35 (1991).
12. S. L. Ball and K. F. Armstrong, DNA barcodes for insect pest identification, Can. J. For. Res., **36(2)**, 337-350 (2006).
13. B. B. Singh and R. Singh, Major Rice insect pests in north eastern UP, Int. J. Life Sci.& Pharm., **3(1),** 124-143 (2014).
14. K. R. Lyla and B. Haseena, Biology and feeding preference of the coccinellid predator, *Coccinella transversalis* Fab, In. Environ., **14(2),** 75-76 (2008).
15. P. F. Rugmanjones, W. Robert, N. Tomvan and S. Richard, Molecular differentiation of the *Psyttaliaconcolor* (Szépligeti) species complex (Hymenoptera: Braconidae) associated with olive fly, *Bactroceraoleae* (Rossi) (Diptera: Tephritidae) in Africa, J. Biol. Control., **49(1),** 17-26 (2009).
16. D. T. Gariepy, U. Kulhmann, C. Gillott and M. Erlandson, Parasitoids, Predators and PCR: the use of diagnostic molecular marker in control of Arthropods, J. Appl. Ecol., **131(4),** 225-240 (2007).
17. D. L. Quicke, M. Alexsmith, D. H. Janzen, W. Hallwachs, T. J. Fernandez, N. M. Laurenne, R. A. Zaldivar, M. R. Shaw, G. R. Broad and S. Klopfstein, Utility of the DNA barcoding gene fragment for parasitic wasp phylogeny (Hymenoptera: Ichneumonoidea): Data release and new measure of taxonomic congruence, Mol. Ecol. Resour., **12,** 676-685 (2012).
18. M. T. Sethusa, I. M. Millar, K. Yessoufou, A. Jacobs, M. Vanderbank, and H. Vanderbank, DNA barcode efficacy for the identification of economically important scale insects (Hemiptera: Coccoidea) in South Africa, African J. Zool., **22(1),** 257-267 (2014).
19. P. D. N. Hebert, M.Y. Stoeckle, T. S. Zemlak and C. M. Francis, Identification of birds through DNA barcodes, Proc. R. Soc. Land. B. Biol. Sci., **2,** 1657-1663 (2004).
20. A. F. M. Aslam, S. Sultana, F. F. Rain, S. Sarker, S. R. Das, A. Siddika and A. J. Howlader, Molecular characterization and identification of three stored grain pests based on mitochondrial cytochrome c oxidase subunit I (*COI*) gene sequences, Bangladesh J. Zool., **47(1)**, 1-11 (2019).
21. O. Folmer, M. Black, W. Hoeh, R. Lutz and R. Vrijenhoek, DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates, Mol. Mar. Biol. Biotechnol., **3(5)**, 294-299 (1994).
22. K. Tamura, D. Peterson, N. Peterson, G.N.M. Stecher and S. Kumar, MEGA6: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol., **28,** 2731-2739 (2013).
23. R. R. Varshney, P. Rachana and R. S. Bishit, Biology and feeding potential of *Coccinella septempunctata* (Linn) against Liphaphis erysimi at different temperature regions, J. Appl. & Nat. Sci., **8(4),** 1762-1765 (2016).
24. S. Unal, A. Er, E. Akkuzu and L. Salek, Predation efficacy of the predator *Coccinella septempunctata* on the aphid species, *Macrosliphum rosae* in kastamonu province, Turkey, Pakistan. J. Zool., **49(1),** 327-330 (2017).
25. H. Sakurai, N. Yoshida, C. Kobayashi and S. Taheda, Effect of temperature and day length on oviposition and growth of lady bird beetle, *Coccinella septempunctata*, Res. Bull. Faculty of Agri. Gifu Univ., **56,** 45-50 (1991).
26. K. C. R. Kerr, M. Y. Stoeckle, C. J. Dove, L.A. Weigt, C. M. Frances and P. D. N. Hebert, Comprehensive DNA barcode coverage of North American birds, Mol. Ecol., **7,** 535-543 (2007).
27. Y. C. Seo, S. Kyong and Y. J. Bae, DNA barcode library and its efficacy for identifying food-associated insect pests in Korea, Entomol. Res., **43,** 253-261 (2013).