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First Record of Mint Leaf Beetle, *Chrysolina herbacea* (Duftschmid, 1825), (Coleoptera: Chrysomelidae) in Iraq

Fayhaa Abbood Mahdi Al-Nadawi

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Abstract:

The insect is diagnosed and named by the National Center of Biotechnology Information (NCBI), USA as the Mint leaf Beetle *Chrysolina herbacea alnadawi* (Duftschmid, 1825), (Coleoptera: Chrysomelidae). The diagnosis was performed depending on the DNA analysis by 73% similarity with *Chrysolina herbacea* (Duftschmid, 1825) sequence, In the present study. It is recorded as a new insect pest on mint plant *Mentha puleguim* (L,1753) (Lamiaceae). DNA analysis confirmend that it is recorded for the first time in Iraq and the Arab world as well as the Middle East. Those insects were observed initially during August 2017 in residential gardens of Al-Bonooq district in Baghdad / Iraq.

Key words: *Chrysolina herbacea,* Chrysomelidae, First record, Mint plant, Sequencing.

Introduction:

The Mint Leaf Beetle was diagnosed and named by the National Center of Biotechnology Information (NCBI) USA, as a *Chrysolina herbacea alnadawi* (Duftschmid, 1825), (Coleoptera : Chrysomelidae) depending on the DNA analysis and the diagnosis was confirmed by a later report (1). The insect was most probably entered to Iraq from neighboring countries with infected plants. The literature showed that *C. herbacea* (Duftschmid,1825) is commonly known as The mint leaf Beetle or Green Mint Beetle (2, 3, 4). It is an aggressive invasive species that is native to the European region and originally described from Austria by Duftschmid in 1825 (5, 6, 7). It has been reported from three countries in various wet biogeographical zones, such as Italy, Portugal (8, 9, 10), Great Britain (11, 12, 13), Caucasus and Western Central Asia (14), Slovania, Spain and Germany(15). *C.herbacea* is a monophagous insect, feeding on various Lamiaceae (Mint, pennyroyal), with a preference for the mint plants (16). Both the larvae and the adult of beetles feed on mint leaves (17). The larvae of *Anaphes chrysoelae* is an egg endoparasitoid of *C. herbacea* (18). The mint leaf Beetle attacks the plant leaves, causing both direct and indirect damage to the plant. Direct damage is caused by chewing the leaves, resulting in many holes in plant leaves by black grubs (larvae) and iridescent green beetles.

The Sciences Department,The Basic Education College, Al-Mustansyriah university .Iraq.
E-mail: lneadawi@uomustansiriyah.edu.iq

Indirect damage is a result of losing the leaves areas which leads to reducing the photo synthesis process and thereby reducing growth, possibly leading to the death of the plant (19). The Mint leaf Beetle has a green or jewel-like iridescent green color which is pioneered with tiny little indentations, with black legs and antennae. Females can range in color from green to purplish grey (20), they can be 8-9 mm long and have black colored larvae (21) that also feed on the mint leaves. Visually it may be confused with the much rarer Tansy beetle (*C. graminis*) (22). There are two peaks in the UK; the first is from march to a the middle of June and the second starts from July to October, sometimes reaching to November. The female can produce two generations per a year (23). There was an outbreak that took place in insect population in the UK in 2016 (24). The Mint leaf Beetle was collected by the author, it was found on the aromatic plant mint Mentha puleguim(L,1753) (Lamiaceae), growing in her private garden at Al-Bonooq district in Baghdad province during August, 2017.

Material and Methods:

DNA was extracted from the insect used a standard DNA extracted from Conda / USA, Intron / Korea, Intron / Korea, Kapa /USA, Intron / Korea, Conda / USA, Integrated DNA technologies /USA, intron biotechnology/Korea companies, as shown in Tables 1 and 2.

Table 1. The Materials of DNA extraction from Insects

	Material	Cat #	company
1	Agarose	8100.11	Conda / USA
2	Red safe staining Solution	21141	Intron / Korea
3	6X Loading dye	21161	Intron / Korea
4	Ladder 100bp	KK6302	Kapa /USA
5	Pre Mix PCR	25025	Intron / Korea
6	TBE buffer 10 X	IBS.BT004	Conda / USA
7	Primer	---	Integrated DNA technologies /USA
8	G- spin DNA extraction kit	17045	intron biotechnology/Korea

DNA extraction from Insects

- Preparation of lyophilized leaf sample

Plant tissue lyophilized after harvesting to allow storage at room temperature (15~20℃). To ensure DNA quality, that samples completed lyophilized within 24 hours of collection. Generally, lyophilized leaves were fine powder form, and therefore it did not any special disruption & homogenization steps. Furthermore, when tissue was used freeze-dried, the samples did not need to be frozen in liquid nitrogen. - Measure 5mg of lyophilized leaf, and then transferring to 1.5ml tube using a spatula.

The leaves were collected and lyophilized then measured It's difficult to handle to measure the fine powder sample due to its static electricity. It can be inhibited by previously chilling the spatula with 1.5 ml tube in liquide nitrogen. that was get result in inappropriate lyses, producing a small amount of DNA with a low purity.

Table 2. List of apparatus used in DNA extraction from Insects.

Company	Origin	Apparatus	No
……	Italy	AURA TM PCR Cabinet	1
Bio San	Germany	Micro spin 12, High-speed Mini-centrifuge	2
Dig system	Germany	V-1 plus, Personal Vortex for tubes	3
Bio San	Germany	Bio TDB-100, Dry block thermostat built	4
Bio San	Germany	Biopette Variable Volume 2-20 ul	5
……	Chain	Mini-Power Supply 300V, 2200V	6
Lab net	USA	Multi Gene Opti Max Gradient Thermal Cycler	7
CBS, Scientific	USA	Electrophoreses	8
Lab net	USA	Document system	9
Vilber lourmat	Farance	UV .transmission	10
Bio san	Lativa	Microspin	11

-Add 390$\mu\ell$ buffer PG, 7$\mu\ell$ enhancer solution, 20$\mu\ell$ Proteinase K, and 5$\mu\ell$ RNase, a solution in to sample tube, and the rotation is strong.

With lyophilized leaf it absorbed lyses buffer and became swollen. It may be difficult to handle plant tissue due to its viscosity.. Furthermore, vortex or pipette vigorously to remove any clumps until any plant tissue clumps was not visible. Clumps of plant tissue Would not lyses adequately and would therefore result in a lower yield of DNA. A disposable micro pestle may be used if clumps were not be removed by pipette and vortex

-Incubation the lyses for 30min at 65.

Completed lyses, mixed 5 or 6 times during incubation by inverting tube. The incubation time can be prolonged for more yields of DN lyses permit you saw pure lyses.

-Add 100 $\mu\ell$ buffer PPT to the lyses , mix very well and put incubation for 5min on ice.

This step precipitates detergent, proteins, and polysaccharides. During incubation on ice, mixed 5 to 6 times by inverting tube. The reaction made clear lyses in to opaque slushy lyses. Generally, plant tissues contained large amounts of polysaccharides and polyphenolics, and were therefore relatively not easier to handle. These impurities may be present in the purified DNA if the amount of starting material was increased. Always kept the recommended amounts of samples.

-Centrifuge the lyses for 5min at 13, 000 rpm at room temperature.

Plant materials create very viscous lyses and large amounts of precipitates during this step. kept our recommended amounts of starting material, optimal results were obtained.,

-Transfer carefully 200$\mu\ell$ of supernatant from step 6 in to a new 1.5ml tube.

Although the supernatant was typically 350~400$\mu\ell$, to recover only 200$\mu\ell$of lyses. More lyses sheared of the DNA and contaminating the next step with impurities. When pipette,

-Add 650$\mu\ell$ buffer PB to the lyses, and mixing well by inverting five to six times or by pipette . Do not vortex.

This step was an equilibration step for binding genomic DNA to column membrane. A precipitated form after the addition of Buffer PB,

- Pipette 650$\mu\ell$ of the mixture from step 8, including any precipitate that may have formed, into the spin column inserted in a 2.0 ml collection tube. Centrifuged for 1 min at 13,000 rpm (RT), and discarded the flow-through. Reuse the collection tube in step 8. If a small amount will not pass through, centrifuged again for 1min at 13,000 rpm.
- Repeated step 9 with remaining sample (maximum 200$\mu\ell$).
- Placed the spin column into a new 2.0 ml collection tube (additionally supplied), added 700$\mu\ell$ buffer PWA, and centrifuged for 1 min at 13,000 rpm. Discarded the flow-through and reused the collection tube altogether.

The centrifuge was returned again. For 1min at 13,000. rpm.

- Add 700$\mu\ell$ buffer PWB to the spin column, and centrifuge for 1 min at 13,000 rpm. the flow-through Discarded, and centrifuged again for additionally 1 min to dry the membrane. Discarded the flow-through and collection tube altogether.

Dried the membrane of the spin column since residual ethanol may inhibit subsequent reactions. Followed the centrifugation, removed carefully the spin column from the collection tube without contacted with the flow-through, since this will result in carried over of ethanol. that 40 ml of ethanol (ETOH) has been added to buffer PWB.

- Placed the spin column into a new 1.5ml tube (not supplied), and 100$\mu\ell$buffer PE directly on to the membrane. Incubated for 1min at room temperature, and then centrifuged for 1min at 13,000rpm to elute.

Elution with 50$\mu\ell$ (instead of 100$\mu\ell$) increased the final DNA concentration, reduced overall DNA yield conventionally. Alternatively, to get a larger amounts of DNA, eluting with 200$\mu\ell$increased generally overall DNA yield.

A new 1.5ml tube can be used for the second elution step to prevent dilution of the first elute. Alternatively, the tube can be reused for the second elution step to combine the elutes.

Agarose gel electrophoresis of DNA

Electrophoresis was performed to diagnosis DNA pieces after the process of extraction or to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the Agarose gel.

Preparation of Agarose gel

According to previous studies (25), the agarose gel has been made in 1.5% condensation by melting 1.5 g of agarose in 100 ml of previously made TBE buffer. Agarose was heated to boil then left to cool down at 45-50°C. The gel was poured in the pour plate in which comb was used to make holes that would hold the samples. The gel was poured gently not to make air bubbles and left 30 minutes to cool down. The comb was removed gently of the solid agarose. The plate was fixed to its stand in the electrophoresis horizontal unit represented by the tank used in the electrophoresis. The tank was filled with TBE buffer until it covered the gel surface.

Preparation of sample

We3 µl Put of the processor loading buffer (Intron / Korea) and 5 µl of the supposed DNA, mixed to be electrophoresis, after the mixing process, the process of loading was to the holes of the gel. An Electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 30µl Red safe Nucleic acid staining solution and 500 ml from distilled water.

Red safe Nucleic acid staining solution

Red Safe Nucleic Acid Staining Solution (20,000x) was anew and safe nucleic acid stain, an alternative to the traditional ethyl bromide (ETBR) stain for detecting nucleic acid in Agarose gels. It emitted green fluorescence when bound to DNA or RNA. This new stain had two fluorescence excitation maxima when bound to nucleic acid, one centered at 309nm and another at 419nm. In addition, it had one visible excitation at 514nm. The fluorescence emission of Red Safe bound to DNA is centered at 537nm. Red Safe Nucleic Acid Staining Solution (20,000x) was as sensitive as ETBR. The staining protocol for Red Safe Nucleic Acid Staining Solution (20,000x) was similar to that for ETBR. Compared to ETBR, known as a strong

mutagen, Red Safe Nucleic Acid Staining Solution (20,000x) caused much fewer mutations in the Ames test. In addition, Res Safe Nucleic Acid Staining Solution (20,000x) had an ergative result in mouse marrow *chromophilous* erythrocyte micronucleus test and mouse spermary spermatocyte chromosomal aberration test. So it is wise to choose Red Safe Nucleic acid Staining Solution (20,000x) instead of ETBR for detected nucleic acid in Agarose gels. (Cat. No. 21141).

KAPA Universal DNA Ladder Fig1 (cat # KK6302)

The KAPA Universal Ladder Kit is designed for determining the approximate size and quantity of double-stranded DNA on Agarose gel. KAPA Universal Ladder kits contain eighteen DNA fragments (in base pairs): 100, 150, 200, 300, 400, 500, 600, 800,1000, 1200, 1600, 2000, 3000, 4000, 5000, 6000, 8000, and 10000. The KAPA Universal Ladder contains four reference bands (500, 1000, 1600, and 4000) for orientation.

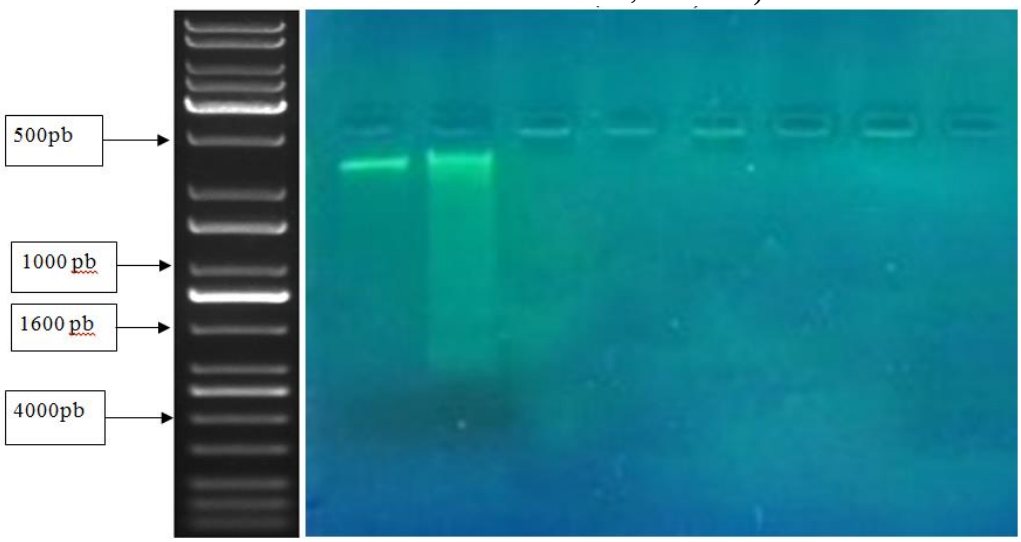

Figure 1. Gel electrophoresis of genomic DNA extraction from bacteria, 1% Agarose gel at 5 vol /cm for 1:15 hours.

The primers used in the interaction

The primers (Table 3) were lyophilized and then dissolved in the free ddH2O to give a final concentration of 100 p mol/µl as a stock solution which was stored at -20. Working primer was prepared in 10 p mol/µl concentration, through adding 10 µl of the stock solution to 90 µl of the free ddH2O water to reach a final volume 100 µl, which was investigated by IDT (Integrated DNA Technologies company, Canada).

Table 3. The specific primer Cox1 of gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'-GTATGAAAGTGTTGATCGTCT- 3'	49.6	38.1	650
Reverse	5'-CCGCATGATCAGGTTAGTAAT- 3'	53.3	42.9	base pair

The optimal condition for initial denaturizing and annealing are identified in tables 5 and 6. Several experiments were performed to gain for this condition; temperature of Gradient PCR was changed throughout the work for all samples to select the optimal condition, DNA template concentration was also changed between 1.5-2µl, where these two factors are considered as important factors in primer annealing with complement (Fig. 4).

Maxim PCR Pre Mix kit (i-Taq) 20µlrxn (Cat. No. 25025)

INTRON's *Maxim* PCR Pre Mix Kit had not only various kinds of Pre Mix Kit according to experience purpose, but also a 2X Master mix solution. *Maxim* PCR Pre Mix Kit (*i*-Taq) was the product what was mixed every component: *i*-Taq DNA Polymerase, DNTP mixture, reaction buffer, and so on-in one tube for 1 rxn PCR. This was the product that got the best result with the most convenience system. The first reason was that it had every components for PCR, so we could do PCR added a template DNA, primer set, and D.W.. The second reason was that it had Gel loading buffer to do electrophoresis, so we did gel loaded without any treatment. It was suitable for various sample's experience by fast and simple used method (Table4,5).

Table 4. The Components of the Maxim PCR Pre Mix kit (i-Taq)

Material	Concentration
i-Taq DNA Polymerase	5U/ µl
DNTPs	2.5mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

Table 5. Mixture of the specific interaction for diagnosis gene

No.	Phase	Tm (°C)	Time	No. of cycle
1-	Initial Denaturation	95°C	3 min.	40 cycle
2-	Denaturation -2	95°C	45sec	
3-	Annealing	62°C	45sec	
4-	Extension-1	72°C	50sec	
5-	Extension -2	72°C	10 min.	

Gel Extraction (Sequencing) Protocol
Absolute ethanol was added to the Wash Buffer prior to initial use. The step of Gel Extraction DNA is as the following (26):

Gel Dissociation

-Agarose gel slica containing relevant DNA fragments were excised and extra agarose was removed to minimize the size of the gel slica.
-Up to 300 mg of the gel silica was transferred to a 1.5 ml micro centrifuge tube.
-500µl of DF Buffer was added to the sample and mixed by vortex.
-The sample was incubated at 55-60°C for 10-15 minutes or until the gel slica has been completely dissolved. During incubation, the tube was inverted every 2-3 minutes.
- The dissolved sample mixture was cooled to room temperature.
DNA Binded
- The DF Column Placed in a 2ml Collection tube.
- 800µl of the sample mixture Transferred from (step1) to the DF Column.
-Centrifuged at 14-16000×g for 30 seconds.
-Discarded the flow-through and placed the DF Column back in the 2 ml Collection tube.
Washed
-Added 600µl of Wash Buffer into the DF Column and let stand for 1 minute.
-Centrifuged at 14-16000×g for 30 seconds and then discarded the flow-through.
-Placed the DF Column back in the 2 ml Collection tube.
-Added 600µl of Wash buffer into the DF Column and let stand for 1 minute.
-Centrifuged at 14-16000×g for 30 seconds and then discarded the flow-through.
-Placed the DF Column back in the 2ml Collection tube.
-Centrifuged 14-16000×g again for 3 minutes to dried the column matrix.
DNA Elution
-Transferred the dried DF Column to anew 1.5 ml micro centrifuged tube.
-Added 20-50µl of Elution Buffer into the center of the column matrix.
-Let stand for 2 minutes the Elution Buffer was absorbed by the matrix.
-Centrifuged for 2 minutes at 14-16000×g to elutes the purified DNA

For this aim, the cox1 sequence was obtained from the NCBI database and primers for PCR were designed manually using the first and the end nucleotides of the desired region. The amplified reactions of the DNA chains of the beet leaf beetle DNA were carried out for PCR techniques, the PCR products were separated on a 2% Agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) after ethyl bromide or Red Stain staining. Sequencing of gene was performed by National Instrumentation Center For Environmental Management (NICEM) online at (http://nicem.snu.ac. kr/main /?en _skin =index.html), biotechnology lab, machine is DNA sequencer DNA3730XL, Applied Biosystem), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http:// www. ncbi. nlm.nih.gov) and BioEdit program**.**

Results and Discussion:

Sequencing and Sequence Alignment

The *Chrysomelidae* (the leaf beetles) is one of the largest families of beetles. The *Chrysomelidae*, as the common name implies, are phytophagous and feed on leaves of plants. Adults of most species are either monophagous or oligophagous and usually use terrestrial

species (27). These findings suggest great regularities in the pattern of assemblage variation at all lineage ages that are best explained by the enduring action of stochastic (neutral) processes of mutation and dispersal. The multi-hierarchical analysis therefore bridges predictions of the neutral theory of molecularevolution and the neutral theory of biodiversity. Neutral processes thus emerge as a unifying principle of ecology and evolution, which has deep implications in biodiversity assessment and conservation. DNA extraction kit (Genomic DNA biotechnology/Korea) was chosen as the DNA extraction method due to the fact that the manual protocols are so slow in speed. The other reason is that the traditional methods such as phenol-chloroform would cause contamination to DNA obtained. On the other hand, the automated method is expensive and requires special instruments. In this study, the isolation of the desired region of cox1 gene from the *C. herbacea* mitochondrion DNA was performed The Mint leaf Beetle is called the *Chrysolina herbacea alnadawi* (Duftschmid,1825) (Coleoptera :Chrysomelidae) recorded as a new insect pest on Mint plant *Mentha puleguim* (L,1753)(Lamiaceae).Those insects were observed initially during August 2017 in residential gardens in Baghdad city, Al- Bonooq district. The results shown in Figures 2 ,3 and 4 along with Tables 6 and 7 indicated that a yield of a single band of the desired product with a molecular weight of 665 bp for *C. herbacea* cox1 gene of *C. herbacea* adult was obtained. The results of sequencing are similar to those of *C. herbacea* voucher BMNH:853152 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial Sequence ID : KF656249 .1Length :655Number of Matches: 1 the range 1: 432 to 607GenBankGraphicsNext MatchPrevious Match, . Alignment statistics for match #(28), These results agree with those of a previous study (29) on mitochondrial DNA heteroplasmy in *C.herbacea* adult. The sequencing of *C. herbacea* cox1 gene amplified product (Fig. 5). Table 8 clarify that the results from 10 samples of *C. herbacea* adult expressed 73% compatibility with that of the standard specimen voucher (BMNH:853152) cox1 gene of *C. herbacea* that was recorded in Spain (Ancares, Lugo ANC results; Sequence ID: gb ID: KF656249.1). The widely sampled species, the score was 102, the range of nucleotide was 432 to 607 and the expect was 6e-25. Multiple hierarchical levels representing haplotype genealogies of various ages showed a similar rate of distance decay of assemblage similarity. In addition, we found strong log-log correlations between hierarchical level (lineage age) and number of lineages, lineage range size and assemblage similarity. Similarity at the species level was strongly correlated to similarity at the haplotype level for the whole assemblage.

Table 6. The optimum condition of detection gene

No.	Phase	Tm (^{o}C)	Time	No. of cycle
1-	Initial Denaturation	95^{o}C	3 min.	40 cycle
2-	Denaturation -2	95^{o}C	45sec	
3-	Annealing	62^{o}C	45sec	
4-	Extension-1	72^{o}C	50sec	
5-	Extension -2	72^{o}C	10 min.	
Gradient Annealing		52	54 56 58 60 62	

Table 7. Sequencing ID in *Chrysolina herbacea alnadawi* gene, score, expects and compatibility of DNA sequences obtained.

No. Of sample	Type of substitution	Location	Nucleotide	Range of nucleotide	Sequence ID	Score	Expect	Identities	SOURCE
1	Transition	436	T>C	432 to 607	ID: KF656249.1	102	6e-25	73%	Chrysolina herbacea
	Trinsvertion	438	A>C						
	Trinsvertion	443	A>T						
	Trinsvertion	451	A>T						
	Trinsvertion	455	A>T						
	Trinsvertion	458	A>T						
	Trinsvertion	459	A>T						
	Transition	466	C>T						
	Transition	471	T>C						
	Trinsvertion	473	G>T						
	Trinsvertion	474	A>T						
	Trinsvertion	476	>T						
	Trinsvertion	478	A>T						
	Trinsvertion	479	A>T						
	Trinsvertion	480	A>C						
	Trinsvertion	481	G>C						
	Trinsvertion	482	C>T						
	Trinsvertion	485	G>T						
	Trinsvertion	486	A>C						
	Trinsvertion	491	A>T						
	Trinsvertion	507	G>C						
	Trinsvertion	508	G>C						
	Transition	509	G>A						
	Trinsvertion	512	G>C						
	Transition	515	C>T						
	Trinsvertion	524	G>T						
	Trinsvertion	535	A>C						
	Trinsvertion	541	A>T						
	Trinsvertion	544	A>C						
	Transition	545	C>T						
	Trinsvertion	549	C>A						
	Transition	550	T>C						
	Trinsvertion	551	G>C						
	Trinsvertion	553	A>T						
	Transition	554	C>T						
	Trinsvertion	557	G>C						
	Transition	559	T>C						
	Trinsvertion	560	G>T						
	Trinsvertion	561	G>C						
	Transition	563	G>A						
	Transition	575	T>C						
	Transition	578	C>T						
	Trinsvertion	580	A>C						
	Transition	586	C>T						
	Trinsvertion	588	G>T						
	Transition	592	T>C						
	Trinsvertion	595	A>T						
	Trinsvertion	597	A>T						

Table 8. Sequencing ID in gene bank, score, expects and compatibility of DNA sequences obtained.

ACCESSION	specimen_voucher	country	Source	Gene	Identities	expect	score	Range
ID: KX943345.1		Spain	*Chrysolina herbacea*	cox1	73%	6e-25	102	2089 to 2264
ID: KF656249.1	BMNH:853152	Spain: Ancares, Lugo (ANC)	*Chrysolina herbacea*	cox1	73%	6e-25	102	432 to 607
ID: KF656217.1	BMNH:853117	Spain: Ancares, Lugo (ANC)	*Chrysolina herbacea*	cox1	73%	6e-25	102	432 to 607
ID: KF655908.1	BMNH:852684	Spain	*Chrysolina herbacea*	cox1	73%	6e-25	102	432 to 607
ID: KF655881.1	BMNH:852655	Spain	*Chrysolina herbacea*	cox1	73%	6e-25	102	432 to 607
ID: KM448445.1	BC ZSM COL 03018	Germany	*Chrysolina herbacea*	cox1	73%	6e-25	102	435 to 610
ID: KM444274.1	BC ZSM COL 03023	Germany	*Chrysolina herbacea*	cox1	73%	6e-25	102	435 to 610
ID: KM442717.1	GBOL_Col_FK_7270	Slovenia	*Chrysolina herbacea*	cox1	73%	6e-25	102	435 to 610
ID: KF656193.1	BMNH:853092	Spain: Ancares, Lugo (ANC)	*Chrysolina herbacea*	cox1	72%	3e-23	96.9	432 to 607

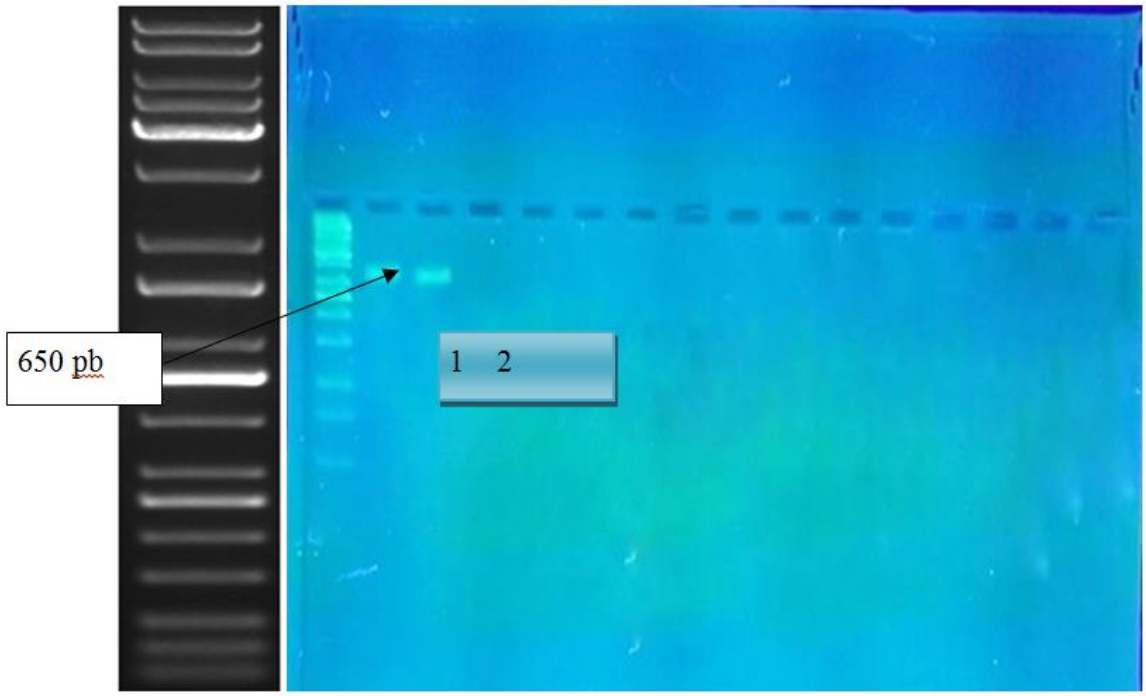

Figure 2. PCR product the band size 650 bp. The product was electrophoresis on 2% Agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

Score	Expect	Identities	Gaps	Strand
102 bits(112)	6e-25	128/176(73%)	0/176(0%)	Plus/Plus

```
Query  1    CAATCACttttttactactatttttttATACATCCAACATTATTTTCCTTATCTCAAT  60
Sbjct  432  CAATTAATTTTATTACTACAATTATTAATATACACCCAATAGAAATAAAGCTAGATCAAA  491
Query  61   TACCTctttttcatccacactcttaattacttcaattcttttccttttttccttacacc  120
Sbjct  492  TACCTCTTTTTTCATGGGCAGTCCTAATTACTGCAATTCTTTTACTTTTATCACTACCTG  551
Query  121  tttaCCCTCTACAATTACAATACTATTCACTGATCTAAACTTTATTACCTCTTTT  176
Sbjct  552  TACTAGCTGGTGCAATTACAATATTACTAACTGACCGAAATTTAAATACCTCTTTT  607
```

Figure 3:*C. herbacea* voucher BMNH:853152 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial Sequence ID: KF656249.1Length: 655Number of Matches: 1 the range 1: 432 to 607GenBankGraphicsNext Match Previous Match, Alinment statistics for match #1

Alignment statistics for match #1				
Score	Expect	Identities	Gaps	Strand
102 bits(112)	6e-25	128/176(73%)	0/176(0%)	Plus/Plus

```
Query  1    CAATCACttttttactactatttttttATACATCCAACATTATTTTCCTTATCTCAAT  60
            ||||  | ||||  |||||||  |||  ||  ||||||  ||||  |  | |     ||  ||||
Sbjct  432  CAATTAATTTTATTACTACAATTATTAATATACACCCAATAGAAATAAAGCTAGATCAAA  491
Query  61   TACCTctttttcatccacactcttaattacttcaattcttttccttttttccttacacc  120
            ||||||||||||||   || ||  ||||||||  |||||||||  |||||  ||  |||
Sbjct  492  TACCTCTTTTTTCATGGGCAGTCCTAATTACTGCAATTCTTTTACTTTTATCACTACCTG  551
Query  121  ttttaCCCTCTACAATTACAATACTATTCACTGATCTAAACTTTATTACCTCTTTT  176
            |  ||  |  ||||||||||  ||  |  |||||  |  |||  ||  |  ||||||||||
Sbjct  552  TACTAGCTGGTGCAATTACAATATTACTAACTGACCGAAATTTAAATACCTCTTTT  607
```

Figure 4. *C. herbacea* voucher BMNH:853152 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial Sequence ID: KF656249.1Length: 655Number of Matches: 1 Range 1: 432 to 607GenBankGraphicsNext MatchPrevious Match

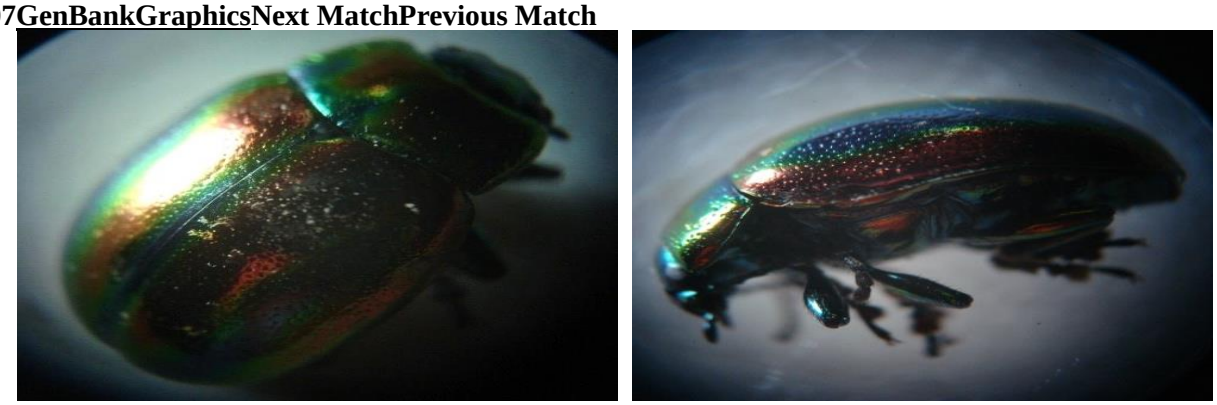

Figure 5. Female adult of *Chrysolina herbacea alnadawi* (Duftschmid, 1825) (125X)

A previous publication reported 139 species of *Chrysomelidae* from New Brunswick, Canada in a catalog of the leaf beetles of North America (30). The presence of *C.staphylaea* (Linnaeus) in Nova Scotia and Quebec, but did not report it from New Brunswick, although it would have likely be found in the province with additional sampling, increasing the number of species of Chrysomelidae known from New Brunswick to 143. The taxonomic circumscription of the large and diverse leaf beetle genus *C. Motschulsky* is not clear, and its discrimination from the closely related genus *Oreina Chevrolat* has classically been controversial. In addition, the sub generic arrangement of the species is unstable, and proposals segregating *Chrysolina* species into new genera have been recently suggested. In this context, the availability of a phylogenetic framework would provide the basis for a stable taxonomic system, but the existing phylogenies are based on few taxa and have low resolution(31).

A phylogenetic analysis based on mitochondrial (*cox*1 and *rrnL*) and nuclear (H3) DNA sequences from a sample of fifty-two *Chrysolina* species representing almost half of the subgeneric diversity of the group (thirty out of sixty-five subgenera) and most of the morphological, ecological and karyological variation in the genus was performed (32). In addition, five *Oreina* species from two subgenera have were analyzed. The resulting phylogeny is used to evaluate some of the most relevant taxonomic hypotheses for *Chrysolina*,. These Studies support the paraphyly of *Chrysolina* as currently defined due to the inclusion of *Oreina*, the monophyly of the *C.*(plus *Oreina*) species including the divergent *C. (Polysticta) vigintimaculata* (Clark,1864), and enable inferences of deep-level evolutionary relationships among the studied subgenera. The plant family Lamiaceae is inferred as the ancestral host of the study group, whose evolution is characterized by continuous host-shifting among pre-existing host plant families. Some *C. clades* include mixtures of species with different levels of diet breadth, indicating that niche width has varied through time. The mitochondrial and nuclear genes used here provided an expanded and better-resolved tree topology for the genus *Chrysolina*, significantly improving previous phylogenetic hypotheses (33).

E Value and Score

The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value

of E. This indicates that the degree of similarity was high between sequences which gives a greater confidence. The value of a very close to zero means that these sequences are identical. The results showed that the insect was identified with the standard species of the insect *C. herbacea* from Slovenia, Spain, Germany, with a frequency of 73%. The evolutionary tree and relations between the isolates were then drawn using the MEGA6 program to draw the evolution tree and the relations between the isolates studied depending on the gene sequence (Fig.6). The PCR amplification technique is a widely used technique because of its ease and speed, as well as the large database available as a result of the expansion of the sequencing study, which allows comparison between stalking and identification of strains (34). This is due to the possibility of analyzing phylogenetic species of closely related and closely related species (35).

Finally this result of the diagnosis of the insect is attributed to the level of the order and family (Coleoptera: Chrysomelidae) and 73% with *Chrysolina herbacea* (Duftschmid, 1825), endemic and registered in Spain, Slovenia and Germany, confirming that it is recorded for the first time in Iraq and the Arab regions as well as the Middle East as appeared by DNA analysis

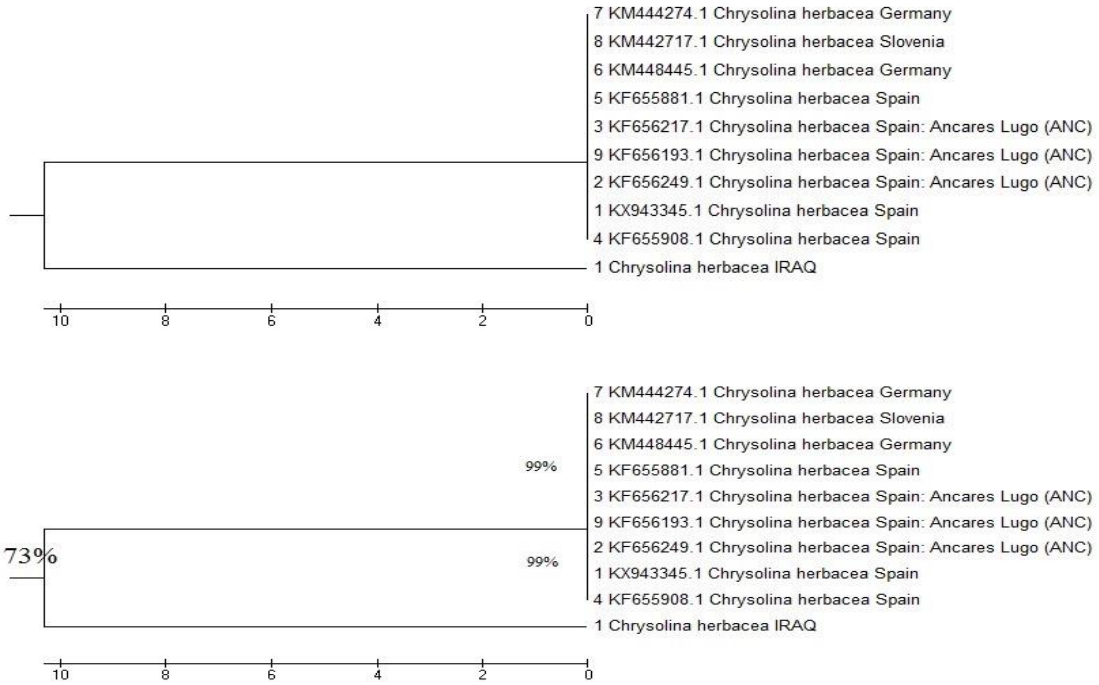

Figure 6. Evolutionary tree and relationships between bacterial isolates using the MEGA6 program.

Conflicts of Interest: None.

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تسجيل أول لخنفساء أوراق النعناع ,(*Chrysolina herbacea* (Duftschmid,1825 في العراق (Coleoptera:Chrysomelidae)

فيحاء عبود مهدي النداوي

قسم العلوم، كلية التربية الأساسية، الجامعة المستنصرية، بغداد، العراق.

الخلاصة:

شخصت الحشرة وسميت بوساطة المركز الوطني للمعلومات التقنية الحياتية على انها خنفساء اوراق النعناع *Chrysolina herbacea* (Coleoptera:Chrysomelidae) *alnadawi* (Duftschmid,1825), بوساطة تحليل الحامض النووي DNA واظهر النتائج تشابة تسلسل النيوكلوتايدات بنسبة 73٪ مع(*Chrysolina herbacea* (Duftschmid,1825، مؤكدة أنها سجلت لأول مرة في العراق والعالم العربي، فضلا عن بلدان الشرق الأوسط بوساطة تحليل الحمض النووي، وقد تم تسميتها خنفساء ورقة النعناع *Chrysolina herbacea* (*alnadawi* (Duftschmid,1825 .سجلت كافة جديدة على نبات النعناع (Lamiaceae)(*Mentha puleguim*(L,1753. وقد لوحظت هذه الحشرات في اول مرة خلال شهر آب / أغسطس 2017 في الحدائق في حي البنوك في بغداد.

الكلمات المفتاحية: التسجيل الأول، تسلسل نيوكلوتايد ، نبات النعناع ، *Chrysolina herbacea*، Chrysomelidae، ،.