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Research Article

Molecular characterisation of tomato leaf miner *Tuta absoluta* populations obtained from different geographical locations of India

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ABSTRACT: The tomato leaf miner, *Tuta absoluta* (Meyrick) is one of the major invasive insect pests on solanaceous crops. *T. absoluta* distribution is observed in European, North African Mediterranean basin and Asian countries. The tomato leaf miner is spreading fast and affecting tomato production in both closed and open-field conditions. This insect pest has recently reported in various tomato growing regions of India and causing significant loss of production. In the present study, occurrence of a tomato leaf miner was studied by undertaking an extensive survey in tomato growing regions of India during September 2017 to May 2018. The infestation of *T. absoluta* was recorded in Maharashtra, Karnataka, Telangana, Tamil Nadu, Haryana and Himachal Pradesh. Severe incidence of *T. absoluta* infestation was recorded in Maharashtra (Nashik district) and Telangana (Mahabubnagar district) followed by Karnataka (Kalaburgi and Raichur districts). The moths collected during the survey were characterized using mitochondrial cytochrome c oxidase subunit I (COI) gene analysis. The *T. absoluta* samples showed maximum genetic similarity with KU565720 Kenya, KT452897 Oman, KY212128 South Africa, KY619687 India, KP814057 India and KJ657679 Florida. Nine *T. absoluta* populations were grouped under a single clade revealing no genetic variation within populations and showed high genetic homogeneity thereby the ideal candidate for sterile insect technique application. Further studies are required on population dynamics, host range, local and area-wide biological control management strategies for the effective management of *T. absoluta*.

KEY WORDS: MtCOI**,** phylogenetic analysis, SIT, *Tuta absoluta*

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INTRODUCTION

Tomato leaf miner, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae) is a major and invasive pest of tomato and other Solanaceous crops in the world (Zappala *et al.,* 2013; Tonnang *et al.,* 2015; Biondi *et al.,* 2018). While, Solanaceae plants are the main host plants for *T. absoluta*, tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), potato (*Solanum tuberosum* L.), pepper (*Capsicum annuum* L.), tobacco (*Nicotiana tabacum* L.) are the major hosts apart from other solanaceous weeds (Desneux *et al.,* 2010; Bawin *et al.*, 2016; Abbes *et al.,* 2016; Biondi *et al.,* 2018). Generally, *T. absoluta* attacks all developmental stages of plants, exhibits high reproduction potential and dispersal ability. Female adult lay eggs on leaves and early fruits, the neonate larvae enter the mesophyll, which forms galleries, thereby making the plant prone to secondary infection by pathogens. The larvae can also enter the stem through buds, flowers, and causes severe damages resulting complete loss of the plant. Eventually, all these events lead to reducing both quality and yield of tomato (Desneux *et al.,* 2010; Ballal *et al.,* 2016). Tomato leaf miner was first reported in 1914 in Peru, and currently this insect is a common pest in South America (Jham *et al.,* 2001). During 2006, *T. absoluta* was reported from Spain and then onwards it rapidly invaded across the Mediterranean coastal tomato-producing areas (Desneux *et al.,* 2010; Desneux *et al.,* 2011). Later, it invaded Europe, Africa and Asia, where it is causing significant damage to tomato crop (Desneux *et al.,* 2011; Ballal *et al.,* 2016; Biondi *et al.,* 2018).

In India, *T. absoluta* was first reported during 2014-15 in and around Bengaluru in Karnataka (Sridhar *et al.,* 2014) and Pune in Maharashtra (Shashank *et al.,* 2015). Subsequently, it has been reported from several states of India causing up to 90.0-100.0% tomato fruit damage (Kalleshwaraswamy *et al.,* 2015; Kumari *et al.,* 2015; Ballal *et al.,* 2016; Swathi *et al.,* 2017; Rasheed *et al.,* 2017; Sidhu *et al.,* 2017; Balaji *et al.,* 2018). Thus, it is considered as a key pest of closed as well as open-field tomato cultivation which threatens tomato growers and allied industries.

Currently, selected insecticides, pheromone traps, biorational insecticides, egg parasitoids and resistant varieties of tomato are being used for suppression of *Tuta* population (Zappala *et al.,* 2013; Ballal *et al.,* 2016; Biondi *et al.,* 2018), but mainly the control is based on the broad spectrum insecticides. However, insecticide-resistance problems have already been reported from several parts of the world (Siqueira *et al.,* 2000; Lietti *et al.,* 2005; Campos *et al.,* 2015). The tomato leaf miner is known to have high reproductive potential, greater adaptability, and invasion capacity, hence considered for a world-wide pest management programme to curtail economic losses. The Sterile Insect Technique (SIT) is an environmentally friendly, sustainable and species-specific method of pest control based on mass rearing of the target pest, sterilization and releasing of sterile males (Dyck *et al.,* 2005). The sterile males will mate with wild females of the target pest population and are unable to produce viable offspring (Dyck *et al.,* 2005). This method has been successfully employed against various lepidopteran insect pests, including *Cydia pomonella* (Linnaeus), *Pectinophora gossypiella* (Saunders) (Tan, 2000; Bloem *et al.,* 2005). The effects of gamma radiation on several moths have also been investigated including *T. absoluta* (Cagnotti *et al.,* 2012; Kuyulu and Genc, 2016).

The success of pest management relies on the proper identification of target insect pests, which are generally identified based on morphological features (Karthika *et al.,* 2016). Molecular characterization and DNA barcoding is a standard taxonomic method that uses a short genetic marker in an insect DNA to identify a species (Jalali *et al.,* 2015). Partial DNA sequences of the mitochondrial gene such as cytochrome c oxidase subunit I (COI), Internal Transcribed Spacer (ITS) regions of rDNA and other molecular markers have been used to assess genetic diversity of *T. absoluta* populations (Suinaga *et al.,* 2004; Cifuentes *et al.,* 2011; Bettaïbi *et al.,* 2012; 2016; Shashank *et al.,* 2018). The study of genetic variability of invasive *T. absoluta* is essential to develop an efficient Integrated Pest Management (IPM) programs (Bettaïbi *et al.,* 2012). In this study, we have made an extensive survey to collect the wild population of *T. absoluta* from different locations of India and identify the *Tuta* samples using *COI* gene analysis.

MATERIALS AND METHODS

Field survey and collection of tomato leaf miner samples and storage

The tomato leaf miner infestation on tomato crop field was identified with the help of National Research Institutes and Agricultural Universities. We used tomato leaf miner pheromone lures from Pest Control (India) Pvt. Ltd. to survey and collect *Tuta absoluta* samples. The samples were collected from various locations of different states during September 2017-May 2018 (Table 1). For each location, 4-5 tomato crop fields were identified and traps were installed. The funnel traps were placed 40 cm above the ground and a spacing of 20 m was maintained between the traps. Individual moths collected from pheromone traps were kept in separate vials with 800 µl of absolute ethanol and kept at -25°C until DNA extraction. *T. absoluta* adults were identified on the basis of morphological descriptions given by Genc (2016) and Visser *et al.* (2017).

Molecular characterization of tomato leaf miner

The incidence of *T. absoluta* in different states of India was confirmed by DNA barcoding technique using standardized DNA barcoding region of mitochondrial *COI* gene (Table 1).

Table 1. Survey locations of tomato leaf miner, *Tuta absoluta* in India

State	Districts	Latitude- Longitude	Crop/Stage of the crop
Maharashtra	Nashik	19°76' N 72°97' E	Tomato/Flowering and fruiting
Karnataka	Raichur	16°21' N 77°34' E	Tomato/Flowering and fruiting
	Kalaburagi	17°32' N 76°°83' E	Tomato/Fruiting
	Vijayapura	16°83' N 75°71' E	Tomato/Flowering and fruiting
Telangana	Mahabubnagar	16°38' N 78°11' E	Tomato/Flowering and fruiting
Tamil Nadu	Coimbatore	11° 01 N, 76.95' E	Tomato/Flowering and fruiting
	Dharmapuri	12°09' N, 78°20' E	Tomato/Flowering and fruiting
Haryana	Hisar	29°14' N, 75°72' E	Tomato/Seedlings, flowering
Himachal Pradesh	Mandi	31°58' N, 76°91' E	Tomato/Flowering and fruiting
Bihar	Bhagalpur	25°34' N, 86°98' E	Tomato/Flowering and fruiting
West Bengal	Nadia	22°97' N, 88°43' E	Tomato/Seedlings, flowering and fruiting

Extraction of total genomic DNA

Total genomic DNA of individual sample moth was extracted (5-10 individuals per location) using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Augustinos *et al.,* 2011). Each moth was individually grounded into fine powder by using TissueLyser II (QIAGEN). 300 µl of pre-warmed (60°C) DNA extraction buffer (5% CTAB, 1M Tris HCl pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl and 4% β-mercaptoethanol) was added to the microcentrifuge tube containing *T. absoluta* powder. The preparation was incubated at 60°C for 30 min by gentle mixing at regular intervals. After incubation, equal volume of 300 µl of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed properly by inverting the tubes several times followed by centrifugation at 8000 rpm for 10 min to remove the aqueous phase. Aqueous phase was transferred to new microcentrifuge tube and 150 µl pre-chilled isopropanol was added and kept at -20°C for 20-30 min to precipitate the DNA. Tubes were then spun at 12,000 rpm for 12 min and supernatant was discarded. The DNA pellet was washed with 70% pre-chilled ethanol, dried and dissolved in 30 µl of Tris EDTA buffer (10mM Tris-HCl pH 8.0 and 1mM EDTA). The quantity and quality of DNA obtained was checked by agarose gel electrophoresis and Nanodrop 2000 (Thermo Scientific NanoDrop™ 2000/2000c Spectrophotometer). The DNA samples were stored at -25°C until further use.

COI gene amplification, sequencing and phylogenetic analysis

The total genomic DNA was extracted from 75 individual adults of *T. absoluta* collected from different locations. PCR was carried out using universal primers, forward primer (LCO1490) 5'-GGTCAACAAATCA TAAAGATATTGG-3' and reverse primer (HCO2198) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer *et al.,* 1994) to amplify a ~710 bp fragment of the mitochondrial gene *COI*. The PCR reaction was performed in 20 µl reaction volume containing 2 µl of 10X reaction buffer with $MgCl_2$ (15 mM), 0.5 µl dNTPs (25 mM), 0.5 µl of each primer (25 µM), 0.5 µl (5U/µl) of *Taq* DNA polymerase (BRIT, India) and 1 µl of template DNA. Amplifications were performed using thermal cycler (Mastercycler gradient, Eppendorf, Germany) with an initial denaturation step of 1 min at 95°C followed by 35 cycles at 95°C for 45 sec, 54°C for 45 sec, 72°C for 1 min and final elongation step at 72°C for 10 min. After amplification, the PCR products were resolved on 0.8% agarose gel to confirm the amplification of *COI* gene. The positive amplicons of *COI* gene from PCR product were purified by Pure Link PCR purification Kit (Invitrogen) and sequenced using Big Dye Terminator V3.1 Cycle Sequencing Kit. The selected purified PCR products were directly sequenced in both direction using LCO1490 and HCO2198 primers in ABI 3730xl cycle sequencer.

COI gene sequences of *T. absoluta* samples from different locations were confirmed through BLASTN in the GenBank database in National Centre of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) for identification purposes. Moreover, additional sequences of COI of *T. absoluta* of different regions of the world were obtained from GenBank database and a multiple alignment was carried out using ClustalW in MEGA version 6 (Tamura *et al.,* 2013). Phylogenetic relationships between *T. absoluta* population of different locations and other sequences of *T. absoluta* were analysed in MEGA version 6 (Tamura *et al.,* 2013). Neighbor-Joining phylogeny was constructed using MEGA version 6 with bootstrap test (1000 replications).

RESULTS AND DISCUSSION

Survey and incidence of tomato leaf miner

The survey was undertaken in 11 districts belonging to 8 states of India during September 2017 to May 2018 (Table 1) for the collection of *Tuta absoluta* samples. The occurrence of *T. absoluta* was recorded in Maharashtra, Karnataka, Telangana, Tamil Nadu, Haryana, and Himachal Pradesh while, infestation was not observed from Eastern Indian states of Bihar and West Bengal during the survey period. Number of *T. absoluta* larvae trapped in pheromone traps varied depending on locations (Fig. 1). The trapped *T. absoluta* samples ranged from 20-100 adults per trap and suggests varied with population size and location to location. Similarly, Mutamiswa *et al.* (2017) observed that trapping of varying *T. absoluta* moths depending on location and whether the traps were installed in open tomato fields or in other habitats. Severe incidence of *T. absoluta* was recorded in Maharashtra (Nashik district) and Telangana (Mahabubnagar district) followed by Karnataka (Kalaburgi and Raichur districts) (Fig. 1). Recently, the occurrence of *T. absoluta* on tomato crop was also recorded from other

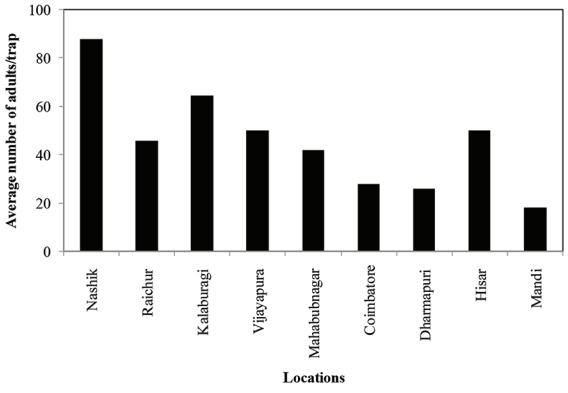

Fig. 1. Average number of *Tuta absoluta* male moths/trap from different locations of India by using *Tuta* pheromone traps.

states of India. *T. absoluta* was reported first time in 2014-15 in and around Bengaluru (Sridhar *et al.,* (2014) and Pune (Shashank *et al.,* 2015). Subsequently, it has been reported from different states including Maharashtra, Tamil Nadu, Andhra Pradesh, Telangana, Gujarat, Chhattisgarh, Punjab, Madhya Pradesh (Kalleshwaraswamy *et al.,* 2015; Kumari *et al.,* 2015; Shashank *et al.,* 2016; Taram *et al.,* 2016; Ballal *et al.,* 2016; Swathi *et al.,* 2017; Rasheed *et al.,* 2017; Sidhu *et al.,* 2017; Balaji *et al.,* 2018;). Recently, extensive survey was carried out for the occurrence of *T. absoluta* in Tamil Nadu and reported that severe incidence was recorded on tomato from Tamil Nadu Agricultural University (TNAU) orchard (92.50 %) followed by Krishnagiri district (89.70%) and Dharmapuri district (82.40 %) (Balaji *et al.,* 2018), while, certain locations of Kalyani (West Bengal), Ludhiana (Punjab), Bhubaneswar (Odisha), Raipur (Chhattisgarh), Hyderabad (Telangana), Rahuri (Maharashtra), Varanasi (Uttar Pradesh) and New Delhi (Delhi) were not infested with *T. absoluta* during 2014 (Sridhar *et al.,* 2014). Later, the incidence of *T. absoluta* on tomato at Vegetable Research Station, Rajendranagar, Telangana State and Ludhiana and Patiala districts of Punjab was reported (Kumari *et al.,* 2015; Sidhu *et al.,* 2017). Therefore, the incidences of *T. absoluta* are more prevalent in Southern and Western states as compared to Northern and Eastern states of India. However, infestation of *T. absoluta* may also be present in other states of India which needs to be monitored.

Tomato crop damage symptoms

The tomato fields showed extensive damage due to *T. absoluta*. The infested leaves showed different sizes of blotches, completely devoid of chlorophyll and dried up appearance in case of severe damage. All ages of tomato fruits showed typical damage symptoms with internal feeding with pinhead exit holes and substantial frass. Early stage fruits were more infested than nearly matured fruits. Most of the matured fruits damaged by *T. absoluta* showed signs of secondary infection thus making the fruit unfit for consumption. The collected *T. absoluta* samples were identified based on the external characters such as moth body length (ca. 5-6 mm) and wing span (ca. 8-10 mm). Forewings narrow, with brown, grey and black mottling; hindwings lanceolate, dark grey with long cilia. Antennae, labial palpi and legs with dark brown and grey banded appearance; antennae long and filiform, labial palpi prominent and curved upward (Genc, 2016; Visser *et al.,* 2017; Sidhu *et al.,* 2017).

Molecular analysis

The sample populations of *T. absoluta* collected from different locations in India were characterized using standardized mitochondrial *COI* gene sequencing approach. DNA of the *T. absoluta* populations from nine locations was extracted and *COI* gene was amplified using *COI* specific primers and sequenced. The PCR amplified product length was approximately 650 bp in all the *T. absoluta* samples. *COI* gene sequences of *T. absoluta* samples from different locations were confirmed through BLASTN in the GenBank database in NCBI. The present study samples show maximum genetic similarity with some sequences like KU565720 (Kenya), KT452897 (Oman), KY212128 (South Africa), KY619687 (India), KP814057 (India) and KJ657679 (Florida). The sequences of present study and certain reference sequences showed 100% identity in *COI* gene sequences. *T. absoluta* samples collected from different states of India also employed for *COI* gene amplification and further confirmed through sequencing (Sidhu *et al,* 2017; Balaji *et al,* 2018). *T. absoluta* samples collected from different locations of Tamil Nadu were identified by using *mtCOI* gene sequencing and sequences were also showed maximum similarity with Oman, Bosnia and Florida (Balaji *et al.,* 2018). So far, *T. absoluta* has been reported from Southern India to Northern India; now it has been stretching to Central and Eastern parts of India (Ballal *et al.,* 2016; Sidhu *et al.,* 2017; Rasheed *et al.,* 2017; Balaji *et al.,* 2018). Recently, it has been reported from adjacent countries like Bangladesh (Hossain *et al.,* 2016) and Nepal (Bajracharya *et al.,* 2016). This indicates that *Tuta* is invading new areas because of its high reproductive capacity and availability of host throughout the year and lack of strong phytosanitary measures during trade or other means of logistic.

The phylogenetic analysis revealed that two main clades were formed based on the *COI* gene sequences of field collected and reference *Tuta* samples (Fig. 2). The sequences from Turkey (MF044028), Serbia (JN417243), Montenegro (KC852870), Bosnia and Herzegovina (KC852869) were clubbed in clade II, while some sequences from Tunisia (JQ749676 and JQ749677) were grouped in clade IB. Several sequences of *COI* of *T. absoluta* from Florida, India, South Africa, Kenya and Oman were grouped in single calde IA (Fig. 2). These sequences showed maximum genetic similarity with present study COI sequences of *T. absoluta*. This indicates that field collected samples of *T. absoluta* populations were grouped under single clade IA revealing no genetic variation within populations and showed high genetic homogeneity, suggesting *T. absoluta* is being expanding to different geographical regions through spread after its introduction. Similarly, high genetic homogeneity was observed in *T. absoluta* samples collected from five locations of India and one from Nepal based on the mtCOI analysis (Shashank *et al.,* 2018). Asma *et al.* (2017) found high genetic homogeneity using mtCOI sequences of seven Tunisian populations of *T. absoluta* and concluded that this

was introduced from a single source in Tunisia. Based on

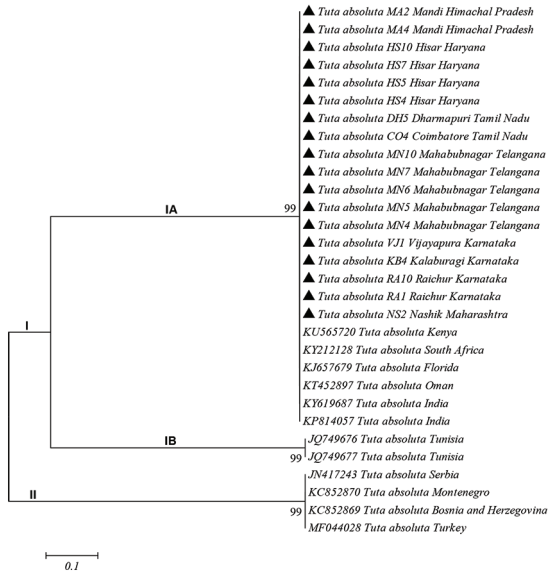

Fig. 2. Phylogenetic analysis based on representative *COI* gene sequences of natural population of *Tuta absoluta* and other sequences of *T. absoluta* using the neighbor joining method ('▲' Represents current study *Tuta* samples).

the ITS 1, 2 and COI sequences of *T. absoluta* populations from the Mediterranean Basin and South America showed high genetic homogeneity (Cifuentes *et al.,* 2011). While, eight Brazilian populations of *T. absoluta* using the Amplified Fragment Length Polymorphism (AFLP) technique showed differences in the populations responses to insecticides as well as host plants (Suinaga *et al.,* 2004). Other studies on Tunisian *T. absoluta* using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) technology resulted high genetic diversity as well as in a significant differentiation between populations (Bettaïbi *et al.,* 2012). Guillemaud *et al.* (2015) reported that the native population of *T. absoluta* in South America is far from genetically homogeneous based on microsatellite markers and illustrated source of invasive population with the hypothesis of single versus multiple introductions. Further research is essential to investigate the genetic variability for *T. absoluta* based on different molecular markers with larger samples from many locations and possible host-plants.

The outcome of this study including distribution and molecular characterisation of *T. absoluta* would be vital for future research for this insect pest. Although, this insect pest has already prevalent in different states of India, continuous monitoring of infestation on tomato and other vegetables crops is required. Currently, tomato leaf miner is being managed locally using field sanitation, sex pheromone traps, augmentation of biocontrol agents, and soft insecticides (Ballal *et al.,* 2016). In addition, this insect pest has been considered in wide area management program including sterile insect technique for suppression/eradication in closed and/or open field conditions. However, further studies on mass rearing, radiation dose optimization and competitiveness of sterile males are needed before field validation. In addition, population dynamics, host range, insecticide resistance and natural enemies of *T. absoluta* would be another important area for future research which will facilitate effective management of *T. absoluta* population using IPM module.

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