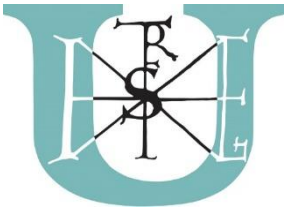

SZENT ISTVAN UNIVERSITY

Molecular identification and assessment of ecological parameters of onion thrips (*Thrips tabaci* Lindeman, 1889)

Thesis of PhD dissertation

FARKAS PÉTER
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PhD School

Name: Doctoral School of Horticultural Sciences

Field: Crop Sciences and Horticulture

Head of Ph.D. School: Prof. Dr. Éva Németh Zámboriné
Doctor of the Hungarian Academy of Sciences
head of department
Department of Medicinal and Aromatic Plants
Faculty of Horticultural Sciences, SZIE

Supervisors: Dr. József Fail
associate professor, PhD
Department of Entomology
Faculty of Horticultural Sciences, SZIE

Dr. Béla Pénzes
professor, CSc
Department of Entomology
Faculty of Horticultural Sciences, SZIE

The applicant met the requirement of the PhD regulations of the Szent István University of Budapest and the thesis is accepted for the defence process.

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Head of Ph.D. School Supervisors

1. INTRODUCTIONS AND AIMS

The onion thrips (*Thrips tabaci* Lindeman, 1889) is 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. Importance of *Thrips tabaci* can be attributed to its wide range of host plants, diverse ecological attributes such as high reproductive capacity, thigmotactic behaviour, virus transmission capacity and resistance to insecticides. The importance of the pest management strategy against onion thrips is enhanced by th fact that, it can cause severe direct and indirect damage due to its ecological attributes. Its direct feeding damage is estimated to cause more than U.S. $1 billion in crop losses annually worldwide (Balan et al., 2018). *T. tabaci* is also a vector of two economically significant viral pathogens, *Tomato spotted wilt virus* (Smith, 1931; Chatzivassiliou et al., 1999) and *Iris yellow spot virus* (Pozzer et al., 1999; Kritzman et al., 2001; Gent et al., 2006; Mandal et al., 2012). The latter pathogen is estimated to cause annual losses of U.S. $90 million to onion production in the USA alone (Gent et al., 2006) while the former can cause over U.S. $1 billion in crop losses annually worldwide (Goldbach and Peters, 1994). Subsequent studies have reported that the spread of the *Tomato spotted wilt virus* on tobacco fields of East-Europe was caused by *T. tabaci*. (Chatzivassiliou et al., 1999).

Severeal studies of the recent decades have pointed out that there are differences in the reproductive biology, host range, virus compatibility, virus transmission capacity and resistance to pesticides between the populations of *T tabaci* originating from different plants (Zawirska, 1976; Chatzivassiliou et al., 2002; Wu et al., 2014). However, the adults of the species complex still remain indistinguishable based on morphological characteristics (Jenser et al., 2001; Jenser et Szénási, 2004; Kobayashi et Hasegawa, 2012). Tha lack of the population genetic studies have impeded the clarification of *T. tabaci*'s taxonomic status. 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 or biotypes based on host preferences: leek-associated types (arrhenotokous L1 and thelytokous L2) and tobacco-associated type (arrhenotokous T). It was also revealed that the different biotypes can co-occure and cause damage on the same host plant at the same time (Nault et al., 2006; Kobayashi and Hasegawa, 2012; Kobayashi et al., 2013). Differences in the ecological attributes of the members in this cryptic species complex will influence the effectivenes of the pest management strategy used agains them. In view of the host plant and composition of the sympatric *T. tabaci*

polulations it would be possible to estimate the expected population dynamics and crop damage. Differences in the ecological attributes could essentially determine the level of risk and damage and the pest management strategy to mitigate the damage.

Studying the literature one will quickly realise that our knowledge regarding the species complex is incomplete, which is especially relevant to the T-biotype. Scientific studies have focused almost exclusively on the different biological aspects of the L1- and L2-biotypes, which were approached from different disciplines. This is partly due to the fact that the leek-associated biotypes can be characterized by a broader host plant range and larger geographical distribution area.

In order to develop a sustainable, long-term effective pest management strategy, it is important to define accurately the ecological parameters that can broaden our knowledge about what biotype or biotypes of the species complex may occur as key pest in cultivated crops.

The aims of my study were the following:

- to identify a molecular marker in order to discriminate *T. tabaci* biotypes unambigously and for developing a simple, rapid laboratory identification method;
- to analyse the genetic variability of the *T. tabaci* cryptic species complex based on mitochondrial COI by using Hungarian field collected specimens which were selected in laboratory and used for setting up mass rearing colonies;
- to determine and compare the life table parameters of the *T. tabaci* cryptic species complex on economically important host plants in laboratory conditions (based on the primary host plant range);
- to assess the sex ratio pattern in the progeny of arrhenotokous *T. tabaci* lineages on preferred and economically important cultivated hosts.

2. MATERIALS AND METHODS

2.1. Establishment of *Thrips tabaci* species complex stock colonies and maintenance

2.1.1. Insect collection

The *T. tabaci* mass cultures were established by the following procedure in 2013 and 2014. The thelytokous *T. tabaci* (L2) samples were collected from different plants in the Botanical Garden of Szent István University located in Budapest and from Tordas located in Central Hungary in July of 2013. Leek-associated arrhenotokous (L1) populations were collected from onion bulbs (*Allium cepa* L.), which were obtained from a traditional onion growing area, Makó Southern-Hungary. Tobacco-associated arrhenotokous (T) speciemens were sampled on tobacco fields by dr. Jenser Gábor (Nicotiana tabacum L.) in Apagy, Pócspetri, Encsencs in East-Hungary.

2.1.2. Reproductive mode identification

The field-collected females of *T. tabaci* speciemens can reproduce by thelytoky and arrhenotoky and most likely are already mated. 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 microcentrifuge tubes on leaf sections and allowed to oviposit through their entire lifespan. 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. Deuterotokous reproduction was not observed at all. The field-collected females were preserved in 75% ethanol until identification. All adult females were slidemounted using Berlese'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). The progeny of the field-collected females was also verified by nucleic acid analysis.

2.1.3. Establishment of laboratory mass rearing colonies and maintenance

The life table paramater experiments of the *T. tabaci* cryptic species complex requires the accurately identified and isolated mass cultures, which can provide thrips specimens

continously to the different treatements. Therefore, stock laboratory mass colonies were set up for each biotype of the *T. tabaci* lineages in 2013 and 2014. The colonies were established based on Steiner and Goodwin's (1998) modified method by using the mixed progenies of each biotypes. The stock thelytokous colony was maintained on cabbage head leaves, the leek-associated colony on leek leaf sections, and the tobacco-associated colony on whole tobacco leaves in ventilated translucent plastic containers. The mass cultures were checked two times per week. The stock colonies were kept in Sanyo MLR-352H (Panasonic Corporation, Osaka, Japan) climate chambers separately in 16 h light and 8 h dark period at 20°C, 70 % relative humidity.

2.1.4. Plant materials

Brassica oleracea L. convar. *capitata* var. *alba* 'Hurricane' F1 cabbage variety was used in both L2-biotype life table studies and mass culture maintenance. Leek plants were obtained from market and used for L1-biotype mass culture maintenance. Onion sets (*Allium cepa* L. 'Senshyu Yellow') were planted for studying the performance of L1-biotype thrips. *Nicotinia tabacum* L. 'Hevesi 9' F1 variety seeds were sown for both T-biotype life table studies and mass culture maintenance. *Phaseolus vulgaris* L. 'Lingua Di Fuoco' Borlotto type seeds were sown for studying the performance of *T. tabaci* biotyes on bean.

2.2. Timatable of the research work

The life table studies were conducted from 13th of July 2015 to 1st of January 2017 in each day on a 12 hours basis. During that period females were randomly chosen from our stock colonies for oviposition and initiatin of the different treatements and the identity of these females were confirmed by nucelis acid analysis. The simple molecular identification method of *T. tabaci* biotypes was developed in 2014. In 2017–2018, we proceeded to evaluate the mortality of eggs in L1- L2- and T-biotypes, slide mounted the collected larvae for sex identification and continued the molecular analysis of thrips females used for treatment initiation.

2.3. Molecular identification of the *Thrips tabaci* cryptic species complex

2.3.1. Genomic DNA isolation

Total genomic DNA was extracted from a single thrips of confirmed origin (host plant) and reproductive mode by a rapid standard method with Proteinase K treatment (De Barro & Driver, 1997). A new thrips-specific primer pair was designed and successfully used for amplifying a fragment of mitochondrial COI gene. The primers amplificated 780 bp long DNA

fragment by using 2x DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Lithuania). The products of the PRC were checked in 2.5% agarose gel. PCR products were purified with High Pure PCR Product Purification Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's protocol and the amplicons were sequenced directly from both directions commercially at BaseClear B.V. (Leiden, The Netherlands).

2.3.2. CAPS marker analysis

The amplified DNA sections can be discriminated from each other by using CAPS method (Konieczny és Ausubel, 1993). The leek-associated arrhenotokous type (L1) has restriction sites both for PsuI and for PsyI-enzymes 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). The amplified PCR products were digested according to the manufacturer's protocol. The digested PCR products were ran in 2.5 %-agarose gel.

2.3.3. DNA sequence analysis, genetic 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). DNA Sequence Polymorphism Software (DnaSP version 5.10) and PoPART softwares (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz) (Bandelt et al., 1999) were used to reconstruct minimum spanning network (MSN). Standard population genetic diversity and haplotype diversity indices were calculated with DnaSP V.5.10 (Librado and Rozas, 2009) and ARLEQUIN v.3.5 software (Excoffer and Lischer, 2010).

Sequence alignments were done using ClustalW algorithm implemented in MEGA6 Software (Molecular Evolutionary Genetics Analysis, Version 6) (Tamura et al., 2013). JModelTest 2.1.10. software (Darriba et al., 2012) was used to specify the substitution model that best fits the data set for the Maximum Likelihood (ML), Maximum Parsimony (MP) and Neighbor-Joining (NJ) analysis. The HKY + G model (Hasegawa et al., 1985) was selected by

JModelTest2 software. Additional Bayes analysis was done by using BEAST V.2.4.8. (Bayesian Evolutionary Analysis Sampling Trees) software (Bouckaert et al., 2014).

2.4. Life table studies

2.4.1. Method of life table studies

For life table experiments, *T. tabaci* speciemns in each treatment were incubated individually in 2 ml translucent microcentrifuge tubes (VWR Collection, SuperClear brand) and held at 23 ±1°C, 70 ± 5% RH, a photoperiod of 16:8 (L:D) h. To start experiments, female adults were collected randomly from stock laboratory colonies and transferred to tubes with a single piece of onion, cabbage, bean or tobacco leaf disc for oviposition. The number of eggs in the leaves was counted using the bottom light of a stereomicroscope Alpha NSZ-606 (Ningbo Yongxin Optics Co., Ltd., Ningbo, China). Leaf tissues with eggs were checked every 12 h for newly emerged larvae. First instars were transferred individually into new tubes with fresh leaf disc. Egg durations and hatching ratio were recorded. Thrips in immature stages were checked every 12 h for development, but the sex could not be determined until adulthood. Survivorship and developmental times at different developmental stages were recorded. When adults emerged, females and males were contained individually in microcentrifuge tubes with a new leaf disc. Arrhenotokous females were paired with males for 12 h. Females were transferred into new tubes at 12 h untill the first egg laid and after this preoviposition period the females were transferred daily. The number of eggs produced by each female was recorded. Males were checked only daily during their lifespan. Preoviposition and oviposition period, adult longevity, lifetime fecundity and mean daily fecundity were calculated.

2.5. Method of sex ratio analysis and egg mortality

For arrhenotokous females, leaf discs with eggs were treated separately for analysis of daily sex ratios. The eggs deposited by mated females during their entiry life span were checked countinously for hatching and the first or second instar larvae were preserved in alcohol during. The unhatched eggs in the leaf discs were also recorded. The preserved larvae were slidemounted in Berlese solution. The sex identification of the larvae were done based on the identification guide of Vierbergen et al. (2010).

3. RESULT AND DISCUSSION

3.1. Genetic analysis of the *Thrips tabaci* cryptic species complex by using CAPS method

Genetic variability was detected in the 116 mtCOI sequences for validating the new CAPS marker. We have identified 106 SNPs (14,32 %) during the assessment of the sequences obtained from the stock laboratory colonies which resulted 21 haplotype sequences in total. 4 haplotypes for L2-, 6 haplotypes for L1- and 11 haplotypes fot T-biotype in nearly the same number of samples per biotype (Figure 1.).

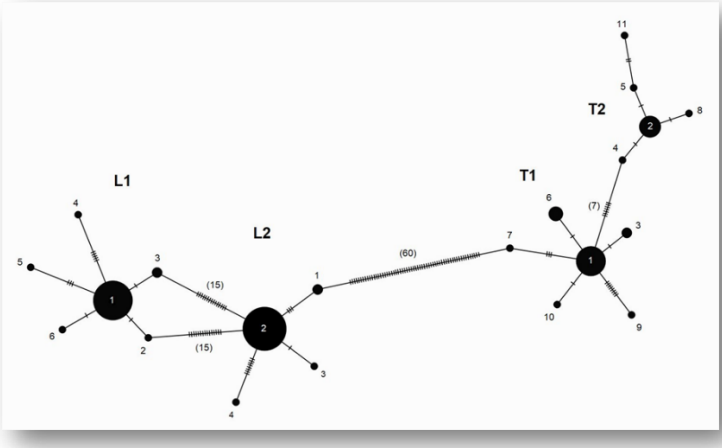

Figure 1. Minimum spanning network (MSN) was calculated based on 116 Hungarian mtCOI sequences using DNaSP (Librado and Rozas, 2009) and PoPART (Bandelt et al., 1999) softwares. Different circle sizes are proportional to the haplotype frequency in the dataset. The numbers within circles are the haplotype designations. Perpendicular tick marks and the numbers in the parenthesis on the lines represent the number of the nucleotide substitutions between the linked haplotypes.

The structure of the MSN confirmed the existence of the three known distinct biotype (L1, L2, T) in Hungary. The high nucleotide variability verified the occurence of two subgroup or biotype within the T-biotype in Hungary, which subgroups were named T1 and T2. Almost exclusively Hungarian sequences clustered together in the T2-biotype clade, only one greece sequence showed closer genetic relationship to the Hungarian haplotype sequences. It suggests, we have found new haplotype sequences in Hungray compared to the international sequences. These new sequences can be characterized higher nucleotide variability than the reference sequences in the group T1. The topology of the MSN and dendograms indicated the same genetic structure which also confirms the genetic structure of *T. tabaci* regarding the distinct monophyletic clades (L1, L2, T) (Brunner és mtsai., 2004; Toda és Murai, 2007; Kobayashi és Hasegawa, 2012; Kobayashi és mtsai., 2013; Jacobson és mtsai., 2013; Nault és mtsai., 2014; Fekrat és mtsai., 2014; Sogo és mtsai., 2015).

Based on the results of the genetic analysis the new CAPS marker allows the simple identification of the three biotypes of *T. tabaci* based on mtCOI gene region by using restriction enzymes to digest the amplfied DNA fragments. The sequence analysis confirmed the result of the restriction enzymes produced banding patterns. The leek-associated arrhenotokous type (L1) has restriction sites for both enzymes, the leek-associated thelytokous type (L2) has only one cleavage site and the tobacco-associated type (T) does not have restriction sites for either of the two enzymes.

3.2. Life table studies of the *Thrips tabaci* cryptic species complex

Based on the laboratory result the different host plants allow distinct growht rates for the different biotypes Differences in the life table parameters suggest different host plant adaptations. Former studies have already reported strong host plant specific (physiological) adaptation in laboratory studies on tobacco, leek and cabbage (Zawirska, 1976; Chatzivassiliou és mtsai, 2002; Chatzivassiliou, 2002; Li és mtsai., 2014). Tobbaco and bean can be defined as good host plants for the T lineage compared the cabbage and onion. Even though juvenile development was completed both on onion and cabbage, it required a longer period of time, fecundity was lower and juvenile mortality was higher resulting in a much slower population growth on thes host plants. Therefore, the host plant designation can be used for those plants, but they can not be defined as good host plants considering the poor population growth. Tobacco can not be classified as host plant at all for the leek-associated biotypes (L1 and L2) because the

adults died within a few days on tobbacco leaf discs. It must be concluded based on the findings that the ancestor of both onion and bean could be the original host plant of the ancient onion thrips taxon. Consequently, other plant species belonging to Alliaceae and Fabaceae might had also been ancestor host plants. The speciation could have started on a currently unidentified possible intermediate host plant or a certain range of host plants, because the taxon could have come to contact with tobacco plants just a few hundreds years ago and during this period of time it could not have developed such an extent in specialzation considering the mutation rate (2 % per million years). This intermediate plant could be the hypotesized *Solnaum nigrum* (Almási et al., 2016), but additional plants in the Solanaceae too. The ancestor plant of the onion thrips taxon could be as well an ancient variant of cabbage, member of the Brassicaceae, because we have found the least mortality of the larvae on this plant.

According Li et al. (2014a) onion is a more advantageous host for the L1-biotype and cabbage allows fast population growth for the L2-biotype. Li et al.'s (2014a) result is confirmed regarding onion is better host for the L1-biotype, however, our findings can not support the same scenario on cabbage for the L2-biotype.

3.3. Sex ratio pattern in the *Thrips tabaci cryptic* species complex

Thrips tabaci has haplodiploid sex determination system, which allows females to allocate the reproduction towards female (diploid, fertilized eggs) or male (haploid, unfertilized eggs) offspring through selective fertilization of the eggs (Flanders, 1946; Cook, 1993). We have found that mated L1 females produced a sex ratio (male:female) of 26,3 % and 73,7 % on onion, 23,2 % and 76,8 % on cabbage, 20,1 % and 79,9 % on bean; and mated T females produced a sex ratio of 29,6 % and 70,4 % on tobacco and 18,1 % and 81,9 % on bean. We have additionally concluded that the sex ratio int he progeny of fertilized females showed an „U' shaped pattern by female age independent of the host plant, suggesting the genetic regulation of age dependent sex ratio. We have proved that the offspring sex ratio at the bottom of the "U" was constant 21 % on onion (30 days long), 17 % on cabbage, 16 % on bean (16 days long) for the L1-biotype females; and 25 % on tobacco (25 days long), 11 % on bean (16 days long) for the T-biotype females, indicating an influence of the host plant quality. We can confirm that the offspring sex ratio is around 30:70 = male:female (Krueger et al., 2015; Li et al, 2015a) and varies around this value depending on the host plant quality.

4. NEW SCIENTIFIC RESULTS

1. We have identified a CAPS marker that allows the simple distinction of the three biotypes of *T. tabaci* based on a region ot the mtCOI gene. The 780 bp long DNA sections were amplified by using TTL-UNIF1 and TTL-UNIR1 primer pairs, which allowed indubitable identification of the biotypes with PsuI and PsyI restriction enzymes produced banding patterns. 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).

2. We have proved that genetic analysis of the Hungarian *T. tabaci* specimens based on mtCOI gene region confirmed the genetic structure and diversity of the three known biotypes (L1, L2, T). Furthermore, we have found novel haplotype sequences in Hungary, which prove the occurence of a new subgroup within T-biotype. We have separated the group T to T1 and T2 subgroups based on the genetic analysis.

3. It has been proved by genetic analysis, that T-biotype speciemens can be characterized higher genetic variability than the leek-associated L1 and L2 onion thrips specimens. We ascertained during further genetic analysis that the populations of leek-associated L1 and L2 might have experienced bottleneck or founder effect, however the T-biotype can be characterized by large and stable population with long evolutionary history.

4. We have compared foremost the life table parameters of molecularly identified *Thrips tabaci* biotypes on four economically significant cultivated host plants (onion, cabbage, tobacco, bean) including male specimens too. There were differences in the performances of the different *T. tabaci* biotypes on the four host plants. Based on the results among tested host plants the tobacco and bean can be defined as good host plants for T-biotype, onion and cabbage can be defined only as host plants. The leek-associated L1- and L2-biotype cabbage, onion and bean also can be classified as good host plants, however, the tobacco can not be classified as host plant at all. The difference between the ecologigal parameters of the biotypes on different hosts, are due to the strong host-related adaptation of the lineages.

5. Our results partially confirmed former results of Li et al. (2014a) considering the host-related performance of leek-associated L1- and L2-biotypes of *Thrips tabaci*. Our results supported the better host adaptation to onion for the L1-biotype, but did not support the better adaptation to cabbage for the L2-biotype.

6. Last but not least, we have assessed the sex ratio pattern of the molecularly identified arrhenotokous biotypes during the complete lifespan of the fertilized females. We have found that the succesfully mated L1 females produced a sec ratio (male:female) of 26.3 % and 73.7 % on onion, 23.2 % and 76.8 % on cabbage,, 20.1 % and 79.9 % on bean; the mated T females 29.6 % and 70.4 % on tobacco and 18.1 % and 81.9 % on bean. We can confirm that the offspring sex ratio of the females are host plant dependent and the sex ratio varies around 30:70 male:female sex ratio depending on the host plant quality.

7. It is proved first within order of Thysanoptera that, the sex ratio in the progeny of the succesfully fertilized arrhenotokous females showed an „U"-shaped pattern plotted to female age independent of host plant, which may refer to the genetic regulation of age dependent sex ratio. Considering the offspring sex ratio, we have found that it was constant 21 % on onion, 17 % on cabbage, 16 % on bean for the L1-biotype females; and 25 % on tobacco; 11 % on bean for the T-biotype females indicating the effect of the host plant quality.

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