Morphological and molecular characterization of groundnut leaf miner (GLM) confirms the occurrence of *Aproaerema modicella* Deventer (Lepidoptera : Gelechiidae) in Tamil Nadu, India

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Abstract

The Groundnut Leaf miner (GLM), *Aproaerema modicella* Deventer (Lepidoptera : Gelechiidae) is a very consequential pest of groundnut and other leguminous crops which causes yield loss of upto 30 to 60 per cent on groundnut in India. Nevertheless, some scientific studies have reported the occurrence of Soybean moth, *Aproaerema simplexella* Walker attacking groundnut in India. The current study was conducted to assess the leaf miner species destructing groundnuts in different regions of Tamil Nadu. Correct identification of an insect species based on both morphological and molecular techniques may pave way for formulating accurate insect pest management strategies. In this view, investigations on the morphological characters of GLM confirmed that the species available at different districts of Tamil Nadu as *A. modicella*. However, BLAST search of GLM sequences indicated cent per cent similarity with *A. simplexella*. Since morphological characters of larva, pupa, adult, and male genitalia clearly indicated the GLM species available in Tamil Nadu, India, as *A. modicella*, the sequences acquired from the current research were submitted as the first entry to the NCBI database for species-level representation of *A. modicella.*

Keywords: *Aproaerema modicella*, Morphological identification, DNA barcoding, *mitochondrial Cytochrome oxidase I (mtCOI)*, first entry in NCBI database

Introduction

Specimen recognition at the species level is vital to recognize species diversity, phylogenetic patterns, and evolutionary associations[1]. Although among millions of species, many are quite unfathomed in the insect catalogue[2]. Conventionally, for the detection of insect pests, various

morphological features are used[3]. Nonetheless, morphology-dependent identification is frequently challenging and time-intensive. Immature stages of insects such as early larval instars and pupae are not recognised by conventional taxonomy because utmost of the taxonomic keys are available only for adult study[4]. Occasionally, phenotypic plasticity hinders conventional morphological identification[5]. Besides this, cryptic species are hard to characterize with the background of morphological characters. In addition, the successful use of taxonomic keys requires a high degree of experience[6]. To overcome this challenge, in recent times, molecular techniques are commonly employed by taxonomists to resolve the ambiguity bound with conventional taxonomic approaches[7]. Amidst these techniques, DNA barcoding is the simplest and extensively adopted approach to discriminate against the species using a short uniform gene region of the mitochondrial cytochrome oxidase subunit I[8,9]. The length of the DNA barcode is around 658 bp that acts as a species tag for an individual animal taxa[3]. Moreover, because of its pertinent sequence length and the strong universal primers, the amplification of this COI segment is very quick and secure[10]. In this regard, the correct identification of an insect species based on both morphological and molecular techniques may pave the way for formulating accurate insect pest management strategies.

Groundnut (*Arachis hypogaea* L.) also known as the 'King' of oilseeds, is an important annual, legume crop cultivated in India, China, Nigeria and Myanmar[11]. In India, it occupies an area of 4.89 M. ha with a production of 9.25 MT and a productivity of 1893 kg ha^{-1}[12]. Groundnut is being ravaged by several insects, of which, groundnut leaf miner (GLM), *Aproaerema modicella* Deventer is a devastating pest on groundnut, soybean, and other leguminous hosts in South and South-East Asia[13,14,15,16,17]. The larvae construct blister-like mines on the upper portion of the leaf near mid-rib and then, the entire leaf becomes brown,

spun, and dried up. The crop shows a burnt-up appearance in serious threat[18]. The leaf miner damage disrupts the photosynthesis in leaves and results in yield losses ranging from 50 to 100 per cent[19]. Initially, the GLM specimen collected from India was described earlier as *Anacampsis nerteria* Meyr., the same pest was referred to by five other binomials: *Biloba subsecivella* Zeller, *Stomopteryx nerteria* Meyr., *Stomopteryx subsecivella* Zeller, and *Aproaerema nerteria* Meyr. The apparent conundrum on the taxonomy was indicative of the presence of two non-congeneric leaf miners: one named *Stomopteryx subsecivella* (Zeller) from South Africa, which is believed to be *Aproaerema simplexella* Walker, the second is *Aproaerema modicella* Walker, the Indian-Indonesian groundnut leaf miner, *Aproaerema modicella* (Deventer)[20,21]. The original description of *A. modicella* was done by Deventer (1904) from a moth collected in Java, Indonesia[22]. There exists documentation that there was a species occurring in Asia alike *A. simplexella*[23]. Nevertheless, no reports confirmed, whether they were the same species. Furthermore, [24] also have suggested that species taxonomy should be re-examined using molecular methods coupled with morphological confirmation based on male genital examination, for potential synonymization of the species names previously used. Taking this into account, the GLM sequences in South Africa were matched with *A. simplexella*, although the symptoms of damage imparted by GLM observed were identical to those of *A. modicella* in Asia. As a consequence, there is a necessity to ascertain whether the two species are genetically distinct or not. In this view, morphological and molecular confirmation of GLM were carried out to ascertain the accurate *Aproaerema* species present in Tamil Nadu, India.

Materials and Methods

Morphological identification

GLM infested groundnut leaves were collected from major groundnut growing areas *viz*., Villupuram (12.147ºN 79.102º E, 138 ft), Cuddalore (12.080º N 79.037º E, 471 ft), Sivagangai (9.710º N 78.285ºE, 299 ft), Coimbatore (11.007º N 76.936º E, 1417 ft), Thiruvannamalai (12.080º N 79.037º E, 457 ft) and Thiruvallur (13.179º N 79.602º E, 364 ft) Districts of Tamil Nadu and brought to the laboratory (Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore) and further reared in a wire mesh cage (60 × 60 × 60 cm). The male, and female larva and pupa of *A. modicella* was distinguished with characters given by[25]. The emerged adults were curated, preserved and examined for morphological characteristics with the help of available literature and taxonomic expert, Dr. S. Jeyarani (Professor, Agricultural Entomology). The morphological characters described by various authors were examined under a stereozoom microscope (Stemi (Zeiss) 2000-C)[25,26,27,28]. The male genitalia of GLM was also dissected based on the previously described methodology[29] and the parts were described as per the genitalial character of GLM. In addition, the adults were preserved for DNA barcoding at -20º C.

Molecular confirmation of Groundnut Leaf miner

Genomic DNA Isolation

CTAB (Cetyl Trimethyl Ammonium Bromide) method was followed to isolate genomic DNA from single adult *A. modicella*[30]. 200 µl of DNA extraction buffer was used for homogenization and incubation was done at 65°C for 1 h in a water bath. Chloroform: Isoamyl alcohol mixture (24:1, v/v) (0.8 volume) was added in an equal proportion and blended upside down for 10 min to obtain an emulsion. After this, centrifugation at 12,000 rpm for 10 min was carried out and the obtained clear aqueous phase was shifted to a new aseptic tube. Following this, Ice-cold

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isopropanol (0.7 volume) was added to the tube, inverted for gentle mixing and stored at -20^0 C for overnight. To pellet the DNA, centrifugation at 12,000 rpm for 10 min was done and the supernatant was dispensed. The acquired DNA pellet was cleansed with 70 per cent ethanol. Then, air drying of DNA pellet was carried out and it was diluted in 20 to 40 µl of Tris-EDTA buffer based on the pellet size and stored at -20°C for further use. Agarose gel **(**0.8 per cent) was employed for checking the quality of genomic DNA. On the other hand, DNA quantification was carried out by employing Nanodrop Spectrophotometer (ND-1000). Following this, depending on the nanodrop readings, DNA dilutions were prepared in TE buffer to attain a final concentration of 50 ng µl^{-1} and stored at 4°C until further use[31].Folmer primers LCOI490 (Forward) and HCO2198 (Reverse)[9]. Forward primer (5'-3') : GGTCAACAAATCATAAAGATATTGG, Reverse primer (3'-5') : TAAACTTCAGGGTAACCAAAAAATCA was employed for amplifying the mitochondrial gene (Cytochrome oxidase I (CO1) across the populations of *A. modicella.* 20 µl of PCR products along with 10 µl each per sample of their respective forward and reverse primers were properly tagged and submitted to Agrigenome Labs Pvt. Ltd., Cochin, Kerala for sequencing. The acquired sequencing data were revived 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. The gene sequences were aligned using the ClustalW algorithm[33]. The phylogenetic tree was built using MEGA version 11.01.13 and the tree was drawn using UPGMA method.

Results and Discussion

The effective execution of an integrated pest management (IPM) programme relies on the precise recognition of agricultural pests. The mainstay of insect pest detection in the former and present has indeed been morphological examinations, which provide complementary, easier, and more reliable choices for species identification[34]. Observations on the morphological characters of GLM larvae disclosed the occurrence of pink coloured gonads on sixth and seventh abdominal segments which was reported to be a discriminating character of a male *A. modicella* larvae. The gonads were clearly evident through the cuticle even with an unaided eye (Fig. 1). Apart from this, a blackspot on 5th and 6th abdominal tergite of male pupa was also observed as per the literature (Fig. 2). One of the defining morphological characteristics of both male and female *A. modicella* pupae is the location of the genital opening. The present study showed that the male pupa's genital aperture is located in the middle third of the last abdominal segment, while the female pupa's genital aperture is located in the one-third of the last abdominal segment, closer to the proximal side of the segment (Fig. 3). Adult moth characters specific to *A. modicella viz.*, single transverse white spot on each forewings, white end at the second joint of the labial palpi were also recorded (Fig. 4). This is in corroboration with [18] who reported that *A. modicella* adult is found to be a grey mottled moth, having transverse white band in each fore wings with a full wing span in India. Examination of male genitalia revealed characteristic bilobed uncus; uncus with dense brush of erect setae, valvae parallel sided with no sharp median tooth on ventral side. Tegumen, Juxta present and gnathos with hook like process, the cucullus arched dorsally and the saccus large, gutter-like. The aedeagus straight tube-like with long basal ansiform process, which is longer than aedeagus. Aedeagus narrowed towards apex with longer rod-like process (Fig.4). The morphological observations clearly indicated that the leafminer species infesting groundnut was *A. modicella*. This corroborates with the findings of[27,28,34.]

Apart from morphological characterization, recognition method employing DNA barcoding contributes in the discovery of novel species as well as the identification of established ones[9]. Molecular characterization of GLM from different districts *viz.*, Villupuram, Thiruvannamalai, Cuddalore, Thiruvallur, Sivagangai and Coimbatore utilizing *mtCOI* primer resulted in amplified product of approximately 680 bp length. BLAST searching of those sequences in GenBank indicated cent per cent sequence similarity with *A. simplexella*. However, based on the morphological observations, the groundnut leaf miner species available in Tamil Nadu, India was confirmed as *A. modicella*. Since, there was no record of molecular sequence of *A. modicella* available hitherto, the sequences acquired from the current research were submitted as the first entry in NCBI database for species level identity of groundnut leaf miner, *A. modicella* and accession numbers were obtained. The sequences of *A. modicella* was compared with other *Aproaerema* spp. available in NCBI and BOLD databases. Multiple Sequence Alignment (MSA) of all the *Aproaerema* spp. was done using UniproUGENE 48.1 software which represented a remarkable nucleotide divergence (Fig. 6). Phylogenetic tree of *Aproaerema* spp. was also constructed with mtDNA (COI) partial sequences using UPGMA method in mega 11.01.13 (Fig. 7). The phylogenetic tree results depicted that *A.* modicella from different regions of Tamil Nadu and *A. simplexella* from Canada and Madagascar has 98 percent homology with *A. anthyllidella* from France and *A. coracina* from Australia. In specific, *A. modicella* from Villupuram and Thiruvannamalai have 74 per cent homology. Nevertheless, the blast analysis of the DNA sequences of GLM showed cent per cent sequence similarity with *A. simplexella.* Also, Phylogenetic tree of *Aproaerema* spp. indicated that *A. modicella* population from Tamil Nadu and *A. simplexella* from Madagascar and Canada clustered together in a same cluster that shows a closer homology . This is in corroboration with [24]who reported the

groundnut leaf miner populations available at Africa, India and Australia as *A. simplexella* through molecular characterization. Nevertheless, they did not publish any morphological characters in support of *A. simplexella* [12,24,35,36] and classified *A. modicella, A. simplexella and S. subsecivella* as conspecific species by employing molecular (mtDNA COI) and ecological study tools. Leaf miner attacking groundnuts and soya bean in Uganda was also documented as *A. simplexella* through DNA barcoding[37]. In contrast, the present study based on morphological characterization clearly confirmed that the groundnut leaf miner species available at all our surveyed location is *A. modicella* and hence, the DNA sequences of *A. modicella* obtained in this study were submitted as the first entry in NCBI database (Sivagangai- MN525164, Thiruvannamalai- MN525165, Thiruvallur - MN525166, Cuddalore- MN525167, Coimbatore - MN525168, Villupuram - MN525169) as a representation for the species level identity of groundnut leaf miner. Thus, traditional taxonomy and DNA barcoding should not be viewed as adversarial tools, but rather as supportive techniques for addressing the taxonomy, phylogeny and nomenclature of living species[38]. Moreover, *A. modicella*, *A. simplexella* and *Stomopteryx simplexella* (Walker) remains conspecific and morphologically similar, it builds conundrum to identify the species. Furthermore, an elaborate study on molecular and phylogeny has to be carried out on a global scale including more sample size of all the species. Hence, different gene regions of mitochondrial and nuclear DNA (COII, cytb, 28S and EF-1 ALPHA) has to be analyzed. Also, the offseason survival methods of various populations of *A. modicella* in the different geographical regions have to be investigated in future.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgement

This research was supported by research grant from the Department of Science and Technology – INSPIRE, Ministry of Science and Technology, Government of India.

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