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GENETIC VARIATION AND PHYLOGENETIC STUDY OF CORN GROUND BEETLE, *ZABRUS TENEBRIOIDES* IN ERBIL PROVINCE-IRAQ

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Abstract

In northern of Iraq, the corn ground beetle (*Zabrus tenebrioides* Goeze) is one of the most significant pests of wheat and the soil-dwelling larvae are the most destructive stage of this pest. The present study aimed to identify this pest using molecular diagnostic PCR and sequence analysis to investigate the genetic variation using a genotype DNA template of the species from twenty localities and two host plants. Further, a comparison study was carried out to examine the relationship between the obtained sequences and sequences recorded worldwide. The results confirmed the identity of the species via the designed species-specific primers. Twenty sequences of the mitochondrial COI gene of Iraqi samples (ON623787- ON623806) were deposited for the first time in the National Center of Biotechnology Information (NCBI). The phylogenetic analysis exhibited diversity in the acquired sequences in accordance with geographical locations as well as the type of diet with an average base substitution of the sequences with the almost equal transition to transversion ratio. The establishment of various clades indicates cluster populations depending on geographic isolation and host plant. The sequences comparison of Iraqi sample genes with worldwide genes exhibited that the Turkish sequence (MN 343842) clustered together with the twenty samples acquired in the study in one clade while the other sequences from other countries clustered together with the exception of Spain (AY551899) which formed one single taxon as an outgroup thus revealing distant relationship with all other sequences.

Keywords: Wheat crop, Transition, DNA extraction, COI gene, sequence alignment

Introduction

The global recession and economic slump will unavoidably have a devastating effect on food production and the global food supply network, particularly for poor nations that struggle to pay import expenses and must rely on their local production, particularly the main food crop. The crops must be adequate to meet both present and future demand, and the emphasis would move to economically significant cereal crops (such as wheat and maize) because of their critical contribution to food security (Welch, 2005, Shiferaw et al., 2011, Tadesse et al., 2018). Diseases and insect pest infestation are the principal causes of poor and unpredictable yields of wheat and maize during pre- and post-harvest practices. *Zabrus tenebrioides* is one of the most dangerous insect pests that feed on cereal crops in all regions of the world. In recent years, it has become a serious pest in the Kurdistan region, where farmers have suffered greatly from it. Both the larval

and adult stages of this pest are capable of causing severe economic damage and a great loss to wheat, barley, and corn crops (Ahmed et al., 2017). An attempt to evaluate the distribution of the genus *Zabrus* in different regions in relation to environmental conditions and identify the primary limiting factors would likely aid in the management of this species. (Avtaeva et al; 2018, Duman ,2019,). The soil-dwelling larvae are most destructive because they feed on the roots of young plants and may burrow as deeply as 40 centimetres. Leaves and stems of vegetation have been destroyed, leaving only parts of veins and the wheat crop will be pulled underground completely via the burrowing larvae (Popov, 2002, Walczak, 2007, Georgescu et al., 2017). There is inadequate research on the genetic diversity of this species despite its widespread distribution and major economic impact on cereal crops worldwide. The availability of molecular primers that can detect a change in the sequence of bases in species-specific DNA, such as mitochondrial genes, enables the successful genetic variation of certain species. Particularly, mitochondrial cytochrome CO1 is functionally conserved and lacks introns in animals, enabling the construction of primers that work with a wide range of species and the alignment of the generated DNA sequences for population genetic and phylogenetic studies (Hebert et al.,2003, Sánchez-Gea et al., 2004). Therefore, due to the lack of corn ground beetle sequencing not only in Iraq but also in the middle east this study has been undertaken in order to sequence the corn ground beetle, which may be a possible explanation for the increasing numbers of the insect pest and their continuous distribution in various areas over the past few years. In addition, building particular primers for species identification can facilitate future molecular research (i.e. DNA sequencing). The insect samples collected from the four directions of Erbil province on wheat and barley plants were sequenced to infer the variety of corn ground beetle populations in diverse geographical locations and their interaction with the host plant.

Materials and methods

Laboratory study/ Collecting and rearing insects

The laboratory experiment was conducted in both the biotechnology and high education laboratories of the Agricultural Engineering Science College at Salahaddin University. Larvae of *Zabrus tenebrioides* were regularly collected from different locations in Erbil province either manually pickings or via using pitfalls traps (diameter 21 cm, height 17 cm) which consider essential in ecological investigations of ground beetles (Gryuntal, 2008, Makarov and Matalin 2009). Samples were collected from 20 different locations that belong to 18 villages on both wheat and barley crops in Erbil province as shown in (Table 1) (Figure 1).

Table (1): Coordinators of cereal fields in various geographical locations of Erbil province where insect samples collected during the study

Sample number	Village name	X coordinate	Y coordinate	Direction	Host plant
1	Qalamurtka	44.0999	36.3528	North	Wheat
2	Shuzal	44.0148	36.3757	North	Wheat

3	Bapirtan	44.0593	36.3464	North	Wheat
4	Kawlokan	44.5361	36.6232	North	Wheat
5	Sreshma	44.4080	36.6447	North	Wheat
6	Balakyan	44.4006	36.6020	North	Wheat
7	Shre	44.2749	36.8093	North	Barley
8	Bestana gawra	44.0872	36.0481	East	Barley
9	Bestana gawra	44.2040	36.0431	East	Wheat
10	Sarmazra	44.0889	36.0532	East	Wheat
11	Kanibzra	44.0729	36.0334	East	Wheat
12	Qoritan	43.8906	36.0511	South	Wheat
13	Koska	43.9382	36.0082	South	Wheat
14	Sherawa	43.9083	36.0315	South	Wheat
15	Tandura	43.8366	36.0750	South	Barley
16	Batrtokh	43.9141	36.0713	South	Barley
17	Qalanchughan	43.8874	36.2741	West	Wheat
18	Sebiran	43.9127	36.2538	West	Wheat
19	Sebiran	43.896	36.2516	West	Barley
20	Gazna	43.9425	36.3024	West	Wheat

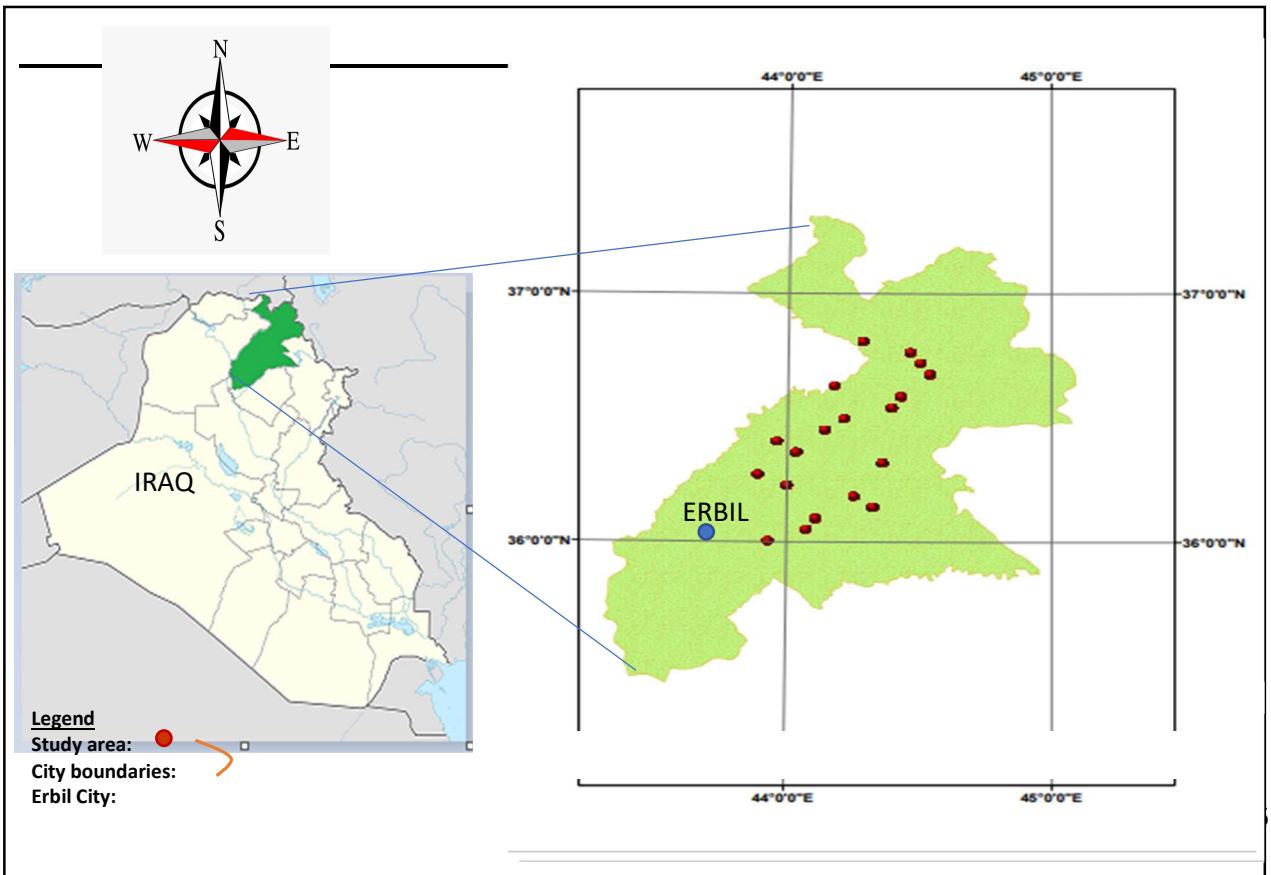

Figure (1): The map of Erbil province illustrating the twenty locations of the infected area by *Zabrus tenebrioides*

Experiment 1: Insect identification through molecular based method (PCR)

Design and preparation of primers

Species-specific primer pairs for the corn ground beetle were created using the Primer3 website (Rozen and Skaletsky 2000). (Table 2). To prepare a 1000x concentration from the new primers the Sigma molecular biology grade water was added to give a stock of 200pmol/ul. The following vertexing and spinning the tubes were kept on ice for 30 minutes to generate a 10x primer stock; 1 µl of the stock was combined with 9 µl SDW for the Forward and Reverse primers, which were placed in a -20 ºC freezer till used for DNA extraction.

DNA extraction

The samples were collected from Qalanchughan and Sherawa villages which was the first site the larvae were observed and consequently used in the experiment. Individuals were immersed into liquid nitrogen and then crushed into a fine powder to isolate the genomic DNA from whole larvae via Beta Bayern tissue DNA preparation Kit ZYMO Quick-DNA Tissue/Insect Microprep Kit manufactured USA- No. D6015 and stored in the freezer at -20ºC until it has been used. The quality and quantity of PCR product were confirmed using a nanodrop Spectrophotometer (NANODROP1000 U.K).

Polymerase chain reaction (PCR)

DNA was amplified using a PCR machine (Bioresearch PTC-200 Gradient thermocycler.) in 25µl reaction volume. The reaction consisted of 12.5 µl of PCR master mix (Ampilqon A/S Stenhuggervej 22, Denmark), 3µl of DNA template (50ng/ µl),7.5 µl of DNase RNase free water (Bioneer); 3 µl of DNA template and 1 µl of both forward and reverse primers (10 Pmol), the reaction in negative control composed of all components except DNA template. PCR reactions were run for 5 min at 94ºC (initial denaturation), followed by 35 cycles of 35 sec at 95ºC (denaturation), 45 sec primer temperature at 59ºC (annealing) and 72ºC for 1 min (extension). The last step was 72ºC for 8 minutes (Final Extension) (Table (2).

Table (2): Primers used to confirm the identification of corn ground beetle, *Zabrus tenebrioides*

Name	(Ps) bp	Length	Sequence F	length	Sequence R	References
Zt1	168	25	5′GATCTGTAGGAAT	20	5′-GATCTCCTC	This study

			AACATTTG ACCG-3′		CTCCTACTGG G-3′	
Zt2	312	20	5′-CAGGTTGA AC AGTGTACC CC-3′	20	5′-GATCTCCTC CTCCTACTGG G-3′	This study
Zt3	420	20	5’GCTCCCG ATA TAGCTTTTC C-3’	21	5’-AGGATCTCCTC C TCCTACTGG-3’	This study
Za	250	23	5′TCCAGGAG CTTT AATTGGTGA TG-3′	20	5′-TCAACCTGTAC C TGCACCTC-3′	This study

Agarose gel electrophoresis

The agarose gel consisted of a 2 % concentration of agarose (Molecular Grad, Bioline) in1x TBE (Tris- Borate- EDTA) buffer followed by the addition of 5µL of ethidium bromide stock before 10 µl of each sample was loaded onto a submerged gel. In the first lane appropriate size marker in (5µl of 3k bp) DNA ladder; New England Biolabs, Ipswich, MA, USA) were loaded and run at 100 Volts for approximately 1 hour. After electrophoresis, results were visualized and photographed under UV Transilluminator Biostep-UST-20M-8K

Experiment 2: Insect sequencing

DNA extraction

A total sample size of 80 larvae on wheat and barley crop was used in the experiment; each pooled samples consist of 4 insects that were collected from a specific village and represent a specific geographical location of the studied insect as aforementioned in the earlier section. Pooled samples of four individuals were immersed into liquid nitrogen and then crushed into a fine powder to isolate the genomic DNA from whole larvae via ZYMO Quick-DNA Tissue/Insect Microprep Kit manufactured USA- No. D6015 with spin column filter method. And the isolated DNA was electrophorized in 1% Agarose gel

CO1 partial genes Sequenced gene

Zabrus tenebrioides specimens were analysed for their mitochondrial CO1 sequence polymorphisms Using only forward prime(5’GCTCCCGATATAGCTTTTCC-3’) and were performed by ABI 3130X

genetic analyser (Applied Biosystem). The PCR products of the 20 samples obtained by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Macrogen Molecular Company of Korea were used as a source of DNA template for sequence specific PCR amplification.

Sequence alignment and phylogenetic analysis

The Chromatograms of the partial gene from the twenty samples of PCR product of CO1 partial genes have been edited and base calls checked using Finch TV program software. The homology search of CO1 partial gene sequences was performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/) and sequence alignment was carried out to compare and align the query sequences in this study with other biological sequences available from other countries via using BioEdit version 7.0.5.3 (Hall, 1999). The phylogenetic analyses were carried out in MEGA v.11.0.13 (Tamura et al., 2013) All the sequences generated in this study were deposited in NCBI-GenBank.

Statistical analysis

The molecular analysis, and sequence alignment were carried out using BioEdit version 7.0.5.3 (Hall, 1999) and the phylogenetic analyses were performed in MEGA v.11.0.13 (Tamura et al., 2013).

Results

Experiment 1: Insect identification through the molecular based method

Polymerase Chain Reaction and Visualization of the DNA fragments

Primers have been designed using the bio-information site Primer3Plus and blasted at NCBI National Centre for Biotechnology Information with a query cover %100 to ensure the species-specific primer. All the designed primers exhibited clear bands on the gel except the primer that designed for *Zabrus aurichalceus* and the negative control as shown in figure (2)

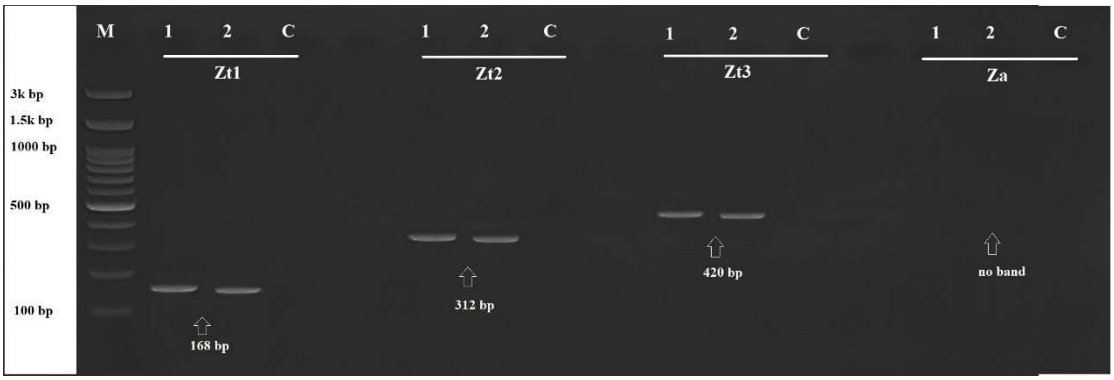

Figure (2): gel plate of PCR product for *Zabrus* sp through using species specific primers (primer labels correspond to those in table 2)

Experiment 2: Insect Sequencing

PCR amplification of CO1 partial gene

The primers of the partial gene were designed for using the sequences of the CO1 partial gene available in *Zabrus tenebrioides*. The primers of CO1 could yield a band size ~420 bp figure (3) after the PCR product was electrophoresed and visualized by 1.5% Agarose gel.

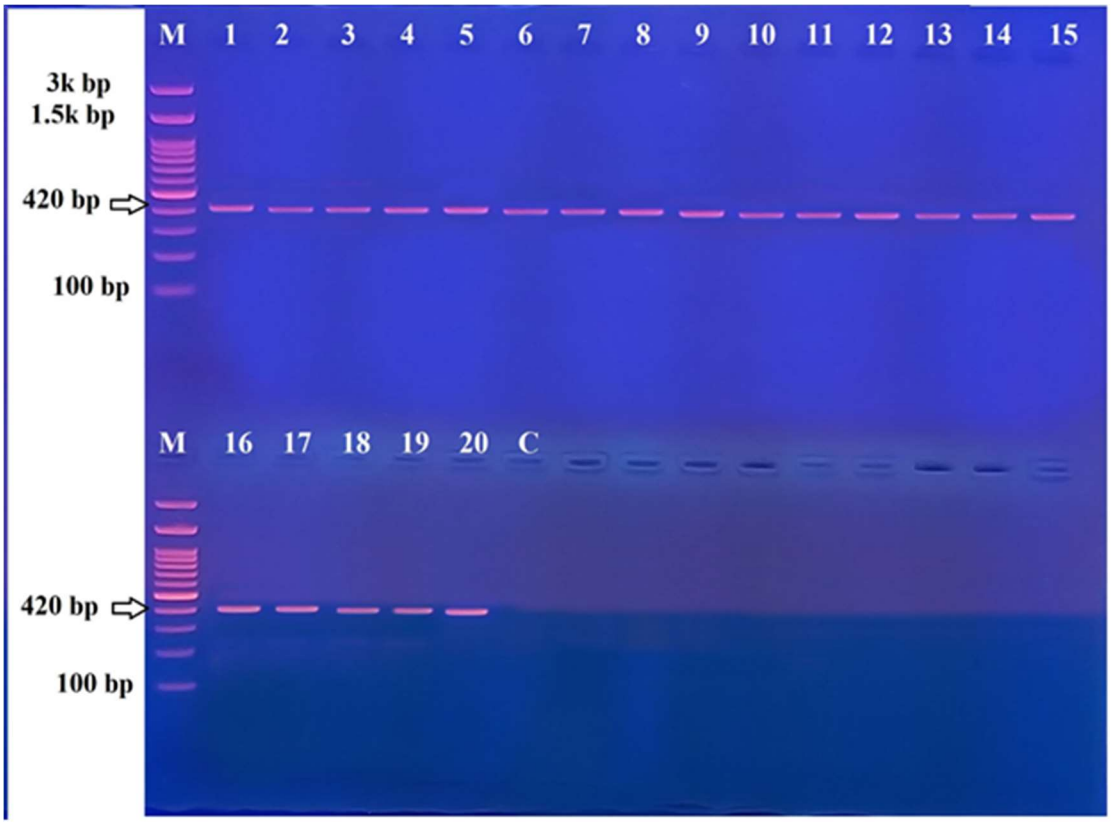

Figure (3): PCR amplification of partial cytochrome oxidase I gene from insects, first lane is the ladder (3k bp-100bp) and sample one to twenty are PCR products with the size of 420 bp and the last lane (C) is negative control without any band.

The nucleotide frequencies of the examined sequences were 29.99 % (A), 38.67 % (T), 16,68 % (C) and 14.63 % (G). The base composition of the CO1 gene fragment constituted 68.67 and 31.13 % A/T and G/C respectively. The average base substitution of the sequences in this study has an overall transition (TS= 10.2) and transversion (TV= 9.7). In addition, no frameshift mutation was observed whether the additions or deletions of one or more nucleotides in DNA sequences. Genetic relatedness within and between the sequences of the species in various geographical locations. The partial gene of CO1 sequences of the twenty samples was alimented by the BLAST program from Gen bank (*http://blast.ncbi.nlm.nih.gov/*) to compare amplified sequences in this study with other GenBank stored species of sequences. The results obtained from the BLAST indicated that the highest query sequence was 98% identical to *Zabrus tenebrioides* Table (3).

Table (3): Percentage distribution of samples of insect species into *Zabrus tenebrioides* according to a blast of GenBank NCBI of partial CO1 gene.

Sample number	Specimen Accession Numbers in the present study	Query Cover %	Identic Number %	GenBank Accession Numbers in other studied areas
1	ON623787	98	99.48	KU910305/ Germany
2	ON623789	98	99.48	KM442411/Germany
3	ON623790	98	99.48	MH300771/Czech Republic
4	ON623795	80	92.48	MN343842/Turkey
5	ON623796	3	80	AY551899/Spain
6	ON623797			
7	ON623791			
8	ON623794			
9	ON623799			
10	ON623798			
11	ON623800			
12	ON623803			
13	ON623802			
14	ON623801			
15	ON623793			
16	ON623788			
17	ON623805			
18	ON623804			
19	ON623792			
20	ON623806			

Phylogenetic inferences

The phylogenetic analysis based on CO1 nucleotide characters was equally weighted to investigate the sequence divergence and similarity. Furthermore, Nucleotide were equally weighted and analysed by maximum parsimony (MP) in paup 4.0b10 (Swofford 1993).

The constructed tree indicated the formation of various clades based on differences in CO1 sequence, and the formation of four clades from samples obtained in this study which show a tendency for grouping the population based on the geographic isolation as well as the host plant. Hence, more than half of the sequences of *Zabrus tenebrioides* locally grouped in to four clades based on village directions of Erbil province which are the (ON623797, ON623796 & ON623789) from the north side, (ON623798 & ON623799) from the east, (ON623802, ON623803) from the

south and (ON623804, ON623805, ON623806) from west direction and all on wheat crop.Further, all the sequences in the Kurdistan region of Iraq along with the Turkish sequence were clustered together and had one common ancestor. Likewise, the sequences from other studies particularly from Germany and the Czech Republic formed a cluster together (KU910305, MH300771, KM442411), while the sequence from Spain (AY551899) is consist of only a single taxon and displayed as an outgroup Figure (4).

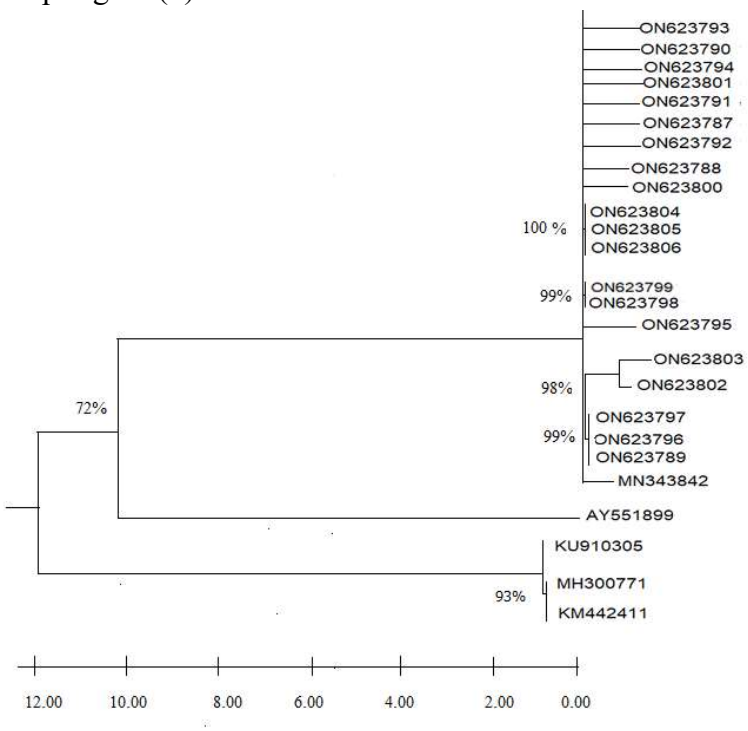

Figure (4): Employing the Maximum Likelihood method based on the Tamura-Nei model in Mega 11 program software and bootstrap analysis, shows phylogenetic positioning of *Z. tenebrioides* of the 20 samples from Iraq: Kurdistan region with similar GenBank sequences of CO1 partial gene that available in GenBank.

Discussion

The corn ground beetle has become increasingly more challenging to control this might refer partially to the lack of gene sequencing in the middle east of this economical species which the populations of have steeply increased recently in the past few years in Iraq. The molecular findings from this study reveal clear genetic variation between *Zabrus tenebrioides* species collected from different locations in Erbil. The cytochrome oxidase subunit I (CO1) gene was successfully sequenced from the species with a total product size of 420 bp and analyzed for the purpose of diversity detection. All four species-specific primers with complete query cover have been designed with a product size ranging between 168 and 420 bp for the purpose of either species identification as the results confirmed that the samples belonged to *Z. tenebrioides* species or for

DNA sequencing, particularly given the fact that no gene sequencing has been conducted on this economically important species not just in the Kurdistan region but also throughout the whole country of Iraq.

The development of a PCR primers capable of amplifying the CO1 barcode region from a diverse array of animals has ensured the widespread use of this region for species discrimination, including arthropods (Sánchez-Gea et al., 2004). As a result, our findings should aid future comparison investigations with other corn ground beetle species located in other geographical sites since they will be placed in GenBank and the Barcode of Life Database within mitochondrial CO1 sequences. Hence, DNA sequences at the mitochondrial CO1 gene of the twenty samples were screened from eighteen villages on both wheat and barley host plants. The sequence data were used to infer the diversity and relationships among the examined species in accordance with geographic location and the type of diet. The observed CO1 sequence variations revealed that geographic separation may have played an important role in the population structure of *Z. tenebrioides* and consequently led to the formation of four clades and revealing genetic variations between geographical locations. The collected samples in various location and direction might be attributed in this diversity since the Kurdistan region experience different climate and other geographic conditions.

In addition, the molecular analyses using the mitochondrial CO1 gene sequences revealed high variation in nucleotide bases in both sequences (ON623802, ON623803) from the south villages of Erbil in comparison with the other sequences from the Kurdistan region. The potential reason for that might refer to the excessive use of chemical pesticides in these villages as there were severely infected with the pest (personal observation during the survey for the pest and from farmers' statements in those villages). The population of species in different locations may have varied biological characteristics and genetic diversity as a result of being geographically isolated, influenced by different environmental factors, and having experienced different selection pressures (Diehl 1984., Lushai2002., Khidr2004).

Furthermore, the analysis revealed that the Turkish sequence (MN 343842) clustered together with the twenty samples acquired in the study in one clade and the potential explanation for this might refer to either the presence of similar environmental factors (e.g. temperature and humidity) since it is geographically close to Iraq and might have experienced similar condition or it might be a population shift with possibilities of migration to the region via imported cereals as it has been discovered and appeared earlier in Turkey (Duman, 2021). The *Z. tenebrioides* sequence (AY551899) from Spain formed one single taxon as an outgroup thus revealing distant relationships and genetic variations with other *Z. tenebrioides* sequences whether in the Iraq country or other countries which clustered similar sequences in a clade.

On the other hand, the diet or the host plant may also have some impact on species diversity since the clades formed within sequences in this study share the same host plant. The degree of population divergence in various species might be related to either the plant type or the diet feeding on (Ferrari et al., 2006, Wang et al., 2017).

The acquired CO1 sequences have a strong bias in the base composition of both A/T (68.67), which is consistent with what has been previously reported for mitochondrial sequences in various

orders of insect species (Liu and Beckenbach, 1992, Wang, 2011. The tendency toward this bias might be due to either the fact that G/C bonds are stronger than A/T bonds or might be by the chance (Zucker et al., 1991). Thus, in both cases irrespective of whether the presence of A+T richness in insect mtDNA was the consequence of chance or and natural selection, the genomes of these insects are now evolved to the presence of high A+T content which might be accompanied by cell metabolism, and selection acts to maintain this adaptation (Jermiin & Crozier, 1994).In concern of the substitution matrix, information on transition and transversion can be used as an indicator of the degree of multiple substitutions and the probabilities based on the data provided have indicated an almost similar ratio of. Sufficient segments of DNA need to be sequenced in order to gain an accurate assessment of the TS:TV ratio and to estimate the genetic distance between taxa (Martin et al, 1990).

Conclusion and recommendation

In the light of the finding, it is possible to draw the conclusion that natural genetic variation in the corn ground beetle, *Zabrus tenebrioides* within different geographical locations locally in the same country is present. Besides, the 420bp primer designed showed clear genetic variation with other sequences located in different countries except for the neighboring country (Turkey) and designing this primer alongside three others in this study might assist in future applied agriculture research for management strategies.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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