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A simple molecular identification method of the *Thrips tabaci* (Thysanoptera: Thripidae) cryptic species complex

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Research Paper

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Abstract

The onion thrips (*Thrips tabaci* Lindeman, 1889) is a key pest of a wide range of crops because of its ecological attributes such as polyphagy, high reproduction rate, ability to transmit tospoviruses and resistance to insecticides. Recent studies revealed that *T. tabaci* is a cryptic species complex and it has three lineages (leek-associated arrhenotokous L1-biotype, leek-associated thelytokous L2-biotype and tobacco-associated arrhenotokous T-biotype), however, the adults remain indistinguishable. *T. tabaci* individuals were collected from different locations of Hungary to create laboratory colonies from each biotypes. Mitochondrial COI (mtCOI) region was sequenced from morphologically identified individuals. After sequence analysis SNPs were identified and used for CAPS marker development, which were suitable for distinguishing the three *T. tabaci* lineages. Genetic analysis of the *T. tabaci* species complex based on mtCOI gene confirmed the three well-known biotypes (L1, L2, T) and a new biotype because the new molecular evidence presented in this study suggests T-biotype of *T. tabaci* forming two distinct (sub)clades (T1 and T2). This genetic finding indicates that the genetic variability of *T. tabaci* populations is still not fully mapped. We validated our developed marker on thrips individuals from our thrips colonies. The results demonstrated that the new marker effectively identifies the different *T. tabaci* biotypes. We believe that our reliable genotyping method will be useful in further studies focusing on *T. tabaci* biotypes and in pest management by scanning the composition of sympatric *T. tabaci* populations.

Introduction

The onion thrips (*Thrips tabaci* Lindeman, 1889) has already been known as a cosmopolitan, polyphagous insect of economic importance due to causing significant damage on cultivated crops globally, mainly on alliaceous crops, cabbage and tobacco (Jenser and Szénási, 2004; Trdan *et al*., 2006, 2007; Shelton *et al*., 2008; Diaz-Montano *et al*., 2011). The onion thrips was known as a single cosmopolitan species before Zawirska (1976) recognized different biological types of *T. tabaci* ('communis type' and 'tabaci type') based on some ecological traits, distinct reproductive modes, different host range, virus transmission capability (TSWV) and morphological differences on the abdominal tergites of second-stage larvae. In the last three decades nucleic acid-based methods have been used to distinguish various population of *T. tabaci*. Klein and Gafni (1996) reported intraspecific molecular variability between *T. tabaci* populations collected from onion fields by using randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique. Kraus *et al.* (1999) found molecular genetic differences between three different populations of *T. tabaci,* however, the computed cluster analysis was not able to distinguish the populations. Jenser *et al.* (2001) used RAPD-PCR to show differences between populations of *T. tabaci* collected from onion and tobacco. Their results indicated characteristic molecular differences among specimens from onion and tobacco. These results, at the nucleic acid level indicated the existence of subspecies within onion thrips, which colonize different host plants. Later Brunner *et al.* (2004) described that onion thrips is a cryptic species complex, based on the DNA sequences of the mitochondrial COI gene and divided the species into three lineages based on host preferences: leek-associated types (L1 and L2) and tobacco-associated type (T). Since then, this genetic divergence was confirmed in many other onion thrips populations as well (Toda and Murai, 2007; Kobayashi and Hasegawa, 2012; Jacobson *et al*., 2013; Kobayashi *et al*., 2013; Westmore *et al*., 2013; Fekrat *et al*., 2014; Li *et al*., 2015).

In the different populations of *T. tabaci* three reproductive modes have been reported: thelytoky, arrhenotoky (Lewis, 1973; Jenser and Szénási, 2004) and deuterotoky (Nault *et al*.,

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2006). In thelytoky (asexual reproduction) virgin females propagate solely females, whereas in arrhenotoky (sexual reproduction) unfertilized females produce only males and fertilized females can produce both sexes (diploid females are produced from fertilized eggs and haploid males from unfertilized eggs) (Moritz, 1997; Jenser and Szénási, 2004) and through deuterotoky virgin females produce both females and males (Moritz, 1997).

Accurate identification of tiny arthropod species such as thrips is often difficult by using exclusively morphological traits, therefore, new identification methods were introduced to separate thrips species based on PCR. Closely related taxa could be identified based on several loci. It has already been demonstrated that the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (rDNA) (Moritz *et al.*, 2002; Toda and Komazaki, 2002; Rugman-Jones *et al.*, 2006; Farris *et al.*, 2010) and mtCOI gene (Brunner *et al.*, 2002; Frey and Frey, 2004; Asokan *et al.*, 2007; Sabahi *et al.*, 2017) can be used to make distinction at species level between thrips. However, other PCR-based techniques also provide opportunity for identifying thrips species (Mehle and Trdan, 2012).

Although, to make a distinction within a species is particularly difficult and require comprehensive knowledge, the leek-associated lineages (L1 and L2) of onion thrips populations can be discriminated by their reproductive mode using PCR-based techniques. Takeuchi and Toda (2011) suggested PCR-restriction fragment length polymorphism (PCR-RFLP) while Kobayashi and Hasegawa (2012) developed PCR-strain specific primers (PCR-SSP) based on sequence differences in the mtCOI gene.

Morphological identification of different lineages within a species is very difficult and problematic. There is no external morphological difference between the adults of the *T. tabaci* 'communis' and *T. tabaci* 'tabaci' according to Zawirska's (1976) experience. However, Fekrat *et al.* (2014) observed differences between tobacco-associated and onion-associated individuals using morphometric analysis between populations from tobacco and onion fields, nonetheless, no external morphological character was singled out which could provide a definite separation.

Despite all the efforts, there is still no available method to discriminate the members of the currently known subspecies in the *T. tabaci* cryptic species complex. The present study describes a CAPS marker system targeting a part of the mitochondrial COI gene, which can be applied to identify the different biotypes in each developmental stages. A better understanding of the genetic diversity of *T. tabaci* cryptic species complex could be useful for improving pest management strategies. The recognition of the different lineages of *T. tabaci* by the presented identification tool is accurate, simple (the method requires basic laboratory skills), rapid (identification without DNA sequencing) and economic (no need for DNA sequencing equipment and purification kit). We investigated the effectiveness of the proposed method by using *T. tabaci* individuals collected in Hungary by analyzing the genetic relationship of haplotypes reported in the present work with published mtCOI haplotypes obtained from NCBI GeneBank.

Materials and methods

Insect collection

T. tabaci specimens were taken from our stock laboratory cultures. The cultures were established with the following procedure in 2013 and 2014. The thelytokous *T. tabaci* (L2) samples were collected from different plants (*Filipendula vulgaris* Moench, *Santolina chamaecyparissus* L., *Lonicera caprifolium* L., *Disaphora Fruticosa* (L.) Rydberg, *Coriandrum sativum* L., *Stenactis annua* (L.) Persoon, *Sorbaria sorbifolia* (L.) A. Braun) from the Botanical Garden of Szent István University (47°28′N, 19°02′E, 115 m altitude), located in Budapest and from cabbage (*Brassica oleracea* L. convar. *capitate* var. *alba*) Central Hungary (47°20′N, 18°44′E, 177 m altitude). Leek-associated arrhenotokous (L1) populations were collected from onion bulbs (*Allium cepa* L.), which were obtained from a traditional onion growing area, Makó (46°14′N, 20°28′E, 76 m altitude) Southern-Hungary. Tobacco-associated arrhenotokous (T) populations were sampled on tobacco fields (*Nicotiana tabacum* L.) in Apagy (47°57′N, 21°55′E, 118 m altitude), Pócspetri (47°52′N, 21°59′E, 133 m altitude), Encsencs (47°44′N, 22°06′E, 153 m altitude) from East-Hungary (fig. 1).

Reproductive mode identification

The collected individuals were kept isolated individually because *T. tabaci* had already been considered as a criptyc species complex with different reproductive strategies. To create pure colonies it was desirable to have thrips specimens collected from known host plants then isolated individually in order to study their reproductive mode by identifying the sex of progeny from virgin females.

To discriminate the reproductive modes, the virgin females of the next generation produced by the originally collected living females were isolated individually in 2 ml centrifuge tubes on leaf sections and allowed to oviposit through their entire lifespan. Small leaf discs (5 mm in diameter) were provided and served as a food source and oviposition substrate to the thrips samples according to the original host plant from which the given samples were collected. If virgin females produced exclusively female progenies the reared specimens were determined as thelytokous. If only males were found in the progenies then the female was considered arrhenotokous (all progenies were kept in isolation also individually and reared to adult stage to confirm the sex). Deuterotokous reproduction was not observed at all. All thrips samples were kept in a climate chamber (SANYO, MLR-352H, Panasonic Corporation, Osaka, Japan) under the identification process in 16:8 L:D cycle at 23°C temperature.

The field-collected females were preserved in 75% ethanol until the taxonomic identification. All adult females were slide-mounted using Hoyer's solution and morphologically identified at species level by using a compound light microscope (LEICA DM LB, Leica Microsystem GmbH, Wetzlar, Germany) based on the identification guide of Mound and Kibby (1998) and the key of Moritz *et al.* (2001).

Stock laboratory colonies

The thelytokous (L2) stock colonies used in this study were established only from the unambiguously identified thelytokous progenies of *T. tabaci* females. The arrhenotokous individuals were split into two groups: those that were originally collected from tobacco were used to establish the culture of the T lineage, whereas those collected from onion were used to establish the pure culture of the L1 lineage.

The stock thelytokous colony was maintained on cabbage head leaves, leek-associated colony on leek leaf sections, and tobacco-associated colony on whole tobacco leaves (*Nicotiana tabacum* L. 'Hevesi 9') that were planted in small glass vials filled with agar medium (Agar powder for microbiology, Merck KGaA, Darmstadt, Germany) in ventilated translucent plastic containers.

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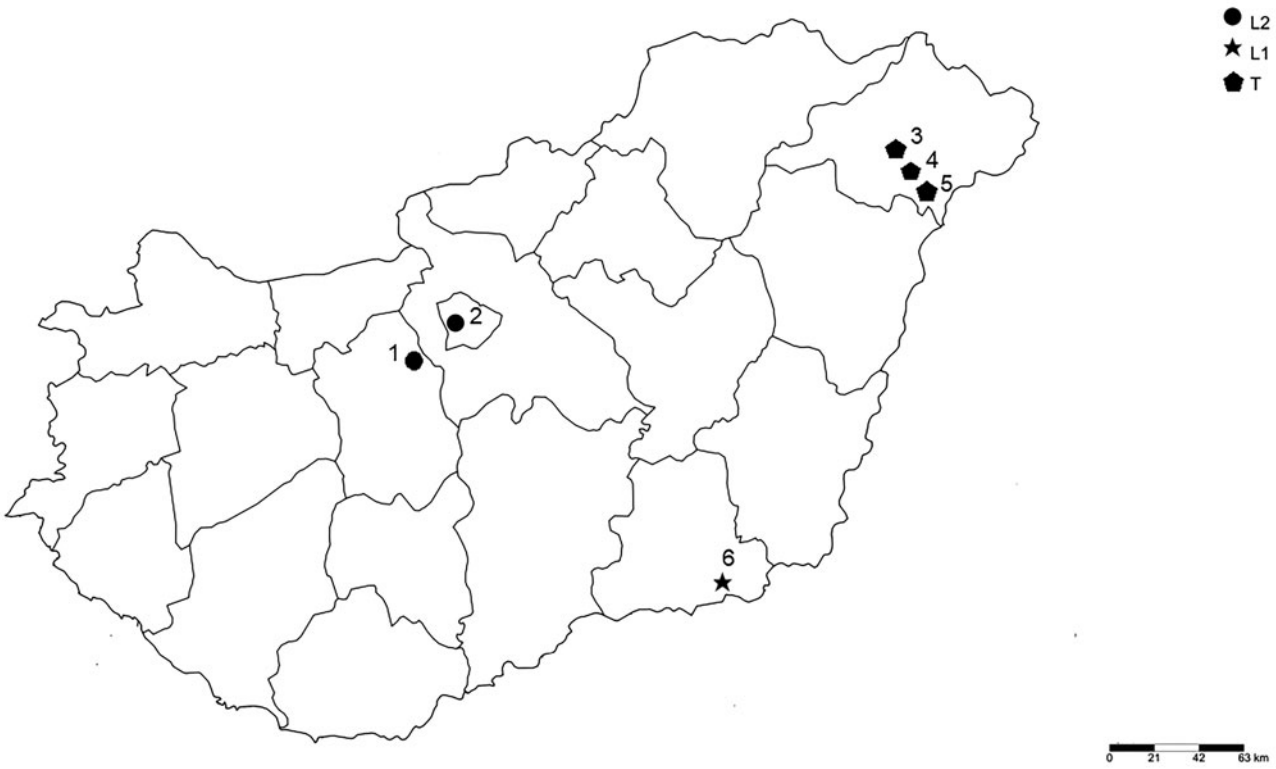


Figure 1. Map showing sampling sites for *T. tabaci* populations in Hungary. 1, Central Hungary - Tordas; 2, Central Hungary - Budapest the source of leek-associated thelytokous (L2) colony. 3, East Hungary - Apagy; 4, East Hungary - Pócspetri; 5, East Hungary - Encsencs the source of tobacci-associated arrhenotokous (T) colony; 6, Southern Hungary - Makó the source of leek-associated arrhenotokous (L1) colony.

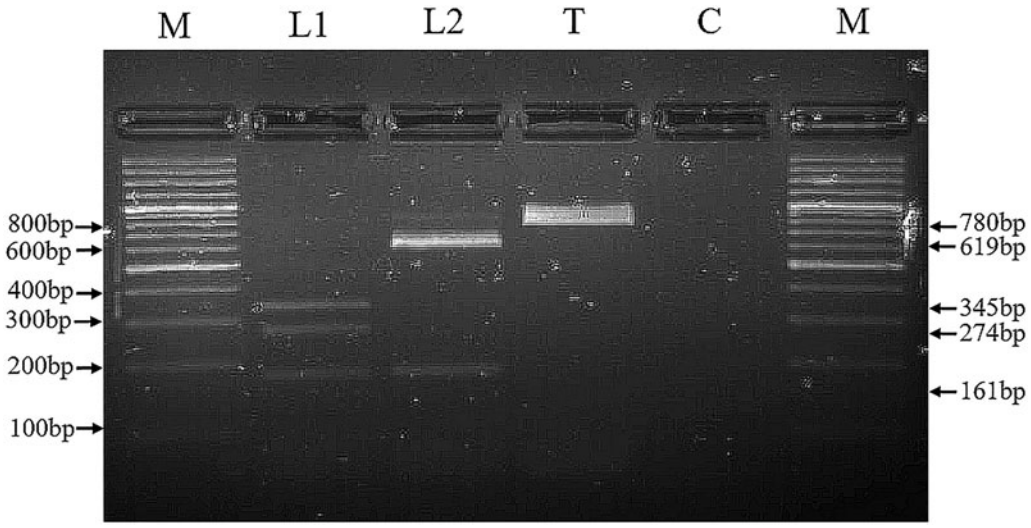
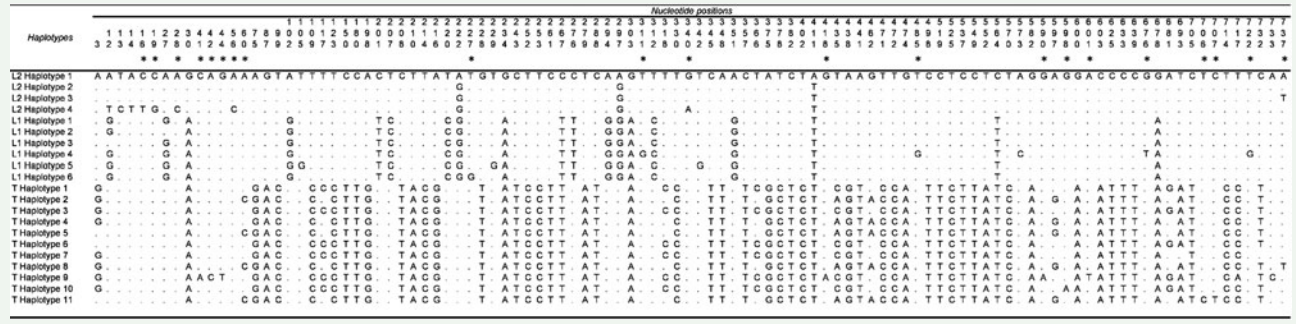


Figure 2. Restriction fragment patterns of the amplified mtCOI gene of *T. tabaci* digested with PsuI and PsyI endonucleases. Lane M is a 100 bp DNA ladder size marker. L1: three fragments of leek-associated arrhenotokous lineage after digestion (345 bp/274 bp/161 bp), L2: two fragments of leek-associated thelytokous lineage after digestion (619 bp/161 bp), T: undigested amplicon of tobacco-associated arrhenotokous lineage (780 bp); C: Negative control with no template DNA.

A double-layered paper towel was placed on the bottom of the plastic containers (under the plant material), which served as a hiding-place for the inactive developmental stages (prepupae, pupae) and it absorbed the surplus humidity as well. The cover of the containers was cut out to provide air ventilation and was covered by thrips-proof densely woven metal mesh to prevent the escape of thrips. All used plant materials (leek, tobacco, cabbage) were grown by ourselves and carefully examined before introduced to the culture in order to prevent unwanted thrips contamination (thrips-free plant material). The stock colonies were kept in climate chambers separately in 16 h light and 8 h dark period at 20°C.

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Table 1. Variable positions in the 740 bp mtCOI defining 21 different haplotypes



Dots indicate nucleotides identical throughout the haplotypes compared. Amino acid replacement substitutions are indicated by asterisks.

Table 2. Comparison of the outputs of the presented identification tool and result of genetic analysis

No. of *Thrips tabaci* individuals	Result of the digested PCR product	Haplotype	Result of the sequence analysis
1–2	2 fragments	Haplotype 1	L2
3–39	2 fragments	Haplotype 2	L2
40	2 fragments	Haplotype 3	L2
41	2 fragments	Haplotype 4	L2
42–72	3 fragments	Haplotype 5	L1
73–74	3 fragments	Haplotype 6	L1
75	3 fragments	Haplotype 7	L1
76	3 fragments	Haplotype 8	L1
77	3 fragments	Haplotype 9	L1
78	3 fragments	Haplotype 10	L1
79–80	1 fragment	Haplotype 11	T
81–106	1 fragment	Haplotype 12	T
107–108	1 fragment	Haplotype 13	T
109	1 fragment	Haplotype 14	T
110	1 fragment	Haplotype 15	T
111	1 fragment	Haplotype 16	T
112	1 fragment	Haplotype 17	T
113	1 fragment	Haplotype 18	T
114	1 fragment	Haplotype 19	T
114	1 fragment	Haplotype 20	T
116	1 fragment	Haplotype 21	T

Molecular protocol

Thrips isolation

Altogether 116 adults were removed from the above mentioned identified stock colonies (L1-, L2- and T-biotype) using a fine brush and placed into 2 ml microcentrifuge tubes containing 96% pure ethanol until processed. The sex of each collected thrips was verified by stereo microscope (ZEISS, Stemi 2000, Carl Zeiss Microscopy GmbH, Jena, Germany) and recorded before molecular analysis.

Genomic DNA isolation

Total genomic DNA was extracted from a single female and male adult thrips of confirmed origin (host plant) and reproductive mode (as described above) by a rapid standard method with Proteinase K treatment (De Barro & Driver, 1997). A single thrips was carefully removed from the 2 ml microcentrifuge tube and transferred into a new 1.5 ml microcentrifuge tube using a micropipette and a fine brush. The ethanol was evaporated from the integument of the thrips before the homogenization. Then the thrips was homogenized in 7 µl of tissue lysis buffer (10 mM Tris-HCL, pH 8.4, 50 mM KCL, 0.45% Tween 20, 0.2% gelatine, 0.45% Nonidet P-40 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany)) and 3 µl Proteinase K enzyme (60 µg ml^{-1} proteinase K) (ThermoFisher Scientific, Waltham, Massachusetts, USA) was added to the crushed sample. The lysate was mixed by vortex and centrifuged for 30 s at 13.000 rpm. The homogenate was incubated at 65°C for 30 min, followed by incubation at 95°C for 15 min to inactivate the Proteinase K. Finally, the DNA was resuspended in 20 µl of TE. The homogenate was stored at −20°C without any further preparation for subsequent PCR amplification.

Development of CAPS marker for distinguishing lineages of the T. tabaci species complex

To make a distinction at species level the mtCOI gene was chosen. PCR primers used in this study were self-designed, based on available *T. tabaci* target sequences in NCBI GeneBank nucleotide database (https://www.ncbi.nlm.nih.gov/genbank/) using OLIGO Primer 7 Analysis Software (Rychlik, 2007). Specific forward primer 'TTL-UNIF1' (5′-ATTAATTATAGGRCTTTAYAAAGAAGG-3′) and reverse primer 'TTL-UNIR1' (5′-GTAGTGAAAGTGAGCT ACAACATAATA-3′).

Amplification was performed in 20 µl PCR mix, containing 10 µl 2× DreamTaq Green PCR Master Mix (ThermoFisher Scientific, Waltham, Massachusetts, USA), 20–80 ng DNA template (1 µl) and 0.5 mM (1 µl) of each primer and nuclease-free water (to make total volume of 20 µl). The PCR protocol comprised initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. The size of the amplified product was 780 bp. The PCR was carried out in an Eppendorf™ Mastercycler™ Nexus Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany). A 100 bp DNA ladder (ThermoFisher Scientific) was used as standard marker in 2.5% agarose gel electrophoresis. The amplified DNA fragment was visualized under UV

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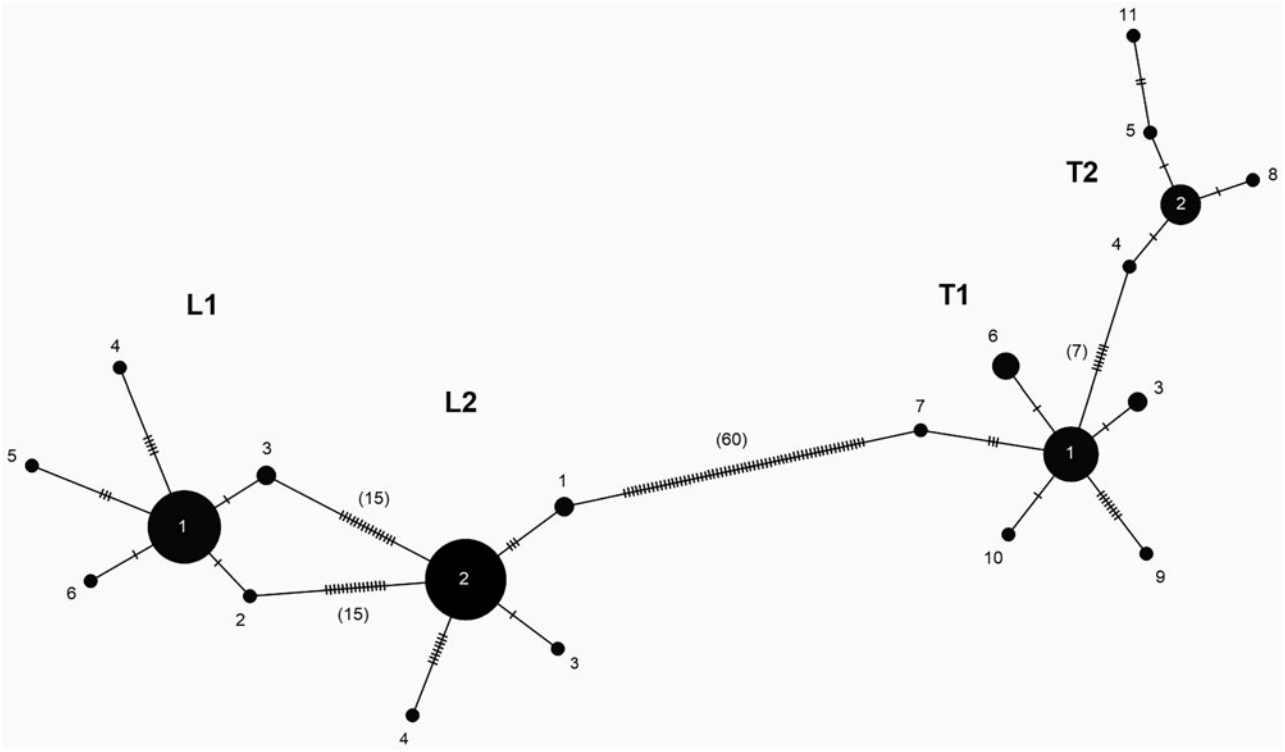


Figure 3. Minimum spanning network (MSN) calculated with mtCOI sequences using PoPART (Bandelt *et al*., 1999). Different circle sizes are proportional to the haplotype frequency in the dataset. The numbers within circles are the haplotype designations as per Table 2. Perpendicular tick marks and the numbers in the parenthesis on the lines represent the number of the nucleotide substitutions between the linked haplotypes.

transilluminator and photographed using gel documentation system (UVP BioDoc-It™ Imaging Systems LMS-20E, Upland, USA). PCR products were purified with High Pure PCR Product Purification Kit (Merck KGaA, Darmstadt, Germany) and the amplicons were sequenced directly from both directions commercially at BaseClear B.V. (Leiden, The Netherlands).

Identification of restriction sites and digestion by restriction enzymes

In order to develop a discrimination method sequences available in NCBI GeneBank were used by BLASTn search tool (Altschul *et al*., 1990) was used, but we filtered the sequences and used only those where reproductive mode was confirmed by checking the sex ratio in the virgin female's progenies.

The restriction site map was generated for each *T. tabaci* biotypes and finally the biotype-specific restriction sites were identified by SnapGene® Software (http://www.snapgene.com/, Free Trial version, GSL Biotech LLC).

Each digestion reaction contained 2 U PsuI and 2 U PsyI FasDigest enzymes (ThermoFisher Scientific), 0.6 µl 10× FastDigest Green Buffer (ThermoFisher Scientific), 3.3 µl unpurified PCR product and 5.7 µl nuclease-free water in 10 µl total reaction volume. The solution was digested at 37°C for 10 min according to the manufacturer's protocol.

Sequence analysis

The raw sequence chromatograms were visually checked, assembled and edited using Chromas Version 2.6.5. (Technelysium Pty Ltd, Australia) (https://technelysium.com.au/wp/) and CLC Sequences Viewer Programme Version 7.8.1. (https://www.qiagenbioinformatics.com/). The identity of the species was checked by BLASTn search tool (Altschul *et al*., 1990) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Haplotype sequence reconstruction was done employing the data generated in this study for each biotype (L1, L2, T), however, other sequences were also used (NCBI GeneBank Accession: MG519810-MG519825) from our previous study (Sojnóczki *et al*., 2015). Haplotype sequences were created as follows: sequence alignments for all biotypes were imported into DNA Sequence Polymorphism Software (DnaSP version 5.10) (Librado and Rozas, 2009) to reconstruct haplotypes and generate haplotype data file. The haplotype data file was used to construct a minimum spanning network (MSN) in PoPART (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz) (Bandelt *et al*., 1999).

Sequence alignments were done using ClustalW algorithm and Maximum Likelihood (ML) and Maximum Parsimony (MP) methods were used to analyze the genetic relationship in MEGA6 Software (Molecular Evolutionary Genetics Analysis, Version 6) (Tamura *et al*., 2013). JModelTest 2.1.10. (Darriba *et al*., 2012) was used to specify the substitution model that best fits the data set for the ML analysis. The HKY + G model (Hasegawa *et al*., 1985) was selected by JModelTest2 and MP analysis was performed under the heuristic search option. *Thrips palmi* (Accession number: AF378690) and *Thrips angusticeps* (Accession number: AF378679) sequences were obtained from NCBI GenBank and served as reference species because they are closely related species (Brunner *et al*., 2002). Branch support was assessed with 1000 bootstrap (Felsenstein, 1985) pseudoreplicates.

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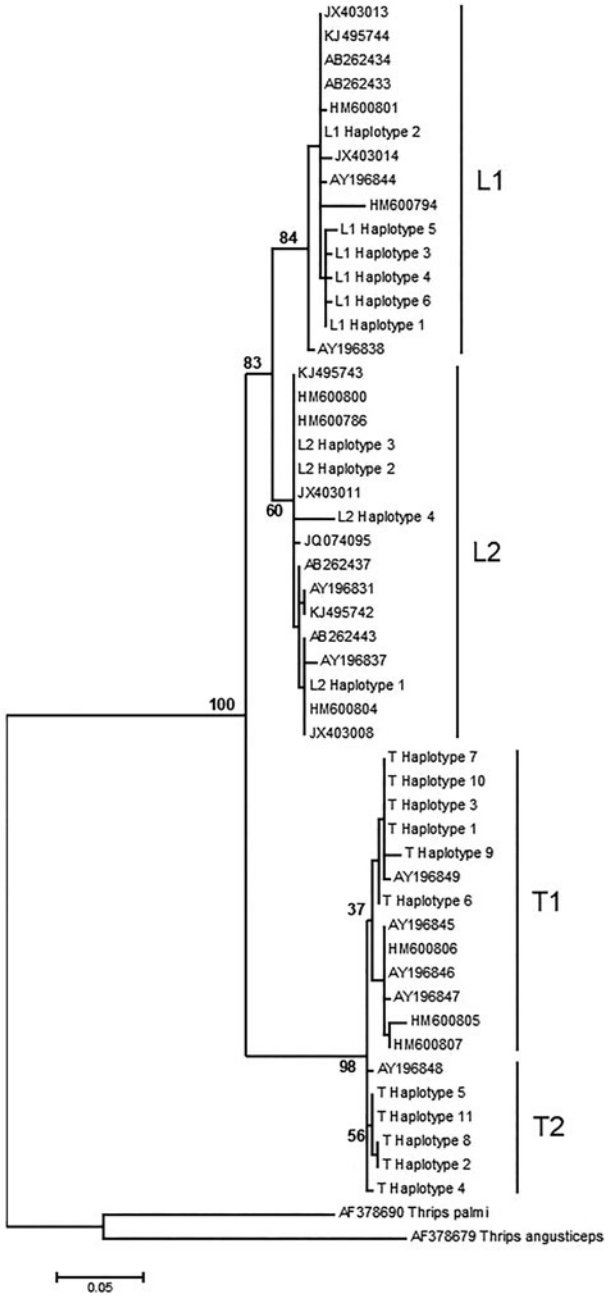


Figure. 4. Dendogram of *T. tabaci* based on 328 bp of the mtCOI gene. The tree is generated by the Maximum Likelihood method based on the HKY + G model (Hasegawa *et al*., 1985) using MEGA6 (Tamura *et al*., 2013). Sequence positions containing gaps and missing data were eliminated. Bootstrap values (percentage of 1000 replicates) are shown above the branches. Accession numbers shows the sequences data of *T. tabaci* obtained from DNA databases. *Thrips palmi* (AF378690) and *Thrips angusticeps* (AF378679) served as outgroups.

Results

Identification of restriction patterns

Restriction enzymes were selected by the following criteria: the enzymes produce easily detectable differences in fragment size and all biotypes of *T. tabaci* can be identified in one process. Among many potential restriction endonucleases based on the summarized restriction site map, PsuI and PsyI endonucleases were tested for restriction patterns preliminary in silico digestions of the amplified fragments of mtDNA gene by using SnapGene® Software (http://www.snapgene.com/, Free Trial version, GSL Biotech LLC). After the *in silico* digestion, the fragment size of the two products was detected by in silico electrophoresis. This analysis indicated that PsuI and PsyI restriction endonucleases facilitate the distinction of the three biotypes of *T. tabaci*. The cleavage with PsuI and PsyI produced banding patterns that allowed indubitable identification of biotypes according to the preliminary test by using double digestion. The leek-associated arrhenotokous type (L1) has restriction sites both for PsuI and for PsyI, which results in three different sized fragments (sized 345 bp/274 bp/161 bp), the leek-associated thelytokous type (L2) has only one restriction site producing two fragments (sized 619 bp/161 bp) because PsuI is a noncutter enzyme for this type. The tobacco-associated type (T) does not have restriction sites for either of the two enzymes thus it remains in one single fragment (sized 780 bp) (fig. 2) (Table 2). The 116 *T. tabaci* COI sequences used in this work were digested by restriction endonucleases and 41 sequences belonged to the leek-associated thelytokous group (L2-biotype), 36 sequences to the leek-associated arrhenotokous group (L1-biotype) and 39 sequences to the tobacco-associated arrhenotokous group (T-biotype) (Table 2).

Sequence analysis

A new thrips-specific primer pair was designed and successfully used for amplifying a fragment of COI gene. Minimum spanning network (MSN) was created by PoPART (Bandelt *et al*., 1999) and produced two main haplotype groups: one group is the leek-associated group, which was divided to arrhenotokous (L1) and thelytokous (L2) subgroups and the other main group is the tobacco-associated arrhenotokous group, which was also divided into two (sub)groups as T1 and T2, which two subgroups were not observed earlier. 21 different haplotypes were identified among 116 *T. tabaci* sequences. The leek-arrhenotokous individuals (L1) clustered together just like the leek-thelytokous ones and these subgroups were connected closer to each other than the leek-associated group and tobacco-associated group. The MSN revealed six haplotypes within the leek-arrhenotokous subgroup, four haplotypes within the leek-thelytokous and 11 within the tobacco-associated arrhenotokous group (fig. 3). The sequences are deposited in NCBI GeneBank under accession numbers *MH782433-MH782453*. L2 haplotype 2 was the most common haplotype within subgroup L2 (37 sequences belonging to this haplotype), L1 haplotype 1 within subgroup L1 (30 sequences belonging to this haplotype) and T haplotype 1 within the group T (17 sequences belonging to this haplotype). Variable nucleotide positions among 21 haplotypes are shown in Table 1. The mtCOI sequences were polymorphic at 105 nucleotide positions (14.19%) and in 21 positions resulted in amino acid replacement. The amino acid replacements occurred mainly at three haplotype sequences, such as L2 haplotype 4, L1 haplotype 4, T haplotype 9 (fig. 3).

Dendograms were prepared based on estimation using ML, MP (Supplementary fig. S1) and Bayesian analysis (Supplementary fig. S2) supported by high bootstrap values and furthermore, the principle coordinate analysis (PCoA) plot of mtDNA data of 116 *T. tabaci* sequences (fig. 5) supported the results obtained by ML analysis resulted in largely congruent topologies, therefore, we show only the ML tree (fig. 4). The genetic tree revealed four distinct subgroups (L1, L2 and T1, T2) based on the host preferences suggested by Brunner *et al*. (2004). The group

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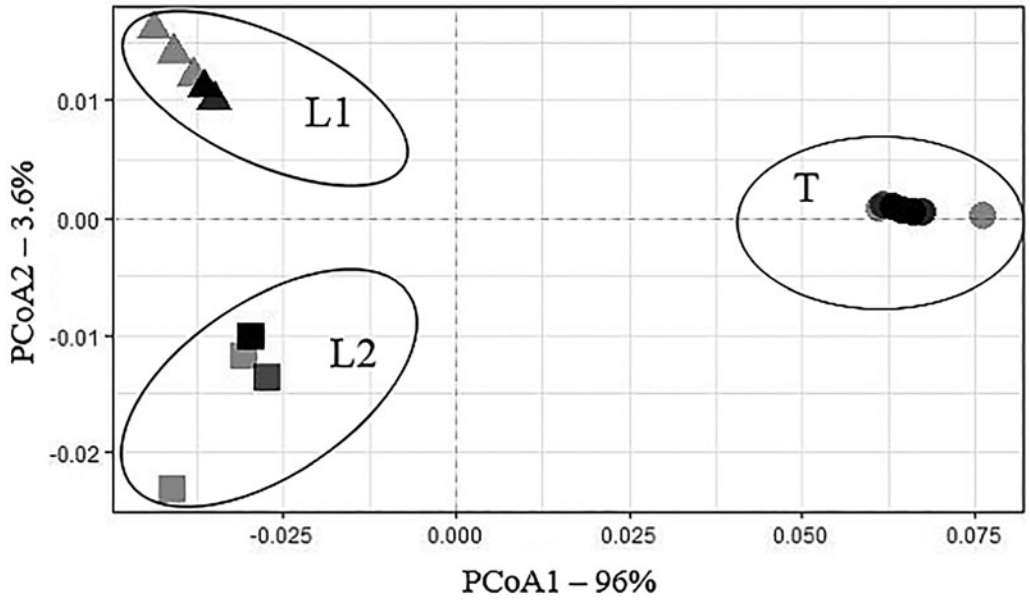


Figure 5. Principle coordinate analysis (PCoA) of mtDNA data (740 bp long mtCOI sequences) of 116 Hungarian *T. tabaci* samples. Pairwise distance matrix was calculated based on the model of Tamura and Nei (1993). First and second axis explains 96% and 3.6% of variability, respectively. The shape of the dots indicates the different biotypes of *T. tabaci*: circle represents T biotype, triangle represents L1 biotype and cubic represents L2 biotype. Darker colour indicates more samples from the same type.

L can be separated to leek-associated arrhenotokous (L1) and leek-associated thelytokous (L2) subgroup, while group T can be segregated tobacco-associated arrhenotokous T1 and T2 subclades (fig. 4). The six L1 haplotype sequences clustered to leek-associated arrhenotokous subgroup L1, while four L2 haplotype sequences to the leek-associated thelytokous subgroup L2 and 11 T haplotype sequences corresponded to tobacco-associated arrhenotokous group. The position of each of our constructed 21 *T. tabaci* mtCOI haplotypes in the genetic tree (fig. 4) confirmed the predicted result of the CAPS marker for all 116 thrips individuals (Table 2). Interestingly, the group T was divided into two subgroups based on the genetic analysis and the haplotype network analysis (fig. 3 and 4), but the PCoA did not show these two groups (fig. 5).

Discussion

A CAPS marker was developed for distinguishing mtCOI sequences of *T. tabaci* lineages and employed to genotype 116 *T. tabaci* specimens from Hungary. Using the new CAPS marker the different *T. tabaci* biotypes (L1, L2 and T) can be unambiguously discriminated, which is particularly relevant for arrhenotokous biotypes. The reliability of the CAPS marker was confirmed by genetic analysis presented in this study. The genetic structure of the tobacco-associated group within *T. tabaci* species complex is limited based on our results and the few available tobacco-associated mtCOI sequences, thus further research is required to make correct consequences and reveal the whole genetic variability.

We consider that population genetic approach using a combination of mitochondrial sequences and nuclear markers (for example ITS) are practical and effective ways to detect the reproductive barriers between *T. tabaci* lineages. Use of these genes would serve directly to detect reproductive barriers between different mitochondrial lineages because mitochondrial lineages are inherited independently from nuclear genes and they do not undergo recombination. Sogo *et al.* (2015) found DNA sequences of two 'new arrhenotokous' specimens in field populations in Japan, which have the same mtCOI characteristics as thelytokous haplotypes in the L2 lineage. It is assumed that these rare arrhenotokous individuals were produced by successful mating between thelytokous females and arrhenotokous males. However, due to the maternal origin of mitochondria in the progeny, it is not possible to identify paternal gene transfer with this technique. It is worth pointing out that in its current form, our method is not able to detect incidental gene flow between thelytokous and arrhenotokous individuals. Li *et al.* (2015) published evidence about interbreeding by detecting gene flow between the progeny of leek-associated arrhenotokous (L1) males and leek-associated thelytokous (L2) females. Due to the successful mating between different lineages, the male-originated nuclear gene allele (histone H3 gene) was present in the progenies, although in only very low proportions. Nevertheless Sogo *et al.* (2015) based on different gene regions (EF, 28S, mtCOI) and Jacobson *et al.* (2016) based on microsatellite and COI markers found similar evidence with regards to potential interbreeding in field-collected populations between L1 and L2 lineages. Recently, heteroplasmy was found in thelytokous (L2) lineages in India and it was also speculated to be derived from paternal leakage, which indicates hybridization events outdoors in the *T. tabaci* species complex (Gawande *et al.*, 2017). Hybridization makes the identification of the different *T. tabaci* subspecies more complicated because of the imperfect reproductive isolation among lineages, especially if they form sympatric populations in the same habitat. Interbreeding among subspecies – besides of increasing genetic diversity – provides opportunities for hybridization in thelytokous and arrhenotokous subspecies as well. In contrast, no research has been carried out about the mating behaviours of the two arrhenotokous strains. This leaves several open questions about this topic, for instance, whether

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tobacco-associated arrhenotokous males can mate with leek-associated arrhenotokous females and vice versa?

Consequently, CAPS markers need to be designed in order to measure the genotypes of nuclear genes within the *T. tabaci* cryptic species complex. However, these procedures are not considered to be difficult when dealing with several closely related biotypes or subspecies. The population genetic approach in the present study is appropriate to recognize species boundaries between closely related (sub)species, however, it is very complicated to determine the boundary between subspecies and species. The population genetic approach used in this study is relevant for studying the ecological traits and evolution of *T. tabaci*, because failure to differentiate between lineages may lead to incorrect conclusions regarding ecological traits (e.g. host associations, virus transmission capabilities, the occurrence of gene flow and insecticide resistance). Therefore, recognizing lineages within a cryptic species complex using specific markers has enormous potential to improve our ecological and evolutionary understanding of the role of insects in natural ecosystems.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485319000762

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