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MOLECULAR IDENTIFICATION OF SOME APHID SPECIES (HOMOPTERA; APHIDIDAE) BASED ON RFLP-PCR TECHNIQUE

Hero M. Muhammad[1], Dilzar H. Mawlud[2], Kamaran M. Taha[3*], Nabeel A. Mawlood[4]

[1,2,4] Department of Plant protection, College of Agricultural Engineering Sciences, Salahaddin University-Erbil, Kurdistan Region, Iraq.

[3,*] Department of Animal Resources, College of Agricultural Engineering Sciences, Salahaddin University-Erbil, Kurdistan Region, Iraq.

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Correspondence Email:
Kamaran.taha@su.edu.krd

ABSTRACT

This work includes, identification eight species of Aphids (Homoptera: Aphididae) which collected from the leaves of different plants in many localities of Erbil governorate Kurdistan region-Iraq from the period May till July 2022, these are: *Chaitophorus salijaponicus*, *Aphis fabae*, *Macrosiphum rosae*, *Capitophorus carduinus*, *Myzus persicae*, *Aphis ruborum*, *Aphis punca, and Aphis gossypii*. The mitochondrial cytochrome C Oxidase subunit I (COI) gene used for identification these species. DNA was isolated, and a band of 550 bp of mt COI gene was amplified during the PCR amplification. The amplicons were digested with *HinfI* and *DdeI* restriction enzymes. The restricted fragments produced by RFLP technique were proved by agarose gel electrophoresis. The results illustrated that digested amplicons were given bands according to their cut sites. This study presented that studying aphids to detect their species through a RFLP-PCR technique by using these restriction enzymes can distinguish some species with reliable results. *HinfI* and *DdeI* REs could not distinguish all species, *HinfI* only discriminated species *Macrosiphum rosae*, *Capitophorus carduinus*, *Myzus persicae* and *Aphis gossypii*, but *DdeI* identified the remain species, *Chaitophorus salijaponicus*, *Macrosiphum rosae*, *Myzus persicae* and *Aphis ruborum*, within and among other species exactly. The study suggested using other restriction enzymes to provide full recognition profile for all species.

College of Agriculture and Forestry, University of Mosul.

INTRODUCTION

Aphids (Homoptera: Aphididae) are the most economical important pests all over word. They transport the viruses and are able to create severe losses in crop yield. They also decrease the quality of crops and then their prices (Kinyanjui *et al.*, 2016). Due of the ease of spreading viruses and using parthenogenetic reproduction, they are nevertheless kept in high regard as serious pests (Foottit *et al.*, 2008). The productivity losses caused by aphids on different crops are estimated to be between 70 and 80 percent worldwide (Aslam *et al.*, 2007). These losses originate from the harm produced by feeding immediately on plant sap, which results in underdeveloped growing crops, plants that turn yellow, which then causes significant losses in vegetable yields, and secondary losses brought on by the spread of plant viruses (Aslam *et al*., 2007).

Prompt and correct aphid species recognition is essential for actual controlling of insects and plant protection methods due to their economic value (Miller and Foottit, 2009 and Lee *et al.,* 2011). By using their physical characteristics, aphid species have conventionally been classified (Emden and Harrington, 2007). However, identification based on physical characteristics is severely hampered by their tiny size and loss or decrease of essential morphological traits (Miller and Foottit, 2009 and Kinyanjui *et al.*,2016).

Due to the obvious variety in each species, discriminating through coloring scheme (morphological) is not only useless for identification of species; it is indeed nearly indistinguishable between the species depends on their morphologies in dried slide preparations due to the absence of characteristic features (Valenzuela *et al*., 2007). In several cases, identification of species based on coloration of pattern is challenging. Because Aphid morphology is influenced by a variety of environmental and physiological factors (Jalalizand *et al.*,2012). In cases when morphological and ecological evidence remain unclear, molecular methods are indeed very helpful (Choe *et al*. 2006 and Valenzuela *et al*., 2007). Since a proper detection of insects is really the first stage in pest management. Precise identification of aphid species is required such as using of molecular techniques (Jalalizand *et al.*,2012). In an effort to identify different aphid species and get over the constraints of morphological identifications, several molecular approaches have been investigated. PCR-RFLP technique relies on the digestion of PCR amplicons with the proper restriction enzymes to yield distinctive polymorphic fragments that are then seen as markers for species identification. For the purpose of identifying different pests of economic value by species, PCR-RFLP has been used in several investigations (Brunner *et al*., 2002 and Kinyanjui *et al*., 2016). Numerous aphid species in the family Aphididae have been identified using RFLPs (Valenzuela *et al.,* 2007, 2009 and Kinyanjui *et al.*, 2016).

No molecular identification tools have been developed to separate these aphid species in Erbil governorate. The goal of the current study was to find a quick method for separating the various aphid species that were gathered from around Erbil. The general objective is to make technologies available that will aid in the prompt and accurate identification of these pests, which should therefore enable speedier and more successful pest control and reinforce of biosecurity protocol in nations where the aphid species is a problem. So that, for this purpose in this study was RFLP-PCR molecular technique have been used to analysis mitochondrial cytochrome c oxidase subunit I gene to distinguish some aphid species, using 2 restriction enzymes (*HinfI* and *DdeI*).

MATERIALS AND METHODS

Sample Preparation and DNA Isolation

This study was done on species of Aphids (aphid species), at genome company laboratory in Erbil. Eight species were collected from different district in Erbil Governorate.

Collected samples of the concerned species were stored in ethanol 70% at 25°C (until the all samples were collected for about a month) for DNA extraction. Genomic DNA was isolated from adult individuals, each specimen was extracted by ZYMO Quick-DNA Tissue/Insect Microprep Kit manufactured USA- No. D6015

according the manufacturer instructions. The extracted genomic DNA was stored at -20°C for the downstream applications. The purity of DNA was checked using Nanodrop spectrophotometer (Thermo scientific UK).

PCR Primers

Polymerase chain Reaction (PCR) arose applying a modification of the restriction fragment length polymorphism (RFLP) method. Table 1 illustrated the specific information on the primer.

Table (1): Pair of primers sequence of Cytochrome Oxidase C subunit I.

Gene name	Nucleotide Sequences	Product size	Reference	TM^{o}
Cytochrome Oxidase c subunit I (COI)	forward primer C1-J-1718 5'GGAGGATTTGGAAATTGA TTAGTTCC-3'	550bp	(Simon *et al.*, 1994).	60 C^{o}
	Reverse primer C1-N-2329 5'ACTGTAAATATATGATGA GCTCA-3'			

PCR Amplification

Mitochondrion gene specific primers were designed for the using the sequences of cytochrome c oxidase subunit I synthesized by Micro-gene Company (South Korea) then for each species were amplified by PCR. The primers yield a band 550bp. PCR amplification for COI partial gene was done in 50 µl of final reaction mixture containing; 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) of pair of primers, DNase free water and template DNA (Table 2), by Bioresearch PTC-200 Gradient thermocycler. The modification temperature profile included step one is an initial denaturation at 95 C for 5 min, step two followed by 35 cycles of a denaturation at 95C for 40 second, a primer annealing at 60C for 40 sec., an extension at 72C for 1 min and final step is an extra extension at 72C for 10 min. then kept at -20 °C for the downstream application. The PCR product was electrophoresed and visualized by 1.5% Agarose gel (Simon *et al.*, 1994).

Table (2): PCR reaction mixture for amplification of COI gene

No.	**PCR components**	**Concentration**	**Volume (µl)**
1	Master Mix	2x	25
2	Forward Primer	20 Pmol	3
3	Reverse Primer	20 Pmol	3
4	DNase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

RFLP Analysis

Assimilation of PCR products for aphid species were done separately with *HinfI* 5'-G/ANTC-3' / 3'- CTNA/G-5' and *DdeI* 5' C/TNAG-3' / 3'- GANT/C-5' (ADR6201 00001211493, Promega- USA) REs. Digestion of amplificons for COI partial gene was done in final volume 20 µl and the reaction covers (2 µl of 10X

Reaction buffer, 0.5 µl (5 U) of Reaction Enzyme, 10 µl PCR product and 7.5 µl of free deionized water), according to restriction enzymes manufacturer's instructions. Then incubated at 37 °C (2 - 3) hours. 10µl of the assimilated products were analyzed in 2.5% agarose electrophoresis. For comparison the sizes of digested products of each species, 100 bp molecular marker (Ladder) RTU (Cat NO. DM012-R500, Promega- USA) was used (Al-Barzinji and Taha, 2017).

RESULTS AND DISCUSSION

Mitochondrion gene specific primers were purified and qualification of DNA ratio checked and ranged from 1.7 to 1.9. The universal cytochrome c oxidase subunit I gene was produced 550bp as shown in figure 1, and run in 1.5% agarose gel electrophoresis (1.5 hours 80V 1X TBE buffer) stained with 5µl ethidium bromide. After that, amplified 550 bp fragment was faced to restriction enzymes *HinfI* and *DdeI*, some restricted sites of amplified DNA were found by both enzymes, and the products ranged from 550 to 50 bps. Bands less than 100 bp were not measured as diagnostic were not shown in agarose gel. Then, bands were electrophoresed by 2.5% agarose. all specimens presented fragment sizes based on their restriction sites for the used enzymes, after they were allied with the molecular marker (ladder 100bp) for comparisons, as shown in Table 3, Figure 2 for *HinfI*, and Figure 3 for *DdeI* restriction enzymes.

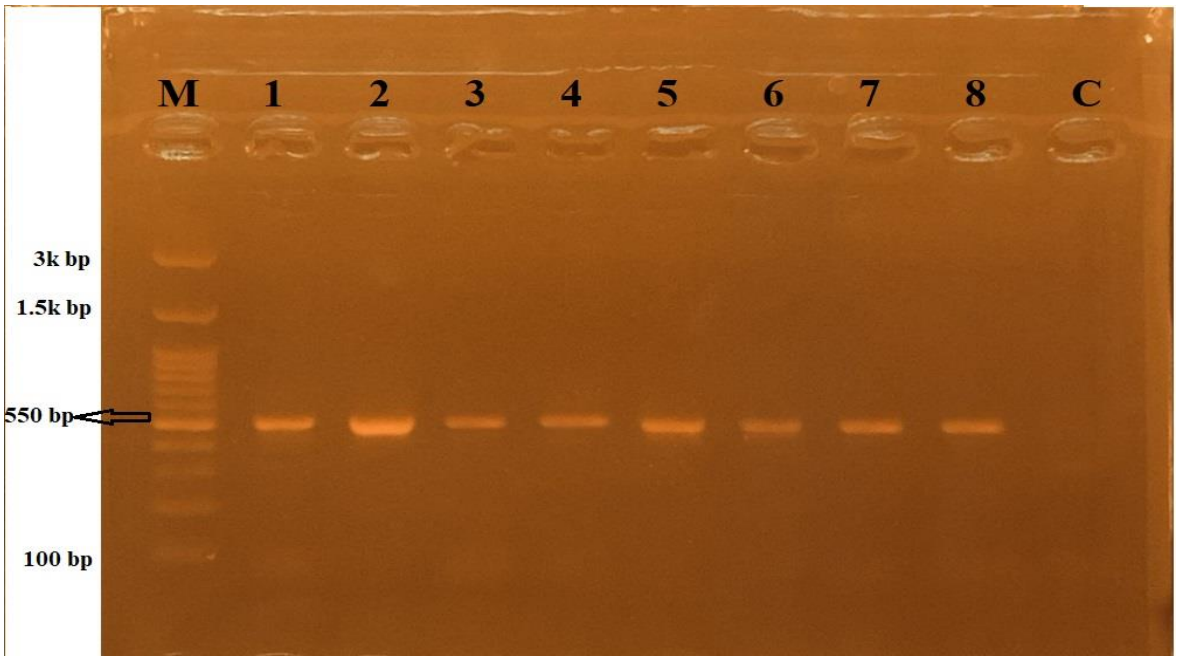

Figure (1): PCR amplification of partial cytochrome C Oxidase I gene from insects. M; indicate: ladder 100 bp, lane 2- 8: 550 bp of PCR products of aphid species (*Chaitophorus salijaponicus, Aphis fabae, Macrosiphum rosae, Capitophorus carduinus, Myzus persicae, Aphis ruborum, Aphis punca, and Aphis gossypii*), and C is negative control.

Table (3): Number of bands and their sizes of Aphid species produced by (*HinfI*, *DdeI*) REs.

Aphid species	***HinfI* fragment size pb**	***DdeI* fragment size pb**
Chaitophorus salijaponicus	2 bands (550,500)	1 band (550)
Aphis fabae	1 band (550)	2 bands (550, 500)
Macrosiphum rosae	2 bands (400,150)	2 bands (300, 200)
Capitophorus carduinus	3 bands (550,300, 200)	2 bands (550, 500)
Myzus persicae	3 bands (550,400,150)	3 bands (550,500,250)
Aphis ruborum	2 bands (550,500)	1 band (500)
Aphis punca	1 band (550)	2 bands (550, 500)
Aphis gossypii	2 bands (300, 200)	2 bands (550, 500)

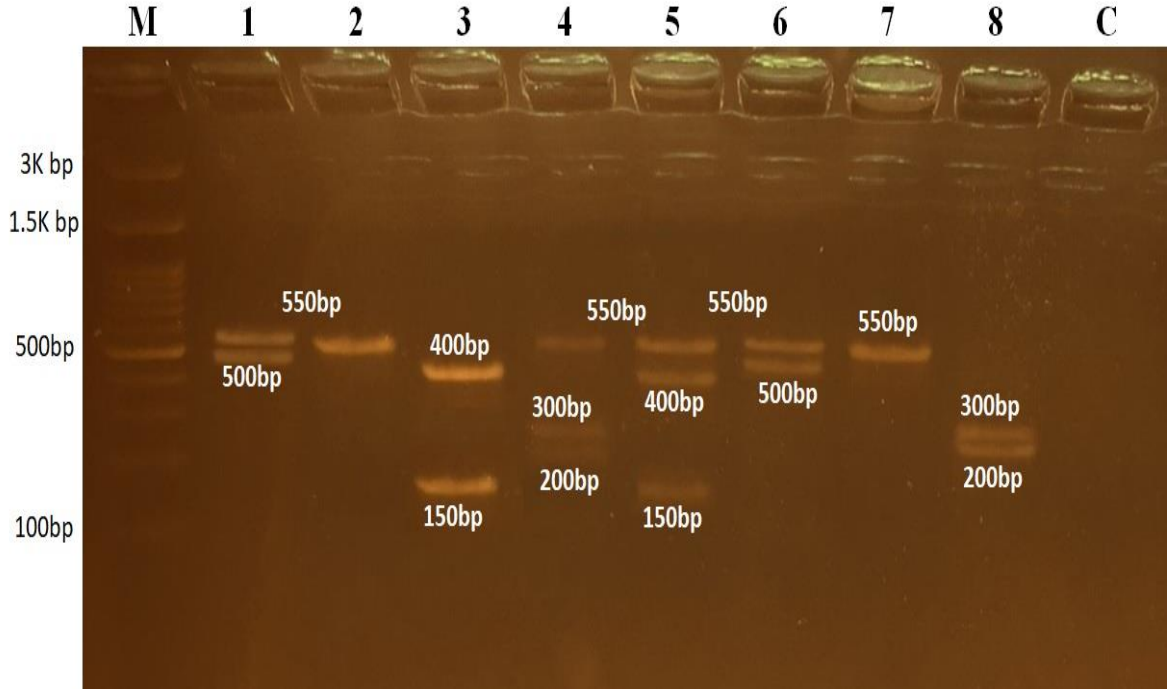

Figure (2): Digestion of PCR product of Aphid species with HinfI restriction enzyme of cytochrome C Oxidase I gene fragments. M; indicate: ladder 100 bp, lane 2- 8: 550 bp of PCR products of aphid species (*Chaitophorus salijaponicus*, *Aphis fabae*, *Macrosiphum rosae*, *Capitophorus carduinus*, *Myzus persicae*, *Aphis ruborum*, *Aphis punca*, *and Aphis gossypii*), and C is negative control.

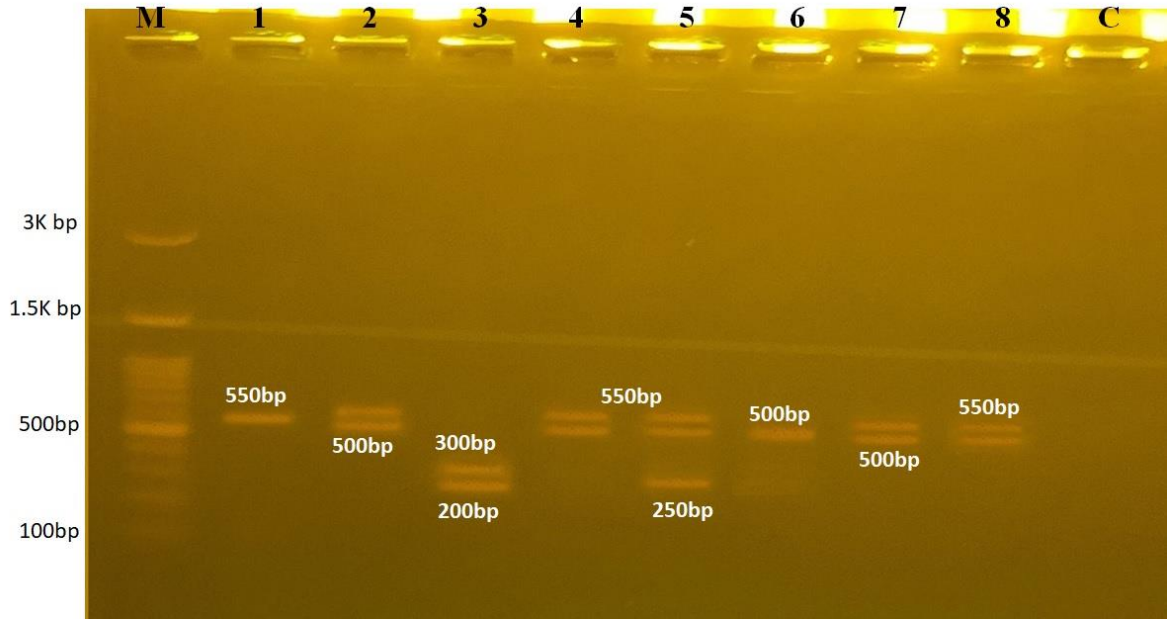

Figure (3): Digestion of PCR product of Aphid species with DdeI restriction enzyme of cytochrome C Oxidase I gene fragments. M; indicate: ladder 100 bp, lane 2- 8: 550 bp of PCR products of aphid species (*Chaitophorus salijaponicus, Aphis fabae, Macrosiphum rosae, Capitophorus carduinus, Myzus persicae, Aphis ruborum, Aphis punca, and Aphis gossypii*), and C is negative control.

Digestion by *HinfI* RE gave bands of 550 and 500 bp in *Chaitophorus salijaponicus* & *Aphis ruborum*. Fragments of 550bp in each of *Aphis fabae* & *Aphis punca*, and fragment sizes of 400 and 150 pb were yielded only in *Macrosiphum rosae*. While formed 550, 300, 200 bp bands in species *Capitophorus carduinus*, species *Myzus persica* gave fragments with the sizes of 550, 400,150 bp, and bands of 300, 200 bp in the *Aphis gossypii* were produced. As a result of the *HinfI* enzyme in figure 2 shown that *HinfI* discriminated species *Macrosiphum rosae, Capitophorus carduinus, Myzus persicae* and *Aphis gossypii*, successfully and exactly within and with other species although failed to differentiate species *Chaitophorus salijaponicus* with *Aphis ruborum* and *Aphis fabae* with *Aphis punca* within species but detected from other species figure 2.

Whereas digestion profile by *DdeI* restriction enzyme gave accurate result and discriminative in *Chaitophorus salijaponicus* and *Aphis ruborum* and could detect them effectively, for *Chaitophorus salijaponicus* species produced band in 550 bp, whereas band in 500 bp were generated for *Aphis ruborum*. DdeI also offered an obvious distinguish between *Macrosiphum rosae* and *Myzus persicae*. Which produced bands in 300 and 200 bp for *Macrosiphum rosae* while bands with sizes in 550, 500 and 250 bp were yielded for *Myzus persicae*. However, DdeI failed to distinguish *Aphis fabae*, *Capitophorus carduinus*, *Aphis punca* and *Aphis gossypii* species that yielded bands of the same sizes in 550 and 500 bps figure 3.

As a result of both restriction enzymes that were done all samples separately at the same time, on one hand, it can be noticed that the *Aphis fabae* and *Aphis gossypii* species were unable to distinguish and marked as same species although their hosts were different, and they were possibly may be visited species that may be stayed on the other host plants for a part of their life span or they may not have restriction sites

for these two restriction enzymes in these two undistinguished species. On the other hand, both enzymes together can provide a clear, effective and reliable identification with each other and the two un distinguished species as well.

The study of the mt COI gene through PCR -RFLP by digesting with *HinfI* and *DdeI* restriction endonucleases demonstrated strong power and together provided a reliable discriminative profile for separating six aphid species such as (*Chaitophorus salijaponicus, Macrosiphum rosae, Capitophorus carduinus, Myzus persicae, Aphis ruborum and Aphis gossypii*) and the bands were generated during digestion were clear and no gloomy amplicons were shown among these 6 species. Therefore, negating the necessity for statistical analysis to determine which species were significant or not. Because restriction endonucleases provided specific fragments for every species (Khan *et al*., 2018). The results were obtained using *HinfI* and *DdeI* restriction enzymes, strongly suggested that they were appropriate for identifying six species and advised to use other enzymes to detect other two undisfigured species. In fact, the most effective way to identify species from all aspects is via the DNA-based methodologies. When tried to compare to molecular markers, earlier identification methods like morphological identification do not produce satisfactory and positive outcomes (Ali *et al*., 2015; Dilger *et al*., 2020 and Kamaran *et al*., 2021).

In overall, aphid species identification in the earlier study was done grounded on morphological and coloration methods (Jalalizand *et al.,* 2012), while in this paper focused on mitochondrion gene via molecular technique which were commonly used for identification species, that not used previously in this region, so that the obtained results were a vital and established a very effective determination in almost all used species by COI mt DNA. Whereas this study concentrated on the mitochondrion gene using a molecular method that was frequently used to identify species "that had not been utilized before in this region". So that results were crucial and recognize a highly successful COI mt DNA determination in practically all species that were used.

Additionally, those studies confirmed that there was a adequate quantity of the targeted DNA to produce amplicons. Then, this is a supporting point to establish the advantages of using mitochondrial DNA more so than nuDNA (Pakendorf and Stoneking, 2005). Kinyanjui *et al*. (2016) used RFLP methods via mt COI gene to spot variation amongst seven species of aphid and grounded on the results *B. brassicae, L. pseudobrassicae, A. pisum, M. persicae and A. gossypii* were differentiated effectively. In a related work, Valenzuela *et al*. (2007) showed how restriction analysis of the PCR-amplified COI gene can be used to identify the immature stages of 25 different aphid species. Jalalizand *et al*. (2012) administrated that *Ericaphis scammelli and wahlgreniella nervata* that recognized via molecular technique were not clearly identified through morphological characters.

CONCLUSION

The results of the present study shown, applying the RFLP-PCR by utilizing the COI gene with the assistance of two restriction digestion (*HinfI* and *DdeI*), is an extremely highly efficient technique for determining aphid species from various hosts. In addition, results demonstrated the applicability of mt COI gene as a most mutable area to distinguish between six aphid species often found in Erbil Governorate. Moreover, it is a rapid technique and does not need hardworking and inexpensive method. This research indicated that, the *Aphis fabae* and *Aphis punca*

species either is not differ with each other and they considered as a visited species or these enzymes failed to recognize restriction sites in these two species. Moreover, the study suggested that two use other enzymes to get full recognition profile for the rest of the aphid species.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

الكشف الجزيئي لبعض انواع المن (Homoptera; Aphididae) باستخدام تقنية تباين اطوال قطع التقييد – تفاعل البلمرة المتسلسل

[1]هيرو محي الدين محمد [2]دلزار حمد مولود [3]كامران مصطفى طه [4]نبيل عبد القادر مولود

[3]قسم الثروة الحيوانية، كلية علوم الهندسة الزراعية، جامعة صلاح الدين – أربيل، إقليم كوردستان، العراق

[1،2،4]قسم وقاية النبات، كلية علوم الهندسة الزراعية، جامعة صلاح الدين – أربيل، إقليم كوردستان، العراق

الخلاصة

هذا العمل يتضمن تشخيص ثمانية انواع من المن التي جمعت من أوراق نباتات عدة من مواقع مختلفة في محافظة أربيل، إقليم كردستان العراق وهي: *Chaitophorus salijaponicus, Aphis fabae, Macrosiphum rosae, Capitophorus carduinus, Myzus persicae, Aphis ruborum, Aphis punca,* and *Aphis gossypii*. جين سايتوكروم المايتوكوندريا المؤكسد هو المستخدم لتحديد هذه الأنواع. وقد تم عزل الحمض النووي الدنا، وتم تضخيم 550 قاعدة زوجية من جين mt COI باستخدام تقنية التفاعل البلمرة المتسلسل. ثم تم هضم القطع المضخمة مع الأنزيمات القاطعة Hinf1 و DdeI. وقد ادى هذا الهضم الى انتاج مجموعات توصيف محددة لكل نوع، ثم تم تحليل القطع المضخمة التي تم انتاجها عن طريق تباين أطوال قطع التقييد بواسطة الترحيل الكهربائي بالهلام. أوضحت النتائج أن القطع المضخمة المهضومة أعطيت حزم حسب مواقع قطع الانزيمات. أظهرت هذه الدراسة أن دراسة حشرات المن لاكتشاف أنواعها من خلال تقنية تباين اطوال قطع التقييد– تفاعل البلمرة المتسلسل باستخدام إنزيمات القاطعة هذه يمكن أن تميز بعض الأنواع بنتائج موثوقة. ان الإنزيمات القاطعة *Hinfl* و *Ddel* لم تتمكن التمييز بين جميع الأنواع. انزيم القطع *Hinfl* ميز بين الأنواع *Macrosiphum rosae* و *Capitophorus carduinus* و *Myzus persicae* و *Aphis gossypii* ، في حين انزيم القطع *Ddel* حددت الأنواع المتبقية، *Chaitophorus salijaponicus*، *Macrosiphum rosae*، *Myzus persicum* بينهما و داخل الأنواع الأخرى بالضبط. اقترحت الدراسة استخدام إنزيمات القاطعة أخرى لتوفير ملف التعرف الكامل لجميع الأنواع المن.

الكلمات المفتاحية: تباين اطوال قطع التقييد– تفاعل البلمرة المتسلسل ، التحديد انواع المن ، الموروث أكسدة سايتوكرومي س الوحيدة الفرعية في الميتوكوندريا، الأنزيمات القاطعة ، القطع المضخمة

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