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Phylogenetic analysis among some species of aphids (Homoptera: Aphididae) using DNA sequencing moleculartechnique

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ABSTRACT

The phylogeny of aphids (Hemiptera: Aphididae) has been studied by molecular technique for the first time in Kurdistan, this study comprises relationship between six species of Aphids collected from the leaves of different plants (Chrysanthemum, Oak, Almond, Pine, Asteraceae and Herbaceous) in many localities of Erbil governorate Kurdistan region-Iraq, started in May to July 2022. For understanding about aphid evolution, phylogenetic of aphid is crucial. However, neither the phylogenetic alterations of the Aphid taxa nor their comprehensive definition have been achieved. Therefore, a unique method has been developed in this study to examine COI gene sequencing and infer the relationships between the major aphid taxa. DNA was isolated, and a band of 550 bp of mt COI gene was amplified. Then the amplicons were sequenced. The part of the COI gene of the insect samples are alignment inside of NCBI GenBank by BLAST program, were used to compare our nucleotide have sequenced with other stored species of insect sequences. This research presented that studying phylogeny of aphid species through a sequencing technique can create a phylogenetic tree for the used species with reliable results. So, our species in Kurdistan region clustered in a monophyletic clade with published from most countries with high identic value (100%). The COI sequence of aphid species were submitted to GenBank with six accessions of OP355287 - OP355292. The composition of nucleotides of the sequence of COI gene was low of G-C base pairing. We were successful in showing that the mt-COI gene can be used as a molecular marker for the identification of related species, as shown by the similarities between the phylogenetic association created by COI.

التحليل النسلي الوراثي لبعض أنواع المن (Homoptera: Aphididae) باستخدام التقنية الجزيئية التتابع النيوكلوتيدي

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الخلاصة

تمت دراسة الشجرة العائلية لحشرات المن (Hemiptera: Aphididae) بالتقنية الجزيئية لأول مرة في كوردستان ، هذا العمل يتضمن العلاقة بين ستة أنواع من المن التي جمعت من أوراق نباتات عدة (أقحوان ، بلوط ، لوز ، صنوبر ، أستراسيا وعشبي) من مواقع مختلفة في محافظة أربيل ، إقليم كوردستان العراق ، التي بدأت في الفترة من شهر الخامس إلى شهر السابع من 2022. حتي نعلم تطور حشرة المن، تعتبرنسالة شجرة عائلة المن ذات أهمية كبيرة. ومع ذلك ، لم يتم اجراء اي التعديل علي شجرة النشوء والتطورانواع المن ولا تعريفها الشامل. لذلك ، تم تطوير طريقة فريدة في هذه الدراسة لفحص تتابع النيوكلوتيدي جين سايتوكروم المايتوكوندريا المؤكسد الوحدة الفرعية (COI) واستنتاج العلاقات بين أصناف المن الرئيسية. وقد تم عزل الحمض النووي الدنا، وتم تضخيم 550 قاعدة زوجية من جين mt COI باستخدام تقنية التفاعل البلمرة المتسلسل. ثم تم تتابع النيوكلوتيدي التضخيم الجين. و من تم استخدامت جزء من جين COI لعينات المن داخل NCBI GenBank بواسطة برنامج BLAST ، لمقارنة التتابع النيوكليوتيدات الخاص بالمن التي جمعنا مع الأنواع الأخرى المخزنة من تتابع النيوكلوتيدي المن. أظهرت هذه البحث أن دراسة الشجرة العائلية لأنواع المن من خلال تقنية تتابع النيوكلوتيدي يمكن أن تخلق شجرة نسالة للأنواع المستخدمة مع نتائج موثوقة. لذلك ، فإن هذا الانواع المن في إقليم كوردستان يتجمع في مجموعة أحادي الشكل مع انواع المنشورة من معظم البلدان ذات القيمة المتطابقة العالية (100٪). تم تقديم التتابع النيوكلوتيدي COI لأنواع المن إلى GenBank مع ستة الارقام مدخلات متتالية منOP355292 - OP355287. كان تكوين النيوكليوتيدات لتتابع جين COI منخفضًا من قواعد نيتروجيني GC. لقد نجحنا في إظهار استخدام جين mt-COI كعلامة جزيئية لتحديد الأنواع ذات الصلة ، كما يتضح من أوجه التشابه بين العلاقة الااشجار العائيلية التي أنشأتها COI.

الكلمات المفتاحية: التتابع النيوكلوتيدي؛ أنواع المن؛ جين Mt COI؛ شجرة النشوء والتطور؛ القطع المضخمة

INTRODUCTION

In the entire world, aphids (Homoptera: Aphididae) are the pests with the greatest economic impact. The small, soft-bodied, and closely related families Adelgidae and Phylloxeridae have an estimated 5000 species. While carrying the viruses, they are capable of leading to huge losses in crop productivity. Furthermore, they reduce crop quality, which drives up agricultural costs (Foottit *et al*., 2008; Kinyanjui *et al*.,2016). Notwithstanding this, they are viewed as harmful pests because of how easily they spread viruses and because they practice parthenogenetic reproduction (Foottit *et al*.,2008). Aphids on different crops are expected to globally decrease productivity by between 70 and 80%. (Aslam *et al*.,2007).

The lack of variety in physical characteristics has made it difficult to determine how different aphid species are related. Aphis species in particular lack distinguishing morphological traits. Many species cannot be differentiated morphologically, despite the fact that some can be quickly identified by a single diagnostic morphological characteristic. As a result, several species have been characterized based on their striking morphological likeness. The resulting entities, referred to as "groups of species," are merely collections of species that are difficult to distinguish morphologically, and thus lack taxonomic validity (Wang *et al*., 2011). Due to the economic

importance of aphids, rapid and accurate species identification is crucial for effective insect control and plant protection techniques (Miller & Foottit, 2009 and Lee *et al*., 2011). The morphological properties of aphid species have traditionally been used to classify them. However, due to their diminutive size and loss or reduction of crucial morphological characters, identification based only on physical criteria is significantly impeded (Miller & Foottit, 2009 and Kinyanjui *et al*.,2016). Additionally, precise taxa are required for the early identification of biological incursions and efficient implementation among those pests (Lozier *et al*., 2008). Stern *et al*. (1997) and Stern (1998) described the first initiatives to use mitochondrial DNA to offer additional information on the phylogenetic and taxonomy relationships within aphids. Since then, a number of mtDNA and nuDNA markers have been used to define the genetic relatedness somewhere at subfamily, tribe, genus, or species levels (Von Dohlen & Moran, 2000; Von Dohlen *et al*., 2006; Rivas and Torres, 2010). Evidence on the time and patterns of divergence, as well as carrier interaction and the bio-geographical origin of aphids, was recently given by Kim *et al*. (2011). In addition to providing accurate and trustworthy information in the form of the sequencing of the mitochondrial DNA to distinguish species of aphids and support phenotypic recognition, molecular procedures can also be a useful tool for building evolutionary relationships. The mt COI, mt COII, and nuclear EF1 DNA sequences for key aphid species have been gathered through numerous molecular phylogenetic investigations on aphids (Moran *et al*., 1999; Von Dohlen *et al*., 2006 and Kim *et al*., 2010).

Molecular approaches that rely on PCR have been crucial in the advancement of biological sciences since they have allowed for the development of reasonable, quick, and reliable approaches and equipment in recent years (Jalalizand *et al*.,2012) Indeed, molecular techniques are highly useful when morphological and ecological evidence is ambiguous (Choe *et al*. 2006 and Valenzuela *et al*., 2007). Since an accurate insect detection is actually the first step in pest management. It is necessary to identify things precisely, for example, by employing sequencing (Jalalizand *et al*., 2012).

It has been difficult to classify between aphid species because of the relative scarcity of unique morphological traits. However, a DNA-based sequencing technique can reveal how aphid species are related with each other. Besides, in the Erbil governorate, no sequencing molecular phylogeny available for aphid species. In the light of this truth, the current study's objective was to provide a rapid method for classifying the various aphid species that were collected near Erbil. The main goal is to develop technology that will make it easier to quickly and accurately phylogeny these aphids. This will facilitate quicker and more effective pest control measures and strengthen biosecurity protocols in countries where the aphid species is an issue. The mitochondrial cytochrome c oxidase subunit I gene was analyzed using the sequencing molecular approach in this study to make phylogenetic for different aphid species.

MATERIAL AND METHODS

Sample Preparation and DNA Isolation

This research was applied on different aphid species in a genome company laboratory in Erbil city. Six aphid species were assembled (by hand picking and vacuum collectors) from many districts in Erbil governorate.

Assembled concerned aphid species were kept in 70% of ethanol at 25°C (until collection was completed an about two months) for isolation of DNA. Genomic DNA includes Mt-DNA was isolated from adult insect; extraction was done utilized ZYMO Quick-DNA Tissue/Insect Micro-prep Kit manufactured in (USA-D6015) based on manufacturer's tips. The isolated DNA was kept at -20°C for the later applications. The extracted DNA including Mt-DNA were purified then quality and quantity of the isolated DNA were measured by Nanodrop 1000 (Thermo scientific UK) by absorbance method, which were fluctuated between 1.7 -1.9. After that, the specific primers for Mt-DNA COI gene were manufactured for sequencing of the used gene in this study.

PCR Primers

Polymerase chain Reaction (PCR) hold and applying a modification annealing temperature program of Thongprem *et al*. (2021), for amplified the following pairs of primer for sequencing. Table 1 exemplified the specific data on the Mt-primers. The COI primers were synthesized in South Korea by a company of Micro-gene.

Table 1: Sequence of primers pairs of Cytochrome Oxidase c subunit I.

Gene	**Nucleotide Sequences**	**Product size PS (bp)**	**TM°**
Cytochrome Oxidase c subunit I (COI)	forward primer C1-J-1718 5'-GGAGGATTTGGAAATTGATTAGTTCC-3'	550bp	60° C
	Reverse primer C1-N-2172(HCO2198) 5'- TAAACTTCAGGGTGACCAAAAAATCA -3'		

PCR Amplification

The primers of specific gene in mitochondria which is well-known as mt COI gene, were well-organized to sequence that is manufactured by a company of Micro-gene in South Korea. Then by the conventional PCR (Bioresearch PTC-200 Gradient thermocycler) the amplification was proceeded. The primers formed a band size of 550bp. The final reaction mixture volume of amplification was hold in 50 µl as in Table 2. The PCR profile Program is exemplified in Table 3. Then saved at -20 °C for the later application. Fragments were fractionated by 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) stained with 5µl Ethidium Bromide.

Table 2: PCR reaction mixture for amplification of COI gene

No.	**PCR components**	**Concentration**	**Volume (µl)**
1	Master Mix (AMPLIQON A/S Stenhuggervej 22)	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

Table 3: PCR Protocol for seven used genes

Step	**PCR temp. (◦C)**	**Time (min.)**	**Cycles**
Initial denaturation	95	5	1x
Denaturation	95	40 sec	35x
Annealing	60	40 sec	
Extension	72	1	
Final extension	72	10	1x
Storage	4	∞	-

DNA Sequencing and submission

The partial of amplificated Mt-DNA-COI gene sequencing was conducted by BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems), at Micro-gene Center in Korea. The product was evaluated utilizing an ABI PRISM 310 (PE Applied Biosystems). The Mt-COI gene sequence chromatograms produced in the current work were collected and by hand edited with Finch TV program software. Then the attained sequence has been placed in GenBank with accession numbers (OP355287 to OP355292) for COI gene which are shown in Table 4.

Sequence Alignment

To achieve sequences of only one consensus, chromatograms were examined using the Maximum Likelihood method based on the Tamura-Nei model in MEGA11 software (Stephen *et al*., 1997). The sequence of mt-COI gene was inputted to Basic Local Alignment Search Tool (BLAST) which is an excellent tool for searching sequence alignment process (https://blast.ncbi.nlm.nih.gov/Blast.cgi), that available at the NCBI (National Center for Biotechnology Information) website as well, for comparison and alignments lab. or query sequence with other downloaded biological sequences to realize further likeness with others.

Results and Discussion

PCR amplification

The primer was amplified using conventional PCR, the amplicon size for the amplified universal cytochrome c oxidase subunit I gene was 550bps as shown in Figure 1, and bands were separated by 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) stained with 5µl Ethidium Bromide.

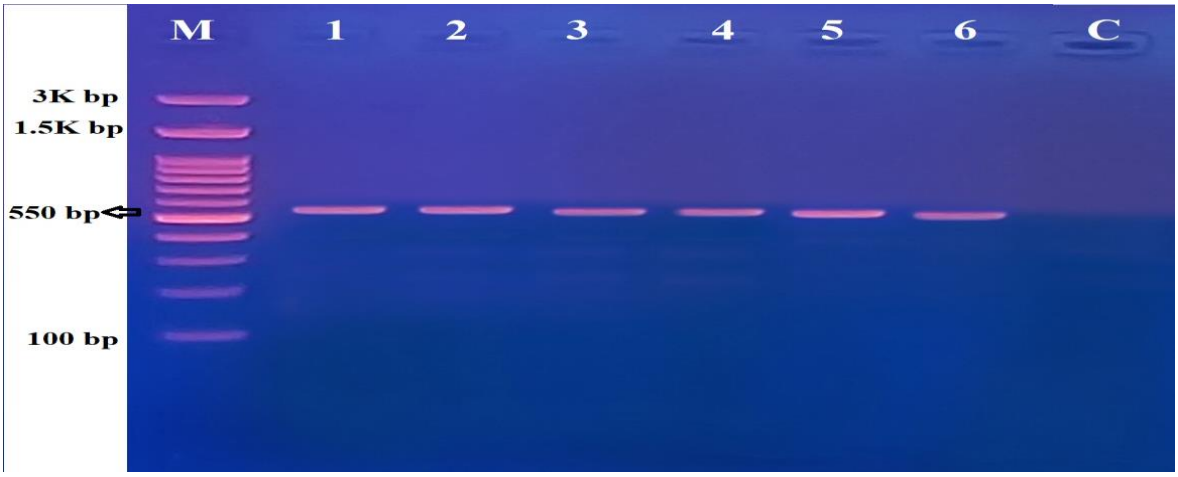

Figure 1: PCR amplification of COI gene from aphid species. M; indicate: ladder 100 bp, lane 2- 4: 550 bp of PCR products of aphid species (*Macrosiphoniella sanborni, Lachnus roboris, Hyalopterus amygdali, Cinara pini, Uroleucon sonchi* and *Aphis nasturtii*) and C is negative control.

Sequence Analysis

Partial and forward primer C1-J-1718 of Mt-DNA COI gene was sequenced, by ABI 3130X genetic analyzer (Applied Biosystem). The Mt-DNA COI gene sequence of aphid sp. is alignment by BLAST database from Gen bank (http://blast.ncbi.nlm.nih.gov/), which was used to show the comparison between our improved sequences with other downloaded sequences of aphid. Then the sequencing result demonstrated that monophyletic in Mt-DNA COI gene sequence was observed in all samples and then no one belonging to specific species. The results got from the BLAST indicated that the highest identity query sequence was 100% that first record in NCBI gene bank for identify of insects. These alignments indicate for inputting our query sequences into NCBI Genbank and given accession numbers as in Table 4. Hence, only one barcode was selected to express each

one during investigates. Mt-DNA COI gene amplicons give rise to produce a 550 bp for all species of aphid. And also, the sequence of Mt-DNA COI gene was plentiful in Adenine and Thiamin.

Table 4: partial COI gene sequences in NCBI and alignment with same sequences after submission

Insect Identified	Accession Numbers	100	100	GU978956
Macrosiphoniella sanborni	OP355287	100	99.75	MN320172
		100	100	JX034913
Lachnus roboris	OP355288	100	98.34	LT600394
		99	100	MT084163
Hyalopterus amygdali	OP355289	99	100	OP103668
		95	100	KF649363
Cinara pini	OP355290	95	100	MN178448
		100	100	KR029937
Uroleucon sonchi	OP355291	98	100	MW596771
		100	100	KC897161
Aphis nasturtii	OP355292	100	100	MW740233

Phylogenetic interpretations

The phylogenic tree was created via the Maximum Likelihood method grounded on the Tamura-Nei model in MEGA11 software (Figure 2), and with previous existent sequence resources analysis. The Linked part of COI mitochondrial gene were applied as entered data. So that, phylogenetic analysis derived from COI nucleotide sequence exposed grouping studied aphid species on regarded lines. From sequence divergence resemblance data and phylogeny created, it was exposed that species fitting to corresponding genera were adjoining to one.

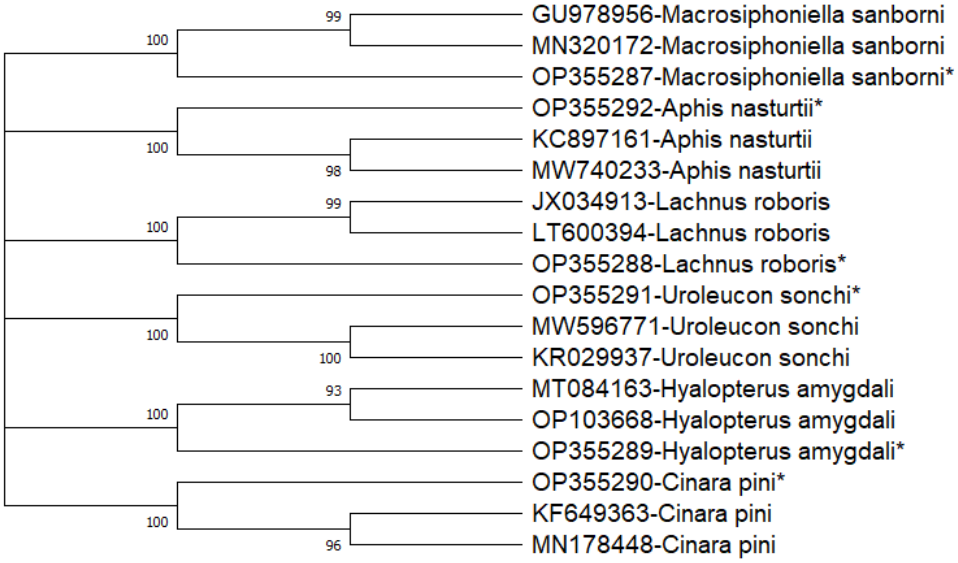

Figure 2: Phylogenic tree of aphid sp. sample from Iraq: Kurdistan region (*).

In overall, aphid species identification and diversity 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 and genetic diversities among 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. Additionally, those studies confirmed that there was an 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).

CONCLUSION

The existing work revealed, using the sequencing technique for gene of mt-COI is a tremendously extremely effective procedure for making phylogeny for species of aphid from several hosts as most of the previous studies showed that the morphological study never gets the reliable result since aphid has small and various size and shape and also even in one type there will be different color. Furthermore, results established the suitability of mt COI gene as a maximum variable part to make phylogenetic trees among six aphid species habitually seen in Erbil Governorate. Additionally, it is a crucial technique, reliable and reasonable method. This study specified that, from sequence divergence resemblance data and phylogeny created, it was exposed that species fitting to corresponding genera were adjoining to one. Moreover, the study recommended that to take more samples in other places to come to be full phylogeny profile for most of the aphid species.

CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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