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## Analysis of genetic variation in an important pest, *Tuta absoluta*, and its microbiota with a new bacterial endosymbiont

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**Abstract:** Tomato leaf miner is an aggressive and invasive pest that causes serious damage in tomato production, resulting in up to 100% yield losses during serious outbreaks. The management of tomato leaf miner with synthetic pesticides is difficult due to its leaf mining habit, short life span, high reproductive rates, and insecticide resistance problem. In this study, sequences of the mitochondrial *cytochrome oxidase I* (mtCOI) gene of *Tuta absoluta* specimens were analyzed and compared with the available COI sequence data members from GenBank. All the specimens of tomato leaf miner were clustered together in a single group with a high support value, suggesting the presence of a homogeneity in the *T. absoluta* specimens. Furthermore, 164 samples were analyzed in order to detect the prevalence of *Arsenophonus*, *Cardinium*, *Hamiltonella*, *Pantoea*, *Spiroplasma*, and *Wolbachia* infection in *T. absoluta* populations. PCR analysis revealed 100% prevalence of *Wolbachia* and *Pantoea*; however, no other symbiotic bacterial infections were observed in *T. absoluta* populations. Finally, *Wolbachia* *wsp*, *Pantoea* 16S ribosomal RNA genes, and mtDNA COI haplotypes were analyzed in order to estimate the mitochondrial effects of these endosymbionts on the population structure of tomato leaf miner. The findings of high genetic homogeneity among *T. absoluta* populations with high prevalence of *Wolbachia* and *Pantoea* infections seem to imply that both bacterial infections have less impact on the mtDNA variations.

**Key words:** Endosymbionts, genetic variation, mitochondrial DNA, tomato leaf miner

### 1. Introduction

Tomato (*Lycopersicon esculentum* Miller) is one of the most widely grown vegetables in the world<sup>1</sup>. Tomato leaf miner, *Tuta absoluta* (Lepidoptera: Gelechiidae) is an aggressive pest that causes serious damage in tomato production, resulting in up to 100% yield losses during serious outbreaks (Flores et al., 2003; Saidov et al., 2018). Among the lepidopteran pests of tomato crop, *T. absoluta* is considered as a highly invasive agricultural pest, which is native to Latin America and has spread through most of Europe, Mediterranean regions, and many other regions worldwide (Hassan and Alzaidi, 2009; Mansour et al., 2018). This pest is known as an oligophagous pest attacking solanaceous crops, including eggplant, pepper, potato, sweet pepper, tobacco (Pereyra and Sánchez, 2006; Kanle Satishchandra et al., 2019), as well as solanaceous weeds (Shashank et al., 2018). Direct feeding of the pest not only damages all parts of tomato plants, but also the entrance of

pathogens through the wounds results in indirect damage (EPPO, 2005). It is difficult to manage tomato leaf miner with synthetic pesticides due to its leaf mining habit, short life span, and high reproductive rates, which results in rapid development of insecticide resistance (Guedes et al., 2019).

Symbiotic relationships between insects and microorganisms have the potential to influence the biology and ecology of their hosts. Depending on the type of symbiotic associations, they can be involved in providing nutritional benefits, host plant preference, reproductive alteration, and adaptation to environmental conditions, as well as in insecticide resistance (Kontsedalov et al., 2008). Detection of microbial fauna of insects is important to highlight the problems caused by these microorganisms to their hosts. The interactions between bacterial microorganisms and their hosts are important in terms of the host's fitness, ecology, and evolutionary biology (Gurung et al., 2019).

<sup>1</sup> Food and Agriculture Organization of the United Nations (FAO) (2012). FAOSTAT [online]. Website <http://www.fao.org/faostat/en/> [accessed 14 March 2020].

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*Wolbachia* is widely distributed with about 70% of all insect species (Werren and O'Neill, 1997), which regulates host reproduction through cytoplasmic incompatibility (CI), feminization, male killing, and parthenogenesis (Werren, 1997; Stouthamer et al., 1999). *Wolbachia* also plays a major role in affecting the mtDNA variation of its hosts (Hurst and Jiggins, 2005; Moran et al., 2008; Charlat et al., 2009; Feldhaar, 2011; Richardson et al., 2012; Bennett and Moran, 2015; Schuler et al., 2016; Schuler et al., 2018). Mitochondrial DNA (mtDNA) has advantages compared to nuclear DNA, such as the absent or very infrequent recombination conjoint with their reduced effective population size and relatively high mutation rates. These properties make this genome suitable for characterization in the level of inter- and intraspecies (Saccone et al., 2000), as well as use in phylogeography (Hurst and Jiggins, 2005).

*Pantoea* is another common bacterium with more than 20 species that have adapted to diverse ecological niches, including humans, insects, plant material, soil, and water (Walterson and Stavrinides, 2015). Recent studies showed that *Pantoea* species contribute to providing essential nutrients limited in the host's diet (Kenyon et al., 2015), participating in creating a nitrogen-rich environment for developing eggs and larvae (MacCollom et al., 2009) and breakdown of plant cell-wall biopolymer (Bozorov et al., 2019). *Pantoea* isolates have been reported from different insect species, such as fruit fly (Lauzon et al. 2009) and Asian citrus psyllid (Guz et al. 2020b).

In this study, samples were collected from different geographic locations of Iran and Turkey in order to investigate genetic diversity and differentiation of *T. absoluta* populations. The nucleotide sequences of the mtCOI gene of these populations were analyzed and compared with the previously reported COI sequence data of tomato leaf miner members from GenBank. Furthermore, 164 samples were analyzed in order to detect the prevalence of *Arsenophonus*, *Cardinium*, *Hamiltonella*, *Pantoea*, *Spiroplasma* and *Wolbachia* infection in *T. absoluta* populations. Finally, *Wolbachia* *wsp*, *Pantoea* 16S ribosomal RNA (16S rRNA) genes and COI haplotypes were analyzed to estimate the mitochondrial effects of these endosymbionts on tomato leaf miner population structures.

## 2. Materials and methods

Samples of *T. absoluta* were collected during 2019 and 2020 from 15 different major tomato-growing areas of West-Azerbaijan of Iran, and Turkey. The collected specimens were transferred to 70% ethanol and preserved at 4 °C until DNA extraction. The details of the collected samples are shown in Table 1.

To avoid any contamination, each larvae and adult sample was exposed to 1% sodium hypochlorite, followed by 2 times distilled water rinse prior to DNA extraction.

**Table 1.** Collection sites.

Location	Coordinates
Salmas	38° 11' 41" N 44° 45' 53" E
Salmas (Kochameshk)	38° 08' 59" N 44° 47' 25" E
Khoy	38° 33' 01" N 44° 57' 08" E
Urmia	37° 33' 19" N 45° 04' 21" E
Urmia (Hesar-e Torkaman)	37° 26' 34" N 45° 13' 08" E
Urmia (Qotlu)	37° 30' 24" N 45° 08' 18" E
Urmia (Tala Tappeh)	37° 43' 13" N 45° 10' 47" E
Urmia (Satlu)	37° 25' 18" N 45° 08' 54" E
Urmia (Kashtiban)	37° 33' 26" N 45° 14' 24" E
Oshnavieh	37° 02' 23" N 45° 05' 54" E
Miandoab	36° 58' 10" N 46° 06' 10" E
Naghadeh	36° 57' 19" N 45° 23' 17" E
Van, Turkey	38° 29' 39" N 43° 22' 48" E
Fethiye, Muğla, Turkey	36° 39' 33" N 29° 7' 35" E
Menemen, İzmir, Turkey	38° 36' 58" N 27° 8' 49" E

Total genomic DNA was isolated individually from 164 specimens, including 19 adults and 146 larvae, using a modified cetyltrimethylammonium bromide protocol (CTAB) with minor modifications (Doyle and Doyle, 1987; Guz et al., 2020a). Briefly, each specimen was homogenized in lysis buffer (100mM Tris, 50mM EDTA, 1.4M NaCl, 2% CTAB) and incubated at 65 °C for 12 h. After chloroform-isoamyl alcohol (24:1) extraction, the DNA samples were precipitated using isopropanol. The quality and quantity of DNA extracts were estimated using electroporation in 1% agarose gel containing Pronasafe Nucleic Acid Staining Solution (Laboratorios Conda, S.A., Madrid, Spain) and NanoDrop2000 spectrophotometer (Thermo Scientific Scientific Inc., Waltham, MA, USA). Each DNA extract was standardized to 100 ng/μL. PCR details used to amplify an mtCOI fragment and bacterial endosymbionts are given in Table 2. Fifteen purified PCR amplicons of COI gene were bidirectionally Sanger sequenced at Macrogen, Inc (Seoul, South Korea).

The forward and reverse nucleotide sequences representing 15 specimens were assembled, edited, and manually aligned, using Geneious R9 (Kearse et al., 2012). Obtained sequences were submitted to the GenBank with the accession numbers of MT328190-MT328204. A sampling dataset of the COI gene region of *T. absoluta* was generated from 594 specimens (15 from this study and 579 sequences from NCBI annotated database representing different localities) (Table 3).

The number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity ( $\pi$ ) were calculated using

**Table 2.** Details of the PCR conditions and primers used in the study.

Target gene	Primer sequence (5'-3')	Tm (°C)	Reference
<i>Hamiltonella</i> 16S rDNA	Ham-F: TGAGTAAAGTCTGGAATCTGG	60	Chiel et al., 2007
	Ham-R: AGTTCAAGACCGCAACCTC		
<i>Arsenophonus</i> 23S rDNA	Ars23S-1: CGTTTGATGAATTCATAGTCA AA	50	Thao and Baumann, 2004
	Ars23S-2: GGTCTCCAGTTAGTGTTACC CAAC		
<i>Cardinium</i> 16S rDNA	CFB-F: GCGGTGTAATAATGAGCGTG	58	Weeks et al., 2003
	CFB-R: ACCTMTTCTTAACTCAAGCCT		
<i>Wolbachia</i> wsp gene	wsp81F: TGGTCCAATAAGTGATGAAGAAAC	55	Braig et al., 1998
	wsp691-R: AAAAATTAACGCTACTCCA		
<i>Pantoea</i> 16S rRNA	Panteo-F: ACGGAGGGTGCAAGCGTTAAT	56	Guz et al., 2020b
	Panteo-R: AGGTAAGGTTCTTCGCGTTGCA		
<i>Spiroplasma</i> 16S rRNA	16SA1: AGAGTTTGATCMTGGCTCAG	55	Tsuchida et al., 2002
	TKSSpR: TAGCCGTGGCTTCTCGGTAA		
Universal COI	dgLCO-1490: GGTCAACAAATCATAAAGAYATYGG	45	Meyer, 2003
	dgHCO-2198: TAAACTTCAGGGTGACCAAARAAYCA		

DnaSP v5 (Librado and Rozas, 2009). The level of genetic differentiation between specimens was quantified via estimating net p-distance value, using MEGA X (Kumar et al., 2018). The principal coordinate analysis (PCoA) was performed using the GenAEx v6.5 (Peakall and Smouse, 2006;2012) based on the matrix of pairwise p-distance values between specimens.

In order to estimate the phylogenetic relationship of the haplotypes of *T. absoluta* specimens, phylogenetic trees were constructed using neighbour-joining (NJ) and maximum likelihood (ML) approaches. *Symmetrischema lectulifera* (KY951829) was selected as an outgroup. For phylogenetic tree constructions, the best-fit model of DNA substitution and the parameter estimates were used in MEGA X program (Kumar et al., 2018). The NJ tree was constructed in MEGA X under the algorithm of Kimura 2 parameter with Gamma distribution (Gamma parameter: 0.11) with 1.000 bootstrapping. ML tree was built using PhyML v3.1 (Guindon et al., 2010) under GTR+G+I substitution model. The visualization of the trees was carried out using FigTree v1.4.2<sup>2</sup>.

### 3. Results

Our dataset consisted of the 594 COI sequences representing 15 specimens from this study and 579 specimens from previously reported studies. After alignment and trimming, the remaining length of sequences was 558 bp. In total, 16 variable positions were detected including 13 singleton variable sites. Diversity indices of haplotype (Hd) and nucleotide ( $\pi$ ) were 0.040

$\pm 0.011$  and  $0.00014 \pm 0.00006$ , respectively. The retrieved haplotype number was 8, and 5 of them were definable by a single mutation difference and were unique to a single location. The haplotype Hap1 was the most abundant and widespread, as it was shared in 97.98% of the sequences.

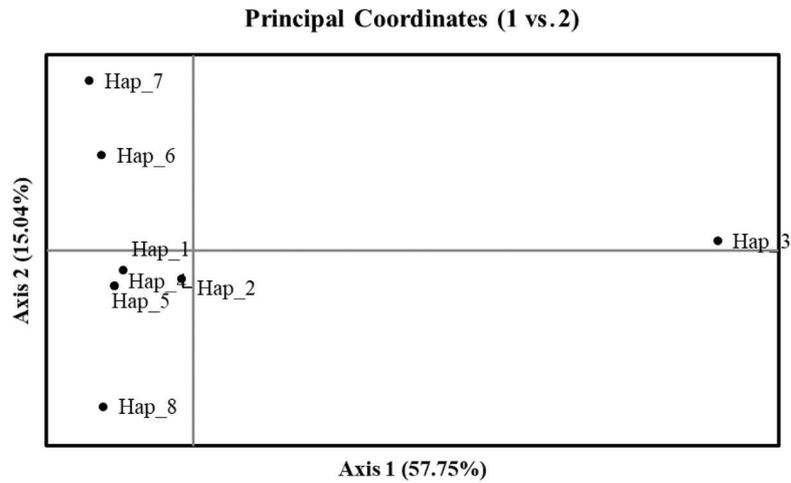
The overall mean p-distance for the COI gene region was found as  $0.0078 \pm 0.0018$ , and the pairwise p-distances was found as 0.0018 between these 5 haplotypes. The first 3 principal axes in the PCoA showed 57.75, 15.04, and 10.76 of the total variation, respectively, which indicated that almost all of the specimens exhibited overlapped clustering except for was clustered together except for the haplotype Hap\_3 (Figure 1).

As NJ and ML analyses have resulted in the same tree topology, the tree produced under the ML approach was presented in Figure 2. All specimens of *T. absoluta* were clustered together in a single group with a high support value (100%, Figure 2) except for the haplotypes Hap\_3, suggesting the presence of a homogeneity in the *T. absoluta* specimens. The tree also indicated no clustering related to geographic origin. Furthermore, no mtDNA polymorphism in entire populations infected by *Wolbachia* and *Pantoea* has been detected.

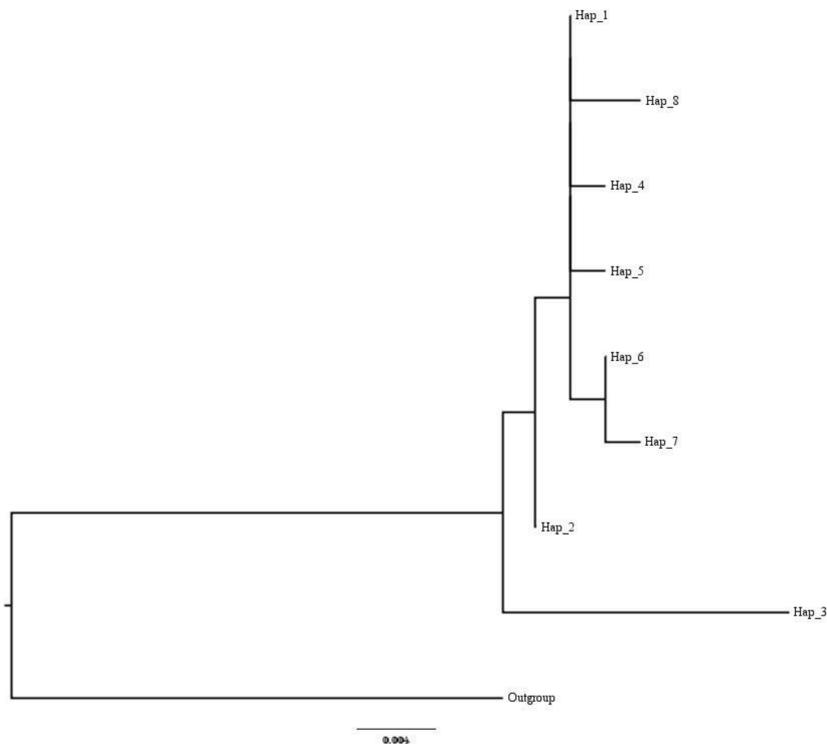
The presence and prevalence of *Wolbachia* infections were detected using the *wsp* gene in *T. absoluta* yielding an approximately 600 bp amplicon in length. The obtained *Wolbachia* sequence from *T. absoluta* has been submitted to GenBank under the accession number MT340264. BLAST analysis revealed that this sequence displayed the highest homology with 99.82% identity to *Wolbachia*

<sup>2</sup> Rambaut A (2014). FigTree v1.4.2 [online]. Website: <http://tree.bio.ed.ac.uk/software/figtree/> [accessed 13 April 2020].





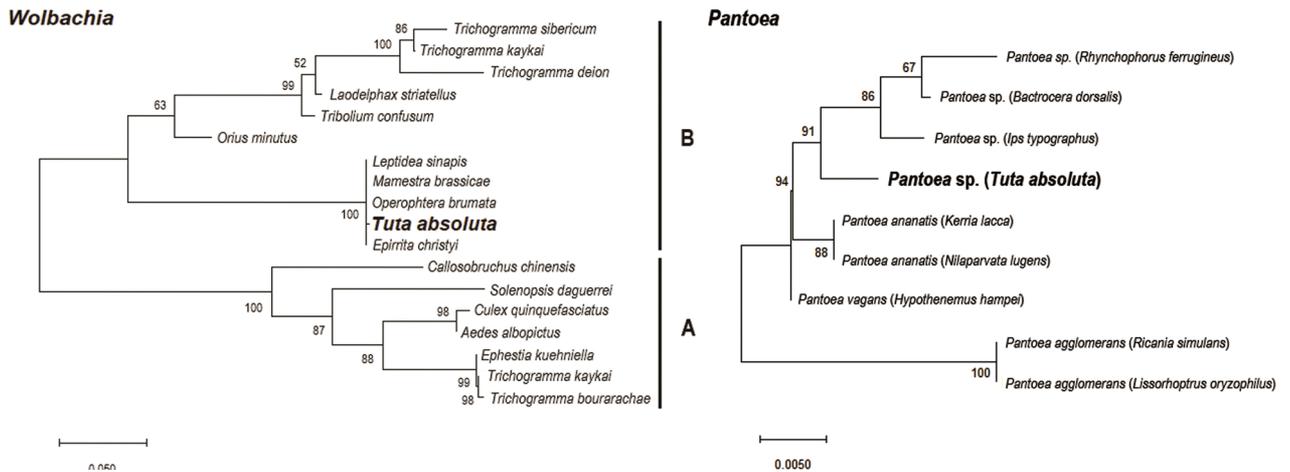
**Figure 1.** Principal coordinate analysis (PCoA) of the original data in 8 haplotypes of *T. absoluta*. Each dot corresponds to a haplotype of *T. absoluta* COI data. The amount of variance explained by each PC is indicated on each axis.



**Figure 2.** ML tree of haplotypes of *T. absoluta* based on the mtDNA COI data. *Symmetrischema lectulifera* was used as an outgroup.

endosymbiont of *Operophtera brumata* (KY587652.1), *Epirrita christyi* (JX310335.1), *Mamestra brassicae* (AB094375.1), *Operophtera brumata* (KY587653.1). To evaluate the phylogenetic relationship between *Wolbachia* strains isolated from various insects, we constructed a phylogenetic tree (Figure 3) of a total of 18 arthropod sequences under the NJ method, and the tree topology

of all subjected sequences revealed 2 supergroups (A and B). The first cluster, supergroup B, consisted of the orders including Hemiptera, Coleoptera, Hymenoptera, and Lepidoptera, whereas the second cluster, supergroup A, exhibited a relationship between Formicidae, Diptera, Lepidoptera, Hymenoptera, and Coleoptera. The phylogenetic tree showed that the *Wolbachia* sequences



**Figure 3.** Phylogenetic analysis of *Wolbachia* and *Pantoea* infections in *T. absoluta* species in relation to other insect species. The species and GenBank accession numbers of the reference sequences used to construct the phylogenetic trees are as follows: Uncultured *Pantoea* sp. (*Rhynchophorus ferrugineus*) KF125498.1; Uncultured *Pantoea* sp. (*Bactrocera dorsalis*) MK749919.1; *Pantoea* sp. (*Ips typographus*) DQ309419.1; *Pantoea* sp. (*T. absoluta*) MT326636; *P. ananatis* (*Kerria lacca*) KF717513.1; *P. ananatis* (*Nilaparvata lugens*) KJ655537.1; *P. vagans* (*Hypothenemus hampei*) KF913828.1; *P. agglomerans* (*Ricania simulans*) KM102494.1; *P. agglomerans*; *Lissorhoptus oryzophilus* (KC754748.1); *Trichogramma sibericum* (AF071923.1); *Trichogramma kaykai* (AF071924.1); *Trichogramma deion* (AF020084.1); *Laodelphax striatellus* (WSTR\_RS03110); *Tribolium confusum* (AF020083.1); *Orius minutus* (AB094363.1); *Leptidea sinapis* (KC137222.1); *Mamestra brassicae* (AB094375.1); *Operophtera brumata* (KY587656.1); *T. absoluta* (MT340264); *Epirrita christyi* (JX310339.1); *Callosobruchus chinensis* (MG255145.1); *Solenopsis daguerrei* (AY878105.1); *Culex quinquefasciatus* (KX650071.1); *Aedes albopictus* (KJ140127.1); *Ephestia kuehniella* (AF071911.1); *Trichogramma kaykai* (AF071912.1); *Trichogramma bourarachae* (AF071913.1).

from *T. absoluta* and different Lepidopteran species were clustered with the *Wolbachia* supergroup B. PCR analysis revealed 100% prevalence *Wolbachia* infection frequencies for the *Wolbachia* out of 164 (including 19 adults and 146 larvae) samples in *T. absoluta* populations.

Investigation of the presence of *Pantoea* in *T. absoluta* populations was performed using a set of primers amplifying the 16S rRNA gene fragment (421 bp) of *Pantoea*. The sequence of *Pantoea* sp. from *T. absoluta* has been submitted to GenBank under the accession number MT326636, which is the first record of infection by *Pantoea* within tomato leaf miner populations. The sequence homology showed >99% nucleotide identity with 16S rRNA encoded with the *Pantoea agglomerans* (NR\_116751.1), *Pantoea brenneri* (NR\_116748), *Pantoea eucalypti* (NR\_116112), and *Pantoea vagans* (NR\_116115) with a variety of different hosts in the database of BLAST. Figure 3 depicts the tree constructed by NJ analysis using sequences of 16S rRNA genes from *Pantoea* originating only from insects. According to the phylogenetic tree, there were 2 major branches based on *Pantoea* sequences. Among these sequences, *P. agglomerans* formed the main branch and differed from all other taxa. *Tuta Pantoea* was grouped in *Pantoea* sp. and diverged from *P. ananatis* and *P. vagans*. PCR analysis revealed 100% prevalence of *Pantoea* infection frequencies for the *Pantoea* out of

164 adult and larval samples of *T. absoluta* populations. On the contrary, no other symbiotic bacteria comprising *Arsenophonus*, *Cardinium*, *Hamiltonella*, and *Spiroplasma* infections were found in any of the tomato leaf miner populations tested.

#### 4. Discussion

The results of this study exhibited high genetic homogeneity with a low nucleotide diversity value among different specimens of *T. absoluta* across their distribution range. The presence of high genetic homogeneity was also observed in the PCoA analysis and phylogenetic tree. This low nucleotide diversity concurs well with previous results of pinworm populations from different parts of its distribution range (Shashank et al., 2018; Guedes et al., 2019). The mtCOI has been shown to be very useful and informative for estimating inter- and intraspecific levels; however, high genetic homogeneity observed in *T. absoluta* populations might be mainly related to the occurrence of genetic drift on this invasive species (Korkmaz et al., 2011; Duman et al. 2015). As reported in many studies on invasive species (Shashank et al., 2018; Hawley et al., 2006; Lindholm et al., 2005; Tsutsui et al., 2000), in small populations, the genetic diversity can be lost due to high genetic drift settled in new parts of the invasive range (Hagenblad et al., 2015). The biggest

limitation of this study is the sole use of *COI*; however, no genetic variation has been observed in *Tuta* populations collected from several countries using mtCOI, ITS rDNA, and microsatellite markers (Cifuentes et al., 2011; Duric et al., 2014; Guillemaud et al., 2015; Yukselbaba and Gocmen, 2016; Shashank et al., 2018; Kinyanjui et al., 2018). Although great variance between nuclear-nuclear and mito-nuclear incompatibilities has been detected in insects (Telschow et al., 2019), further analyses with a wide range of samples need to be performed to enable a better estimation of the degree of population differentiation of *T. absoluta* using nuclear genes and microsatellites, which has already been developed (Bettaibi et al., 2013; Tabuloc et al., 2019).

Recently, insecticide resistant phenotypes have been discovered in *T. absoluta* in different studies (Siqueira et al., 2000; Lietti et al., 2005; Yalcin et al., 2015; Karaagac et al., 2015; Zibaee et al., 2016; Barati et al., 2018; Guedes et al., 2019). The populations examined in this study needs to be confirmed in terms of insecticide susceptibility since a single genetically uniform *Tuta* population has been related to higher insecticide resistance in Mediterranean Basin and South America (Cifuentes et al., 2011).

On the other hand, mtDNA is still commonly used as a molecular marker for identification, phylogeny, and population structure of insect species (Guz et al. 2013; Hebert et al., 2003; Krishnamurthy and Francis, 2012; Yatkin and Guz 2018). This is of significant importance as the spread of *Wolbachia* might influence polymorphism in mtDNA (Turelli et al., 1992; Solignac et al., 1994; Ballard et al., 1996; Keeling et al., 2003; Charlat et al., 2009; Graham and Wilson, 2012; Richardson et al., 2012; Schuler et al., 2016). In this study, the findings of high genetic homogeneity among *T. absoluta* populations with high prevalence of *Wolbachia* and *Pantoea* infections seems to imply that both bacterial infections have less impact on the mtDNA variations. Our results are in accordance with the findings about the populations reported in Brazil (Carvalho et al., 2018). Even though these results differ from previous studies that report that *Wolbachia* spread affects the mtDNA diversity of its host through indirect selection, which means if a population gains any reproductive advantage or fitness from *Wolbachia*, the initial mtDNA type will hitchhike through the population and change the uninfected haplotypes (Hurst and Jiggins, 2005; Schuler et al., 2016). Therefore, the inference about lack of genetic diversity is more precise when the uninfected *Wolbachia* population of *T. absoluta* is analyzed. To our knowledge, this is the first report for *Pantoea* in terms of mtDNA haplotype diversity, which needs to be interpreted with the insights into the temporal and spatial spread of *Pantoea* in *T. absoluta*.

*Wolbachia* is the most common bacterium with an approximately 66% infection in all insects (Hilgenboecker et al., 2008). In a broad survey, *Wolbachia* infected majority of neotropical insect orders, including Hemiptera, Orthoptera, Diptera, Hymenoptera, Coleoptera, and Lepidoptera (Werren et al., 1995). Other comprehensive studies about *Wolbachia* infection in Lepidopteran families showed 58.3% in 120 species of Siberian populations (Ilinsky and Kosterin, 2017); 45% of 49 Japanese species (Tagami and Miura, 2004); 52% of 29 Indian species (Salunke et al., 2012), and 79% of 24 *Acraea* species from Uganda (Jiggins et al., 2001). In our study, all tomato leaf miner populations displayed 100% of *Wolbachia* infection, which is consistent with the populations from Brazil, Croatia, and Montenegro (Škaljac et al., 2012; Carvalho et al., 2018). Although *Wolbachia* is mostly known bacteria, *Spiroplasma* were present at low levels while *Rickettsia* was also not commonly found with only one species across Lepidopterans. However, none of the samples harbored *Arsenophonus*, *Cardinium*, or *Hamiltonella* in any host (Russel et al., 2012; Weinert et al., 2015). On the other hand, *Bacillus*, *Enterobacter*, *Enterococcus*, *Pseudomonas*, and *Staphylococcus* were detected as the most common bacterial genera >70% of the 30 different caterpillar species (Voirol et al., 2018). In particular, in *T. absoluta* *Rickettsia*, *Hamiltonella*, *Arsenophonus*, *Cardinium*, and *Fritshea* were not detected in Croatian populations (Škaljac et al., 2012). These findings are congruent with our results since all the other bacteria we have tested comprising *Arsenophonus*, *Spiroplasma*, *Hamiltonella*, and *Cardinium* were negative in *Tuta* populations.

In this study, we have detected 100% *Pantoea sp.* in *T. absoluta* populations for the first time. The *Pantoea* is a member of the *Enterobacteriaceae*, with more than 20 species, which are adapted to diverse ecological niches, including humans, insects, plant material, soil, and water (Walterson and Stavrinos, 2015). It has been reported to colonize in several Lepidopteran species (Broderick et al., 2004; Robinson et al., 2010; Pinto-Tomás et al., 2011; Tang et al., 2012; He et al., 2013; Chen et al., 2016; Sevim et al., 2016). Recent studies have shown that *Pantoea* species contribute to the provision of essential nutrients limited in the host's diet (Kenyon et al., 2015), participating in creating a nitrogen-rich environment for developing eggs and larvae (MacCollom et al., 2009) and the breakdown of plant cell-wall biopolymer (Bozorov et al., 2019). To our knowledge, no previous research has investigated the impact of *Pantoea* on *T. absoluta*, which would be of interest to further determine the ecological benefits of this symbiotic bacteria.

Currently, based on phylogenetic reconstructions, 17 *Wolbachia* supergroups (A-Q) have been recognized (Glowska et al., 2015). The multilocus sequence typing

(MLST) system became a standard protocol in determining *Wolbachia* strains using the substitutions in housekeeping loci (*ftsZ*, *gatB*, *coxA*, *hcpA*, and *fbpA*) (Baldo et al., 2006). In this study, we have detected *T. absoluta* using *wsp* gene sequencing, which has been used in phylogenetic studies and for microtaxonomic subdivision (Van Meer et al., 1999). Our result is congruent with other pinworm populations in which 2 *Wolbachia* strains were detected, including ST41 and ST354, and classified in the supergroup B (Carvalho et al., 2018). *Wolbachia* sequences of *T. absoluta* analyzed in this study are grouped with the other Lepidopterans belonging to supergroup B, which is suitable with Croatian populations, as well (Škaljac et al., 2012). However, the predominance of supergroup B in Lepidopteran species is still discussed to determine whether there is a congruence between Lepidoptera and B group or whether the groups are ancestrally associated (Choi et al., 2015, Ahmed et al., 2016). Therefore, for *T. absoluta* populations, pyrosequencing-based MLST method for large-scale screening of multiple *Wolbachia* STs is suggested to be fully investigated due to the limitation in application in infections with a single strain of *Wolbachia*.

In Lepidoptera endosymbiotic bacteria are known to alter host reproduction via feminization, male killing, and CI (Duplouy and Hornett, 2018). *Wolbachia* induced feminization occurred in *Bombyx mori*, *Eurema*, and *Ostrinia* species (Hiroki et al., 2002; Fujii et al., 2001; Kageyama et al., 2003, 2004). Male killing has been reported in Lepidoptera generated by *Wolbachia* as well as *Spiroplasma* (Duplouy and Hornett, 2018). CI has been observed at high frequency in various Lepidopteran families (Sasaki et al., 2002; Hornett et al., 2008). *Wolbachia* strains detected in Brazilian pinworms found to be associated with CI (Carvalho et al., 2018). Apart from *Wolbachia*, tomato leaf miner populations showed deuterotokous parthenogenesis in mating experiments under laboratory conditions (Caparros Megido and Haubruge, 2018). The low genetic variation has been attributed to parthenogenetic reproduction; however,

more genetic polymorphism was observed in asexual species than sexual species (Chevasco et al., 2012). The fitness of parthenogenetic ancestries are clearly needed to confirm the deuterotokous parthenogenesis in *T. absoluta*. Moreover, endosymbiont manipulation, impact upon host fitness, and additional effects of the bacterial microbiota in *T. absoluta* need to be further assessed.

Here, we detected *Wolbachia* and *Pantoea* infection in both adult and larval stages in the entire populations of *T. absoluta*, verifying the findings that some bacterial taxa can persist through the life cycles of insects (Hammer et al., 2014). However, in a large-scale study, it is outlined that host phylogeny, developmental stage, environmental habitat, and diet could influence the host's microbiota (Yun et al., 2014). As expected, the bacterial communities might substantially differ between larval and adult stages of the same species (Staudacher et al., 2016, Xia et al., 2017) since almost all lepidopteran larvae feed on plant material and the majority of adults feed on floral nectar and other liquid substances (Krenn et al., 2010). The widespread abundance of *Wolbachia* infection in *T. absoluta* might be due to the shared natural enemies and food sources as potential routes of horizontal transmission (Ahmed et al., 2016). Although whiteflies and tomato leaf miner populations share common habitat, host plant, and natural enemies, we confirmed in this study that the symbiotic bacterial composition of 2 pests differed except *Wolbachia*. It remains unclear why the composition of bacterial communities varies in distinct phytophagous species. Further experimental investigations are needed to estimate the transmission routes, which might have a critical role in evolutionary biology of *T. absoluta* populations.

As a result, characterization of the microbiota of the tomato leaf miner as well as factors influencing symbiotic composition and the impact of symbiotic bacteria to its host, such as nutrition, physiology, and behavior, are important in developing alternative control strategies for this invasive pest.

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