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A novel capillary gel electrophoresis based fragment analysis method for the rapid detection of important thrips species on alfalfa in Turkey

Türkiye'de yoncada önemli thrips türlerinin hızlı tespiti için yeni bir kapiler jel elektroforez tabanlı fragment analiz yöntemi

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ABSTRACT

Thrips cause significant yield reduction in several industrial crops. Since these pests are also included in the quarantine organisms of Turkey, the rapid detection of agents is important to prevent their spread to new areas. Mitochondrial cytochrome oxidase I (COI) barcoding gene assay; one of the molecular methods is widely used in thrips identification. However, as the COI gene has a very short fragment length, it is very difficult to distinguish fragment sizes on agarose gel after PCR. In this study, a new identification method was developed by integrating the Capillary Gel Electrophoresis (CGE) system for *Thrips tabaci Lideman, Frankliniella occidentalis* (Pergande) and *Frankliniella intonsa* (Trybom) species, using primer pairs previously used by different researchers. The assay produces strong signals obtained by minimizing the margin of error in the separation of fragment lengths close to each other, especially in the short fragment length COI gene. Therefore, by eliminating the gel electrophoresis step, reliable detections could be obtained without exposure to hazardous chemicals. The novel method shortened the detection time and minimized the process mistakes on the detection of a single thrips with a low DNA concentration. Total 83 thrips individual (52 *F. intonsa*, 31 *F. occidentalis*) were able to be detected with this capillary gel electrophoresis based fragment analysis. The novel method is evaluated as unique, specific and quick for the detection of three different thrips species. It is also thought to be able to utilize for identification of different thrips species with short fragment sizes in the foreseeable future.

INTRODUCTION

There are many harmful organisms in alfalfa cultivation areas. Clover proboscis beetle [*Hypera variabilis* Hebst. (Coleoptera: Curculionidae)], 24-spot ladybird with main pest [*Subcoccinella vigintiquatuorpunctata* (Linnaeus 1758) (Coleoptera: Coccinellidae)], the Clover leaf beetle [(*Gonioctena fornicata* (Brüggem) (Coleoptera, Chrysomelidae) and thrips come primary pests on causing yield losses in alfalfa production. *Thrips palmi* Karny, 1925 and *Frankliniella occidentalis* (Pergande, 1895) are quarantine pests for many countries. It is essential to detect these pests quickly and accurately. It has been emphasized in different studies

that these pests need rapid detection to understand their biology and prevalence for developing a strategy in pest management (Danks 1988). Identification of thrips at genus level requires expertise and in most cases can only be conducted only by experienced people (Przybylska et al. 2015). However, although there are reports that thrips have been successfully identified at the larval stage (Skarlinsky and Funderburk 2016), this identification method is still not preferred (Mound 2013). As there are not enough diagnostic keys to identify at the level of eggs, larvae or pupae by morphological examination, adult individual thrips are generally preferred for identification (Mound 2013, Skarlinsky and Funderburk 2016). Besides identification studies of some thrips species such as *F. occidentalis* may cause doubtful results due to the high morphological diversity among species. Therefore, the use of molecular techniques is beneficial in reliable identification (Bravo-Pérez et al. 2018, Rugman-Jones et al. 2006). The use of molecular techniques gives more reliable results, especially in identification of the quarantine pests as mentioned above.

Hebert et al. (2003a, 2003b) designed a primer set to amplify the 648-nucleotide region of the mitochondrial cytochrome-c oxidase subunit I (COI) gene for rapid and accurate identification of a wide variety of biological samples. The method called "DNA barcoding" has several advantages compared to other methods. The standard DNA barcode region of the COI gene is very effective for species identification and discrimination. This region has good discrimination against most animal groups. These universal primers, originally designed for marine invertebrates, can be applied to all animal phylums (Folmer et al. 1994, Hebert et al. 2003a, 2003b, 2004). The 648-nucleotide fragment has sufficient information and can be sequenced directly. The alignment process is not difficult because it is a protein-coding region. Because of these advantages, the COI region is used for standard DNA barcoding studies. DNA barcoding is a simple and powerful method for routine identification by researchers, especially for large numbers of samples (Jinbo et al. 2011). DNA barcoding using sequence data obtained from a standard region of the COI gene offers important solutions for the identification of thrips species (Glover et al. 2010). The main reason for the use of this particular gene region is to obtain more phylogenetic signals with the presence of robust universal primers covering many animal branches (phyla) (Folmer et al. 1994, Glover et al. 2010, Zhang and Hewitt 1997). Mitochondrial DNA (mtDNA) is abundant, has a relatively fast evolution rate, and amplification of degraded mtDNA samples is possible. In contrast, nuclear genes have relatively slow evolution rates and are usually single copies (Glover et al. 2010). As a result of the analysis of the COI gene, three of *T. tabaci* (Brunner et al. 2004, Toda and Murai 2007) and two of *T. palmi* (Glover et al. 2010, Karimi et al. 2010) were identified. Similarly, two species of *F. occidentalis* were successfully determined (Iftikhar et al. 2016, Rugman-Jones et al. 2010). It has also been reported that the COI provides sufficient variation to be used in future DNA barcoding studies within the thrips species (Glover et al. 2010). Identifying economically important thrips species at the larval stage can be difficult and usually needs culturing (Jinbo et al. 2011). Especially in invasive quarantine organisms, the COI gene is very advantageous in terms of rapid pest identification (Glover et al. 2010). However, PCR products obtained from COI gene are usually required sequence analysis (Bravo-Pérez et al. 2018). In a study in 2017, *Thrips tabaci, T. palmi, F. occidentalis* and *Frankliniella intonsa* were identified by multiplex PCR in a single reaction using specific primers (Sabahi et al. 2017). As a result of the study, the researchers proved that the primers could detect these four species. The method was used for thrips identification at different developmental stages and reliable results were obtained for all samples examined. They reported that this method is simple to be applied by non-expert taxonomists and can also be detected quickly and reliably without sequence analysis (Sabahi et al. 2017). As interest in biodiversity has increased in the fields of ecological evolutionary biology, agriculture and economics, reliable identification of the organisms has been rising in importance (Jinbo et al. 2011). On the other hand, the number of taxonomists has decreased significantly, especially in the identification area of quarantine thrips pests. As a result, the necessity of alternative and reliable identification methods is getting increase day by day especially in absence of experts. Sabahi et al. (2017) could detect four different thrips quickly and reliably. However, it was determined in the study that the electrophoretic separation of *T. palmi, F. occidentalis* and *F. intonsa* fragments on agarose gel is quite difficult due to closely sized fragment lengths. The main reason of confusion is that 30-40 nucleotide differences are not clearly separated in the gel electrophoresis.

In this study, the amplicons obtained as a result of PCR analysis performed using primers developed by Sabahi et al. (2017) were analyzed using a high-efficiency DNA Fragment Analyzer instead of agarose gel. The closely sized fragments are separated with a DNA fragment analyzer and the analysis time is considerably shortened.

MATERIALS AND METHODS

Samples and DNA extraction

A total of 123 individual thrips were collected with a mouth aspirator from an alfalfa cultivation area in Aksaray province of Turkey. While 40 thrips were used for morphological identification, the rest of them were used for molecular identification. Collected samples were stored in ethyl alcohol before use. Positive controls DNA of *T. tabaci, F. occidentalis* and *F. intonsa* used in the molecular study were provided by the Plant Protection Central Research Institute, Ankara. Morphological identification was realized by Prof. Dr. Ekrem Atakan at Çukurova University. The samples were examined under a stereoscopic microscope and placed in AGA (9 parts of 60% ethyl alcohol, 1 part of glacial acetic acid, 1 part of glycerine) for identification. Thrips samples kept in this solution for one or two days were then taken and labelled in small plastic tubes containing 60% ethyl alcohol. To facilitate the preparation, the thrips samples, which were kept in AGA fluid for 2 days and then taken into alcohol (60% ethyl alcohol), were kept in a 5% NAOH fluid until individuals had a slight color change and the body content was cleaned by allowing this fluid to enter the body. After the samples were kept in 96% ethyl alcohol for 5 minutes, their preparations were made by taking them into Hoyer medium. The preparations (microscope slides) were kept in the oven at 45 °C for about 3 weeks to dry. Thysanoptera species were identified by the author using identification keys of Zur-Strassen (2003) and Balou et al. (2012).

Thrips samples were used in molecular studies for quick and economical DNA extraction as described in Sabahi et al. (2017). For this purpose, thrips individuals were ground with the help of a drill attached to the tip with a needle. The concentration and purity of the obtained total DNA was measured in an electrospectrophotometer (Nanodrop 2000-Thermo) and kept at -20oC for later use in the PCR stage.

Multiplex PCR

For the detection of *T. tabaci, F. occidentalis* and *F. intonsa* species in multiplex PCR, one general forward primer and three reverse primers (given in Table 1) specific to Cytochrome Oxidase I (COI) region were used. For PCR optimization studies, first, gradient PCR was performed with primers separately and optimal annealing degrees were determined.

Table 1. Primers and fragment sizes used in the detection of thrips species by multiplex PCR (Sabahi et al. 2017)

Name	Specificity	Sequence	Product length
tabR (reverse)	*Thrips tabaci*	5'-TGTGAT-AGCTCCCGCTAAC-3'	360 bp
occiR (reverse)	*Frankliniella occidentalis*	5'-GGTCCAGAGTGA-TAAAAAGTTGAC-3'	163 bp
intR (reverse)	*Frankliniella intonsa*	5'-AGGTATTTAAGT-TTCGATCTGTAAG-3'	390 bp
Common forward		5'-YTWGGAGCHCCH-GAYATAG -3	

In 25 µl total volume for multiplex PCR; 5 µl 5X GoTaq Buffer (Green), 1.25 µl MgCl2 (25 mM), 0.7 µl dNTPs (10 mM), 1 µl each of the specific reverse primers (10 µM), 2.5 µl common forward primer (10 µM), 0.5 µl DSMO (2%), 0.25 µl GoTaq Flexi DNA Polymerase (5 µl), 2.5 µl DNA template (20 ng/µl) and finally 8.3 µl nuclease free water were added. After 3 minutes of pre-denaturation at 94 oC, PCR was performed at 94 °C for 30 seconds, at 56 °C for 30 seconds, at 72 °C for 1 minute (35 cycles) and then at 72 °C for 1 minute. First amplicons were visualized on agarose gel before fragment analysis. PCR products were analyzed at 80 V for 60 minutes on a 1.5% agarose gel prepared with Pronosafe (Conda, Madrid, Spain) DNA dye and visualized under UV transilluminator.

Fragment analysis

After the gel electrophoresis process, 2 µl of the PCR products were mixed with 20 µl of dilution buffer for dilution of samples. The diluted PCR products were placed on the reading plate of the capillary gel electrophoresis (CGE) based analyzer (Qsep-100 ™, Bioptic, Taiwan). A high resolution cartridge with a capacity of 200 samples was placed to start the reading process on the device, and appropriate markers (marker created with quantitative markers and DNA amplicons determined for this study) and other buffer solutions (distilled water, separation buffer) were added. The reading procedures of 83 individuals were completed after the sample injection protocol at 8 kv for 10 seconds with a high resolution cartridge, following a 300 s separation process at 5 kv.

RESULTS AND DISCUSSION

Many of the thrips species are polyphagous pests and have similar host ranges. Although there are morphologically diagnostic keys (Glover et al. 2010, Rebijith et al. 2014), it is known that there are not sufficient criteria for the identification from egg, larva or pupa (Kadirvel et al. 2013).

This study morphological identification that 28 individuals (22♂ and 6♀) of 40 examined thrips were *F. intonsa* and the other 12 individuals (4♂ and 8♀) were *F. occidentalis*. In molecular studies, 11 larvae and 72 adults were used.

The economical DNA extraction method (20 µl of nuclease-free water) was performed for all individuals, including larvae. Approximately 5-25 ng/µl DNA concentration from each of the individuals was obtained. Multiplex PCR produced 163 bp and 390 bp fragments for *F. occidentalis* and *F. intonsa*, respectively (Figure 1). However, fragments of T. tabaci (360 bp) were not detected in the tests.

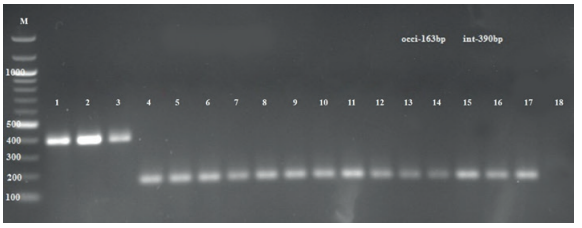

Figure 1. Agarose gel electrophoresis of the multiplex PCR products. (M: DNA 1-kb Ladder; line 1-3 *Frankliniella intonsa*; line 4-17, *Frankliniella occidentalis*; line 18, water control)

As the 360 bp and 390 bp fragments were found very closely to each other, capillary gel electrophoresis (Qsep-100 ™) was applied for these two primer pairs for quick and reliable results. It was observed that detection range of the commercial quantitative marker (20-1000 bp) in the range of 300-400 bp (*marked with) is quite wide (Figure 2). This marker was not used in the study due to the detection thresholds were in this range and deviated ± 10 bp instead of this marker, a new reference marker was created with previously determined DNA amplicons. The extreme signal values were obtained from the readings with this reference marker and they were recorded in the system. The markers used in the study showed that they are rather suitable for the identification of thrips.

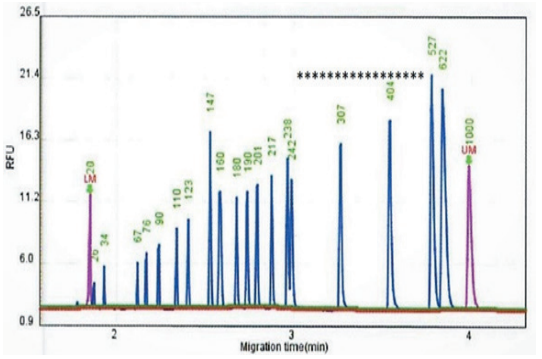

Figure 2. The image of the signal values of the commercial quantitative marker was used first in the study

Effective signal values in expected sizes were obtained with a deviation of approximately ± 2 bp in each reading with the values of the reference marker and commercial quantitative marker recorded in the Qsep-100 system. As a result of the signal values the samples were separated and identified in a more detailed form for three thrips species (Figure 3).

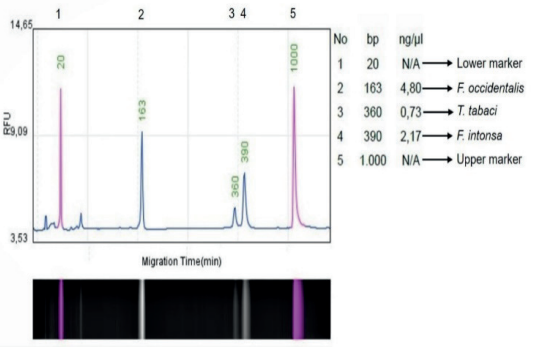

Figure 3. Capillary gel electrophoresis-based fragment analysis of multiplex PCR amplicons of thrips species (No 2, *Frankliniella occidentalis*; No 3, *Thrips tabaci* and No 4, *Frankliniella intonsa* positive control DNA)

COI was used as a universal DNA barcode (Hebert et al. 2003a, 2003b). The DNA barcode was considered as the official protocol for the identification of insects, not only as a competitor to traditional taxonomy, but also as a powerful tool to assist in detection and identification of new species (Leite 2012). Herein, some thrips individuals could not be morphologically identified by experts. The study brought out that the CGE method was highly beneficial in cases of difficulties during morphological identification.

Molecular methods support morphological identification and both methods yield consistent results in many studies (Marullo et al. 2020, Xie et al. 2019). Meanwhile, the number of morphological identification experts in quarantine laboratories is getting decrease.

Thrips are significant pests in plant quarantine (Fekrat et al. 2015, Haung et al. 2009, Sabahi et al. 2017). So, quick identification of these harmful organisms in trade is very important (Danks 1988). The combination of different detection methods gets more reliable results. Combined molecular methods for identifying thrips species are a valuable alternative when morphological identification is difficult or almost impossible. Modern methods used together with morphological keys to identify thrips species are highly useful in identifying species in all life cycles of thrips (Mehle and Trdan 2012). So far, many studies have been performed by using different molecular techniques for eliminate of the morphological identification problems (Fekrat et al. 2015, Gariepy et al. 2005, Huang et al. 2010, Mehle and Trdan 2012, Przybylska et al. 2016, Sabahi et

al. 2017, Saccaggi et al. 2008, Toda et al. 2013, Zhang et al. 2012). In these studies, it was aimed to establish faster and more accurate identification criteria with the primer pairs developed by Sabahi et al. (2017) for the rapid detection of thrips. In this novel method presents a different diagnostic assay with a new fragment analysis method without the need for sequence analysis and gel electrophoresis. Different primer sets were used to amplify the 648-base pair (bp) region of the mitochondrial cytochrome-c oxidase subunit 1 (COI) gene identified by Hebert et al. (2003a, 2003b). However, either this gene region is subjected to sequence analysis or it is used in identification by using specific primers.

Specific primer pairs can lead to misleading results on agarose gel, due to amplification of a 648-base region. Przybylska et al. (2016) showed that close amplicon length leads to unreliable results. With the CGE method, these misleading results can be eliminated and identification studies can be performed with ± 2 bp deviation values for each thrips species. In identification studies, it has been observed that very successful results have been obtained with CGE assay. Similar results have also been obtained in Kerékgyártó et al. (2013). The methods used in molecular analysis are still in progress. Especially, the CGE technology (Qsep100) method is very advantageous in terms of time, cost, convenience and accuracy. With this novel method developed for thrips, it has become the primary tool, especially in quarantine and research studies. Many advantages of CGE technology have been mentioned in different studies. Agarose gel electrophoresis is a widely used method because it is cheap and simple, but the deterioration during electrophoresis significantly affects the analysis. In some cases, it may not be possible to obtain the correct amplicon sizes on visual inspection (Yokoyama et al. 2006). It has been reported that it is possible to determine the length of the pieces without bioinformatics experience and to share the obtained data with other laboratories thanks to CGE. Similarly, it has been reported that many steps of the current method, including gel electrophoresis, can be carried out reliably without the need for steps with harmful chemicals (Karakuş et al. 2017). In different studies, it has been reported that the signals obtained from electropherograms in CGE technology can be detected as low as 0.01 ng/µl and 0.002 ng/µl after the injection of samples with different concentrations (Kerékgyártó et al. 2013). In this study, successful results were obtained from nucleic acids with low concentration obtained from a single individual (larva) regardless of their developmental level. The samples even with a concentration of 0.73 ng/µl had a Relative Fluorescence Units (RFU) value of 5>. One of the strongest aspects of the method is that the cost is reduced by significantly reducing the total volume in PCR.

This study revealed production of each thrips species effective signals at very low concentrations and likewise, the bands were quite clear in the agarose gel. Fragment analysis results showed that 52 individuals were *F. intonsa* and 31 individuals were *F. occidentalis*. Based on morphological and molecular analyses, their results were found compatible. In the results of both analyses, all collected samples were *F. intonsa* and *F. occidentalis*.

CGE technology is used in many areas of molecular studies (genotyping, PCR, RFLP, SNP, SSR, etc.) it provides benefits in terms of sensitivity, reliability, convenience, cost and time. In this new capillary gel electrophoresis analysis method, *T. tabaci*, *F. instonsa* and *F. occidentalis* the rapid identification of species without the use of any commercial extraction kit is described. This method can be easily applied by taxonomists who are not experts in quarantine analysis. Also, provide a reliable diagnosis without exposure to carcinogenic chemicals used in gel electrophoresis.

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ÖZET

Thripsler, birçok endüstriyel üründe önemli verim kayıplarına neden olur. Bu zararlılar Türkiye'deki karantina organizmaları arasında yer aldığından, etmenlerin hızlı tespiti yeni alanlara yayılmalarını önlemek için önemlidir. Mitokondriyal sitokrom oksidaz I (COI) barkodlama geninin analizleri; moleküler yöntemlerden biri olarak thrips teşhislerinde yaygın olarak kullanılmaktadır. Ancak COI geninin fragman uzunluğu çok kısa olduğundan, PCR sonrası agaroz jel üzerinde fragman boyutlarını ayırt etmek çok zordur. Bu çalışmada, daha önce farklı araştırmacılar tarafından kullanılan primer çiftleri kullanılarak *Thrips tabaci Lideman, Frankliniella occidentalis* (Pergande) ve *Frankliniella intonsa* (Trybom) türleri için Kapiler Jel Elektroforez (CGE) sistemi entegre edilerek yeni bir tanımlama yöntemi geliştirilmiştir. Analiz, özellikle kısa parça uzunluklu COI geninde birbirine yakın parça uzunluklarının ayrılmasında hata payını en aza indirerek, elde edilmiş güçlü sinyaller üretir. Bu sebeple, jel elektroforezi adımı ortadan kaldırılarak, tehlikeli kimyasallara maruz kalmadan güvenilir tespitler elde edilmiştir. Yeni yöntem, tespit süresini kısaltmış ve düşük DNA konsantrasyonuna sahip tek bir thripsin saptanmasındaki işlem hatalarını da en aza indirmiştir. Kapiler jel elektroforezi tabanlı fragman analizi ile toplam 83 thrips bireyi (52 *F. intonsa*, 31 *F. occidentalis*) tespit edilebilmiştir. Yeni yöntem, üç farklı thrips türünün tespiti için benzersiz, spesifik ve hızlı olarak

değerlendirilmektedir. Ayrıca yakın gelecekte kısa fragman boyutlarına sahip farklı thrips türlerinin tanımlanmasında da kullanılabileceği düşünülmektedir.

Anahtar kelimeler: *Frankliniella intonsa, Frankliniella occidentalis*, mitokondriyal sitokrom oksidaz I, multipleks polimeraz zincir reaksiyonu, Thysanoptera

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