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DNA Barcoding Reveals the First Occurrence of Chrysanthemum Leafminer, *Chromatomyia syngenesiae* Hardy (Diptera: Agromyzidae) in India †

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† Presented at the 1st International Electronic Conference on Entomology (IECE 2021), 1–15 July 2021; Available online: https://iece.sciforum.net/.

Abstract: Chrysanthemum is ravaged by numerous agromyzid leafminers. Of which, *Chromatomyia syngenesiae* Hardy (Diptera: Agromyzidae) is an invasive polyphagous leafminer which causes heavy infestation by mining the green leaf tissue and weakens the plant by reducing the photosynthetic activity that results in unmarketable flowers. Hitherto, no further information is available from India on *C. syngenesiae* apart from just new record. The present study was undertaken to assess the damage potential and to identify the leafminer species attacking chrysanthemum. In this view, Survey was conducted in chrysanthemum growing polyhouse of Nilgiris, Tamil Nadu, India during 2016 to 2018. The results indicated that the damage potential caused by *C. syngenesiae* was 75.40 % and 69.00 % in 2016 to 2017 and 2017 to 2018, respectively. The highest parasitization of *Diglyphus isaea* (Eulophidae: Hymenoptera) Walker on *C. syngenesiae* was recorded as 18.27 % and 20.80 % in the respective years. In addition, *C. syngenesiae* and its parasitoid were characterized at molecular level and species specific DNA barcodes using *mitochondrial Cytochrome Oxidase I* (mtCOI) were developed. BLAST searching of GenBank showed 100 per cent sequence similarity with *C. syngenesiae* and clearly indicated the presence of *C. syngenesiae* at Nilgiris, Tamil Nadu, India. The sequences obtained from the present study was submitted to NCBI database as the first entry for species level representation from India and the accession numbers (*C. syngenesiae* -MN969922 and *D. isaea* -MN525179) were obtained.

Keywords: First report; Survey; DNA barcoding; mitochondrial Cytochrome Oxidase I (mt COI); Chrysanthemum; *Chromatomyia syngenesiae*; *Diglyphus isaea*

Citation: Murugasridevi, K.; Jeyarani, S.; Mohankumar, S. DNA Barcoding Reveals the First Occurrence of Chrysanthemum Leafminer, *Chromatomyia syngenesiae* Hardy (Diptera: Agromyzidae) in India, in Proceedings of the 1st International Electronic Conference on Entomology, 1–15 July 2021, MDPI: Basel, Switzerland, doi:10.3390/IECE-10569

Published: 2 July 2021

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1. Introduction

Chrysanthemum is ravaged by numerous agromyzid leafminers *viz.*, *Chromatomyia syngenesiae* Hardy, *C. horticola* Goureau, *Liriomyza trifolii* Burgess, *L. sativae* Blanchard, *L. huidobrensis* Blanchard and *Nemorimyza maculosa* Malloch [1-3]. Leafminer species that attack chrysanthemum are morphologically similar, specifically *C. syngenesiae* and *C. horticola*. In general, all of the *Chromatomyia* spp., seem to be quite indistinguishable and are frequently confused with one another. Regarding an accurate identification, a thorough examination of the leaf mines at all phases of development is mandatory [3]. Although *Chromatomyia* spp. have substantial physical similarities, only taxonomic experts can accurately identify specific infestations [4]. Consequently, limitations associated with traditional alpha taxonomy *viz.*, stage-specific taxonomic keys, phenotypic plasticity of key characters, failure to demarcate cryptic species and limited dipteran taxonomists, lead to

problems about unambiguous agromyzid species identification. DNA barcoding has now developed as a unique method for accurately identifying species in the shortest duration, even by non-insect taxonomists [5]. It not only acts as a supplement to traditional alpha taxonomy in verifying species recognition, but it also has an advantage over it in identifying cryptic species in the population [6]. Hence, the present study was undertaken to determine the leafminer species infesting chrysanthemum, reckon its damage potential and explore its associated parasitoids in India. To establish the right identity of our specimens, we attempted to build a cytochrome c oxidase I (COI)-based DNA barcode for leafminer infesting chrysanthemum and its concomitant parasitoid in India.

2. Materials and methods

2.1. Assessment of damage potential of chrysanthemum leafminer

Surveys on damage potential of chrysanthemum leafminer, *Chromatomyia* spp., and its associated parasitoids were carried out on chrysanthemum in Nilgiris district of Tamil Nadu, India (11.449º N, 76.886º E) during the period of 2016 to 2018. Damage potential of leafminers on chrysanthemum was assessed from 20 leaflets per leaves at top, middle, bottom of five randomly selected plants in each location [7].

2.2. Assessment of per cent parasitization of parasitoids attacking chrysanthemum leafminer

The leafminers infested chrysanthemum leaves were brought to the laboratory and examined under microscope for live mines and parasitized mines and were kept in plastic containers (7. 5 cm dia, 21 cm ht.) for the emergence of parasitoid adults. The per cent parasitization was calculated from number of parasitoid adults emerged to the total number of mines [7]. The emerged adult leafminer and parasitoid were preserved in 70 per cent ethanol and identified by the taxonomic expert, Dr. A. P. Ranjith, University of Calicut, Kerala and Dr. Alfred Daniel, Tamil Nadu Agricultural University.

2.3. Genomic DNA extraction

Genomic DNA was isolated from single adult leafminers and its parasitoid by following the CTAB (Cetyl Trimethyl Ammonium Bromide) method [8]. The DNA extraction buffer contained 100mM Tris-HCl (pH 8), 10mM EDTA, 1.4M NaCl, 2.0 per cent CTAB and 5.0 per cent β-mercaptoethanol. Individual insect sample was homogenized with 200 µl of DNA extraction buffer and incubated at 65°C for 1 h. The tubes were removed from the water bath and allowed to cool at room temperature. Equal volume of Chloroform: Isoamyl alcohol mixture (24:1, v/v) (0.8 volume) was added and mixed by inversion for 10 min to form an emulsion. It was centrifuged at 12,000 rpm for 10 min and the clear aqueous phase was transferred to a new sterile tube. Ice-cold isopropanol (0.7 volume) was added and mixed gently by inversion and was stored at -20^0 C for overnight. It was then centrifuged at 12,000 rpm for 10 min to pellet the DNA and the supernatant was discarded. The DNA pellet was washed with 70 per cent ethanol. After washing, DNA pellet was air dried and dissolved in 20 to 40 µl of Tris-EDTA buffer depending on the size of the pellet and stored at -20°C until use. Quality of genomic DNA was checked by 0.8 per cent agarose gel. Agarose at0.8g was dissolved in 100 ml of 1X TBE (Tris Borate EDTA) buffer. After cooling, 1 to 2 µl ethidium bromide was added from the stock (10 mg ethidium bromide / ml H_2O). Then the mixture was poured into a preset template kept with appropriate comb to make wells. 2µl DNA added with 2 µl loading dye (6X loading dye) were loaded in each well. Electrophoresis was carried out at 65 V for 1 h. Amplified genomic DNA was visualized on UV transilluminator (Bio-Rad, USA) and documented using Gel documentation system (GELSTAN 1312). The quantification of DNA was done using Nanodrop Spectrophotometer (ND-1000). Based on the nanodrop readings, DNA dilutions were made in TE buffer to make a final concentration of 50 ng μl^{-1} and stored at 4°C for further use [9]. A fragment of the mitochondrial gene (Cytochrome Oxidase 1 (CO1) was amplified across the populations of leafminers and parasitoids using Folmer primers

LCOI490 (Forward) and HCO2198 (Reverse) [10]. Forward primer (5′-3′) : GGTCAACAAATCATAAAGATATTGG, Reverse primer (3′-5′) : TAAACTTCAGGG-TAACCAAAAAATCA. Polymerase chain reactions were performed with 25µl volumes in PCR machine (Sure cycler 8800, Agilent Technologies). The optimized PCR cocktail mixture (for 25 µl reaction mix) is narrated as follows: (25 µl) using 0.5µl Taq polymerase (1.5U/ µl), 2.5µl of 10X PCR reaction buffer with 0.5µl of 25mM $MgCl_2$,1.0µl of 250 µM of each dNTPs, 1.0µl of forward and reverse primers,16.5µl of nuclease-free water. The PCR conditions were as follows: the initial denaturation for 3 min at 95 °C followed by 35 cycles of denaturing for 20 s at 95 °C, annealing for 0.30 sec at 52 °C, and extension time of 40 s at 72 °C, with a final extension for 10 min at 72 °C. The PCR products were visualized using UV transilluminator on agarose gel (1.5%) after electrophoresis and the gel was documented using gel documentation system (GELSTAN, 1312). PCR products (20 µl) and their respective forward and reverse primers (10 µl each per sample) were labelled appropriately and sent to for sequencing at Agrigenome Labs Pvt. Ltd., Cochin, Kerala. The PCR products were sequenced by double pass method in both forward and reverse direction. The PCR products were purified using Pure Link PCR purification Kit and the sequencing PCR were set up by using Big Dye Terminator V3.1 Cycle Sequencing Kit. The resulting sequencing information were retrieved from the client database of Agrigenomelabs online portal. Then the sequences were aligned, edited and trimmed using the programme Geneious and outgroups obtained from GenBank using the *blastn* algorithm to search for nucleotide (nr/nt) data base. The nucleotide sequences were compared to identify the similarity between each host by Basic Local Alignment Search Tool (BLAST) and Barcode of Life Database.

3. Results

3.1. Damage potential of C. syngenesiae and its parasitization by D. isaea

The survey carried out on the chrysanthemum during 2016 to 2017 indicated highest incidence of *C. syngenesiae* at Erumaipatti village (75.40 %) of Nilgiris district followed by Kothagiri (69.00 %). The survey also revealed 18.27 per cent parasitization at Erumaipatti village followed by Kothagiri (15.72 %) (Fig. 1). The eulophid parasitoid, *Diglyphus isaea* Walker was found to be predominantly associated with *C. syngenesiae*. Survey conducted during 2017-2018 also revealed highest damage by *C. syngenesiae* (69.00 %) and parasitization by *D. isaea* (20.80%) at Erumaipatti village (75.40 %) of Nilgiris district followed by Kothagiri (Fig. 2).

3.2. DNA barcoding of C. syngenesiae and D. isaea

DNA barcoding of chrysanthemum leafminer and its parasitoid confirmed the occurrence of *C. syngenesiae* and its parasitoid, *D. isaea* populations at Nilgiris, Tamil Nadu, India using *mitochondrial Cytochrome Oxidase I (mtCOI).* BLAST searching of GenBank indicated 100 per cent sequence similarity with *C. syngenesiae* and *D. isaea,* respectively. The sequences obtained from the present study was submitted to NCBI database as the first entry for species level representation from India and the accession numbers (*C. syngenesiae* -MN969922 and *D. isaea* - MN525179) were obtained. The amino acid sequence of the *C. syngenesiae* and *D. isaea* COI fragment does not have any stop codon and the uninterrupted ORF confirmed the quality of the DNA barcode generated in this study. The first-ever DNA barcode of *C. syngenesiae* and *D. isaea* representing India was submitted in the Barcode of Life Database (BOLD systems) and an illustrative barcode was obtained (Fig. 3 and 4).

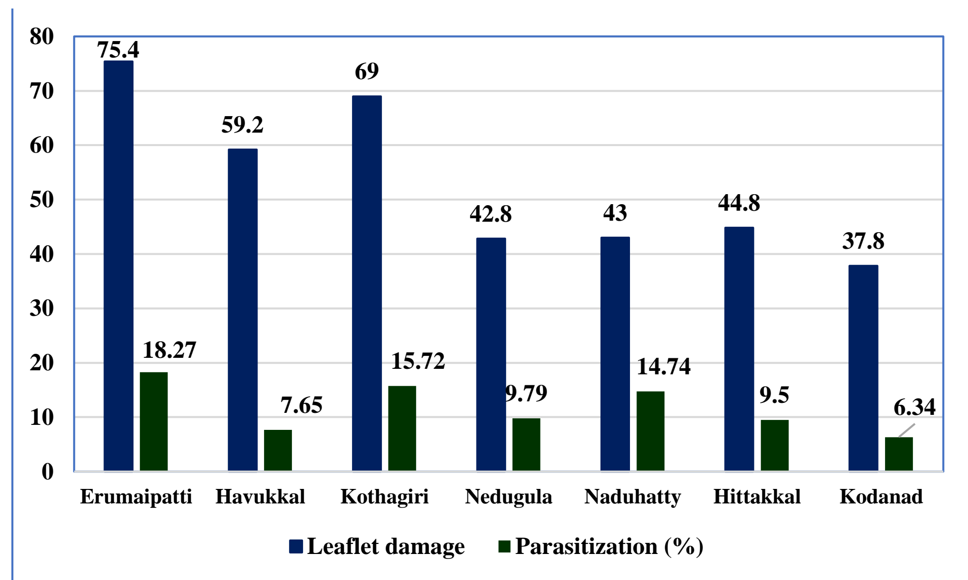

Figure 1. Occurrence of *C. syngenesiae* and its associated parasitoid on chrysanthemum in India during 2016 to 2017.

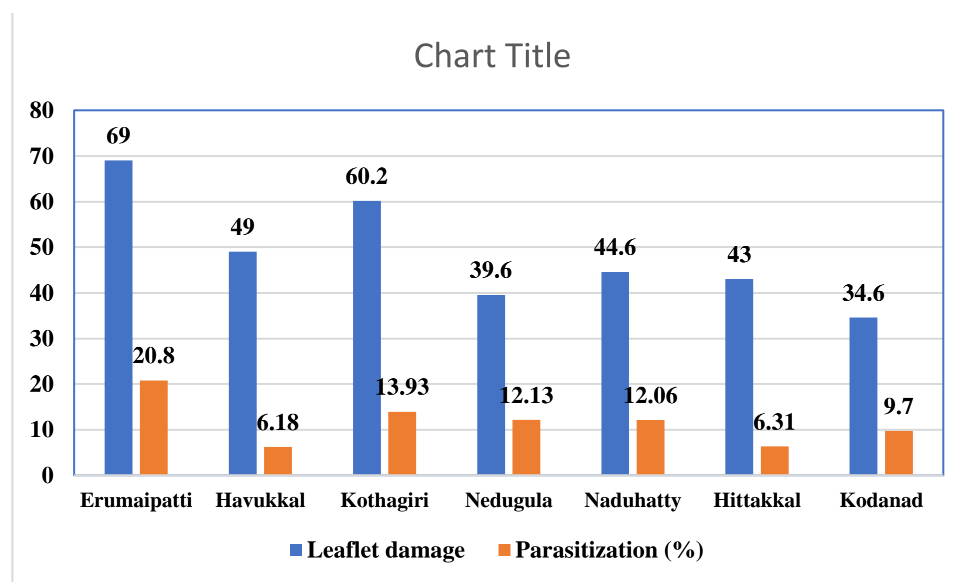

Figure 2. Occurrence of *C. syngenesiae* and its associated parasitoid on chrysanthemum in India during 2017 to 2018.

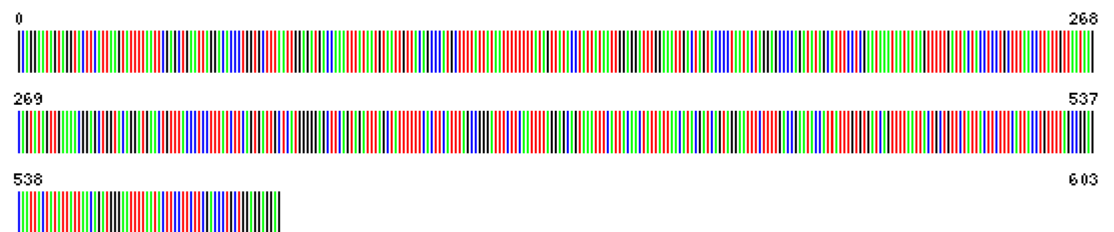

Figure 3. An Illustrative barcode of *C. syngenesiae* (MN969922).

Figure 4. An Illustrative barcode of *D. isaea* (MN525179).

4. Discussion

4.1. Occurrence of C. syngenesiae and its parasitoid on chrysanthemum

The present results revealed the occurrence of *C. syngenesiae* infesting chrysanthemum in Nilgiris district, Tamil Nadu, India during 2016 to 2018. *C. syngenesiae* is an invasive polyphagous pest very common in Europe and was introduced to both East and West coasts of the U.S.A. and then to Australia, New Zealand and Srilanka [19,20]. Previous studies have indicated that *C. syngenesiae* can quickly achieve damaging levels in newly invaded areas irrespective of the use of insecticides [11,12]. Infact, the pest is difficult to manage due to protection conferred by its leaf mining habit. High reproductive capacity, short developmental time, high survival, rapid spread and resistance to insecticide makes this pest more challenging [13-15].

The highest parasitization of *D. isaea* on *C. syngenesiae* was also recorded in Erumaipatti, Nilgiris district during 2016 to 2017 (18.27 %) and 2017 to 2018 (20.80 %). These results are in accordance with previous investigations which have reported the natural parasitization of *C. syngenesiae* on chrysanthemum by 21 species of parasitoids in Southern England [16]. In addition, the maximum parasitization of 58.13 per cent on *C. horticola* by *D. isaea, D. minoeus* and *Chrysocharis pentheus* Walker was noticed [17]. The eulophid parasitoid, *D. isaea* was reported as the dominant larval parasitoid of *C. syngenesiae* and *C. horticola* resulting in 40 and 52.50 per cent mortality of the pest [18-20]. The results of the survey also imply that parasitoids play a significant role in the natural management of leafminer densities on crops.

4.2. Firstever DNA barcode of C. syngenesiae and D. isaea in India

DNA barcoding using *mitochondrial Cytochrome Oxidase I (mtCOI)* primer confirmed the occurrence of *C. syngenesiae* and its parasitoid as *D. isaea* at Nilgiris, Tamil Nadu, India. Thus, the COI sequences we generated for *C. syngenesiae* and *D. isaea* would be suitable for an accurate and unambiguous species identification. The COI-based DNA barcoding has proven to be a useful, trustworthy, cost-effective, and complimentary approach to classical alpha taxonomy. The COI sequences have been reported to be extremely efficient in detecting insect species, regardless of which orders they correspond to. Earlier workers have successfully utilized the DNA barcoding approach to discriminate the closely related species of diptera from horticultural ecosystem. The present study corroborates with earlier studies which have explored the outbreak populations of invasive *Liriomyza* spp. leafminer pests in the Philippines through DNA Barcoding [21]. The species specific DNA barcodes of *L. sativae* by using *mt COI* gene was developed in North Eastern India [22]. In contrast, some reports discovered the existence of *C. syngenesiae* in India, despite the lack of morphological or molecular evidences [23]. However, our study unveiled the incidence of *C. syngenesiae* for the first time in the country through DNA barcoding approach.

Funding: This research was funded by DST-Inspire, Ministry of Science and Technology, Government of India

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

Data Availability Statement: Data are available on request from the corresponding author

Conflicts of Interest: The authors declare no conflict of interest.

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