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MOLECULAR STUDY OF THE RED-LEGGED HAM BEETLE NECROBIA RUFIPES (DE GEER, 1775) (COLEOPTERA: CLERIDAE) FROM THE ERBIL PROVINCE OF THE KURDISTAN REGION, IRAQ

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Abstract

Necrobia rufipes (Coleoptera: Cleridae) are economically essential since most species are predaceous on other insects in both adult and larval stages. Molecular investigation is useful for identifying species and other downstream analysis. In addition to identification, this research addresses past gaps in knowledge through establishing the sequence of current insect COX genes and other investigations. A novel method was developed in this study to examine COX gene sequencing and to determine the relationship between Cleridae subfamilies. The DNA was isolated, and a band of 550 bp of the mt COX gene was amplified. Then the amplicons were sequenced. Parts of the COX gene were aligned with Clustal Omega and visualized and edited via Jalview software. This research presented the Ts:Tv mutation ratio through a sequencing technique for the species with reliable results. The species from the Kurdistan region in this study were clustered in a monophyletic clade published in Germany (KU916741.1) with a high identity value (100%). The COI sequence of the species

was submitted to GenBank with the PP175929.1 accession number. The base composition of the COX gene sequence had a higher AT figure at 67.89% compared to the GC's 32.1%. These results offer new valuable insights on the *Necrobia rufipes* and may provide useful data for its identification, bioinformatics uses, and management applications. The results show that the mt-COX can be used as a marker for further molecular experiments.

Keywords: Alignment, Genetic variation, Bioinformatics, Phylogenetic.

دراسة جزيئية لخنفساء لحم الخنزير ذات الأرجل الحمراء Necrobia Rufipes De Geer 1775 (Coleoptera: Cleridae) من محافظة أربيل إقليم كردستان – العراق

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الخلاصة

تعتبر حشرة *Necrobia rufipes* (Cleridae :Coleoptera) هي حشرة مهمة اقتصاديا، حيث أن معظم الأنواع تكون مفترسة للحشرات الأخرى في مرحلتي البالغة واليرقة. يعد التشخيص الجزيئي طريقة ذا قيمة لتحديد الأنواع تم في الدراسة الحالية تشخيص الحشرة اضافة الى سد الفجوات التي توفر معلومات قيمة حول طبيعتها الجزيئية من خلال استخدام تسلسل جين COX. لذلك، تم تطوير طريقة فريدة في هذه الدراسة لفحص تسلسل جين COX واستنتاج العلاقات بين عائلات Cleridae الفرعية. تم عزل الحمض النووي، وتم تضخيم شريط من 550 bp من جين mt COX. جرى عمل تحديد التتابعات لناتج تفاعل البلمرة (الأمبليكونات). تم عمل اصطفاف التسلسل الناتج من الدراسة الحالية مع انواع اخرى تم الحصول عليها من موقع ncbi عبر برنامج Jalview. وتم الحصول على نسبة طفرة Ts: Tv تجمعت أنواعنا في إقليم كردستان في مجموعة واحدة مع نوع الماني (KU916741.1) بنسبة تشابه عالية جدا بلغت 100٪. تم تسجيل النوع بموقع GenBank تحت رقم انضمام (PP175929.1). كان التركيب الأساسي لتسلسل جين COX حاوي على القواعد AT بنسبة بلغت 67.89٪

بينما بلغت 32.1٪ للقواعد GC. تقدم هذه النتائج معلومات قيمة جديدة حول *Necrobia rufipes* وقد تفيد البيانات الناتجة في امكانية مكافحة الحشرة. كانت النتائج إيجابية في رؤية أنه يمكن استخدام mt–COX كعلامة جزيئية لمزيد من التجارب الجزيئية.

كلمات مفتاحية: المحاذاة التسلسلي، التباين الوراثي، معلومات الحيوية، النشوء والتطور.

Introduction

Family Cleridae, commonly known as checkered beetles, is a cosmopolitan group (except for the Antarctic) with approximately 4000 species and 320 genera (5) with (3) listing 291 species in North America alone. The family Cleridae beetles are a diverse group of insects with wide geographic distributions (10). The morphology-based classification of Kolibáč (17) reduced the number of its subfamilies to four by employing the Transformation Series Analysis method, namely Tillinae, Hydnocerinae, Clerinae, and Korynetinae (20).

The Cleridae family are extremely important economically as they prey on other insect species at both adult and larval stages. Most species in this family exist on plants and tree trunk (11), but the Necrobia species are scavengers and can infest carcasses and the hides of vertebrate carrion (2). The Cleridae larvae are generally predacious and feed upon wood and bark-boring beetles. Several examples have been verified on flowers, vegetation, dead and dying trees infested with borers, as well as on the bones and skins of dead animals (8).

Molecular methods using PCR have been instrumental in the development of biological sciences as they allow for the development of satisfactory, quick, and reliable approaches and equipment. Accurate insect detection is the first step in pest management and it is necessary to identify target organisms by sequencing (13). Indeed, molecular techniques are highly useful when morphological and ecological evidence is ambiguous (26). In addition to providing accurate and reliable information for identification when sequencing the mitochondrial DNA, molecular procedures can also be useful tools for building evolutionary relationships. The mt COI, mt COII, and nuclear EF1 DNA sequences for key species have been attained through numerous molecular phylogenetic investigations (16 and 27). Except for the differences in higher classification, the phylogenetic relationships within the Cleridae are relatively different.

The sub-familial relationships are covered in each subfamily with the other three subfamilies (18), then in turning around among all 4 subfamilies on the molecular data (4 and 19). It has been argued that morphology-based analyses are weighted towards the characters used as taxonomic discriminators between lineages and do not provide an unbiased assessment of phylogenetic relationships (10). Although some molecular phylogenies have been attempted, they were all reconstructed on the basis of short nucleotide fragments of mitochondrial cox1, cytb, 12S, and 16S rRNAs as well as nuclear 18S and 28S rRNAs (4, 7 and 19), which may not contain enough phylogenetic information as well as provide no information on the genetic variations among them.

Databases or publications on *Necrobia rufipes* molecular studies in the Iraq - Kurdistan region in particular are limited. Besides, in the Erbil governorate, no

sequencing molecular phylogeny is available for this species. There is also scant information on the extent of the genetic relationship among the Cleridae subfamilies to establish any similarities and genetic variations among their offspring. Thus, it has been difficult to obtain molecular information on this species. However, DNA-based sequencing techniques can independently show how this species behaves with others. Some studies have highlighted the use of molecular phylogeny with some genes other than cytochrome oxidase I, for the purpose of species identification only. This study aims to provide comprehensive information on the molecular experiments and bioinformatics used on this species obtained from various locations in Erbil. The main objectives are to align and detect genetic variations, make an accurate phylogeny of the *Necrobia rufipes* species with the offsprings in other subfamilies, as well as develop restriction mapping using the mitochondrial COX subunit I gene.

Materials and Methods

Sample Collection, Preparation and DNA Isolation: Sixty specimens of the male red-legged ham beetle *Necrobia rufipes* were taken (based on the morphological identification keys) from carrion and carcasses in contact with soil during March-April, 2023 from various localities in the Erbil Province of the Kurdistan region in Iraq. Ten specimens each were placed in plastic bags and stored at -20°C in the refrigerator to be anaesthetized which is a less painful way than other killing methods. The samples were then sent to a private molecular laboratory in Erbil where the first process of DNA isolation was done under controlled and uncontaminated conditions. For the DNA isolation procedure, only parts of the body (head, thoraxes, and abdomen) of the male adult insects were used to avoid contaminating other external part.

Total genomic DNA was extracted from the insects in each separate plastic bag according to the instructions of the ZYMO Quick-DNA Tissue/Insect Micro-prep Kit (USA-D6015). The isolated DNA was kept at -20°C for downstream applications. The quality and quantity of the isolated DNA was inspected by Nanodrop 1000 (Thermo Scientific UK), and ranged from 1.7 to 2. All samples met purity isolated DNA standards in Nanodrop except for one which was excluded due to low purity of under 1.6 (reference, ZYMO kit instruction procedure).

PCR Primers: The primers of a specific gene in mitochondria, known as the mt-COI gene, was chosen for PCR amplification using the pairs of primers shown in Table 1, which exemplified the specific data on the Mt-primers (24). The COX primers were synthesized at the Micro-gene Center in South Korea.

Table 1: PCR primer COX gene used in the study.

Gene	Nucleotide Sequences	Product Size	TMº
***Cytochrome Oxidase c subunit I* (COX)**	Forward primer C1-J-1718 5'- GGAGGATTTGGAAATTGATTAGTTCC-3'	550bp	60º C
	Reverse primer C1-N-2172(HCO2198) 3'- TAAACTTCAGGGTGACCAAAAAATCA-5'		

PCR Amplification: The polymerase chain reaction (PCR) was done using a thermal cycler (Bioresearch, PTC-200, Gradient thermocycler) while the final amplification volume for the reaction mixture was done in 50µl (Table 2). The PCR was adjusted according to the selected primer by applying a program following (1) as in Table 3. The 550 bp-sized amplicon were pictured in a 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) stained with 5 µl EtBr. Then the PCR products were stored at -20°C for future use.

Table 2: PCR amplification components.

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	Template DNA	50ng/µl	4
5	DNase free Water	-	15
	Total		50

Table 3: PCR program COX gene.

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 Sanger sequencing of a partial gene of Mt-DNA-COX was done at the Micro-gene Center in Korea via a BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems) and the product was evaluated utilizing an ABI PRISM 310 (PE Applied Biosystems). The mt-COX gene sequence chromatograms produced in this work were collected, manually examined, and inspected via Finch TV v1.4 (accessible at https://digitalworldbiology.com/FinchTV). Then the attained sequence was submitted and confirmed on the NCBI nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in GenBank and received the accession number PP175929.1 for COX gene for comparison and alignment query sequencing with other downloaded biological sequences to determine further similarity with others.

Alignment and Phylogenetics: To achieve consensus for only one sequence, the DNA sequence was translated to the protein sequence and the frequency of amino acids for the transcribed sequence was calculated based on the MEGA11 software. Then the alignments for the protein sequences were done using the Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/). The phylogenic tree was created via the Maximum Likelihood method grounded on the Tamura-Nei model in MEGA11 software (23) with the available sequences of the COX gene of the Cleridae family belonging to the Korynetinae, Tillinae, Hydnocerinae, and Epiclininae sub-families taken from the NCBI nucleotide for phylogenetic study.

Restriction Mapping and Theoretical Digestion: The physical mapping and theoretical digestion procedures were used to detect the virtual position of restriction

sites on COX gene fragments to produce a restriction map and digested fragments. For that purpose, a popular program (NEB cutter V.3.0.17, New England Biolabs) tool was used to obtain the virtual maps and their digested fragments. This software can be downloaded at https://www.neb.com/en or directly from http://tools.neb.com/NEBcutter. We selected 1 Cutters restriction enzymes for creating restriction mapping for identifying a precise palindromic on our gene and makes specific cuts. Then the ratio of each nucleotide's frequency and base compositions of AT% and GC% were determined based on the differences in nucleotides among alignment sequences.

Results and Discussion

DNA Extraction and PCR Amplification: Based on the proposed methods 20-25µg of genomic DNA including mt-DNA was successfully isolated from the male insects (as we did not work on the sex chromosome the insects' gender did not influence the study results) with a purity of 1.7 - 2 (one sample was excluded because its purity was less than 1.6). Then two pulled samples were prepared for the downstream application. After that, amplification was carried out by traditional PCR aided by a pair of primers for the mt-COX gene. The amplified amplicon size was 550bps and the bands were separated by 1.5% agarose gel electrophoresis (45 seconds, 75V 1X TBE buffer) stained with 5µl ethidium bromide, as shown in Figure 1. The size of the amplicons is correct and accurate based on (1)

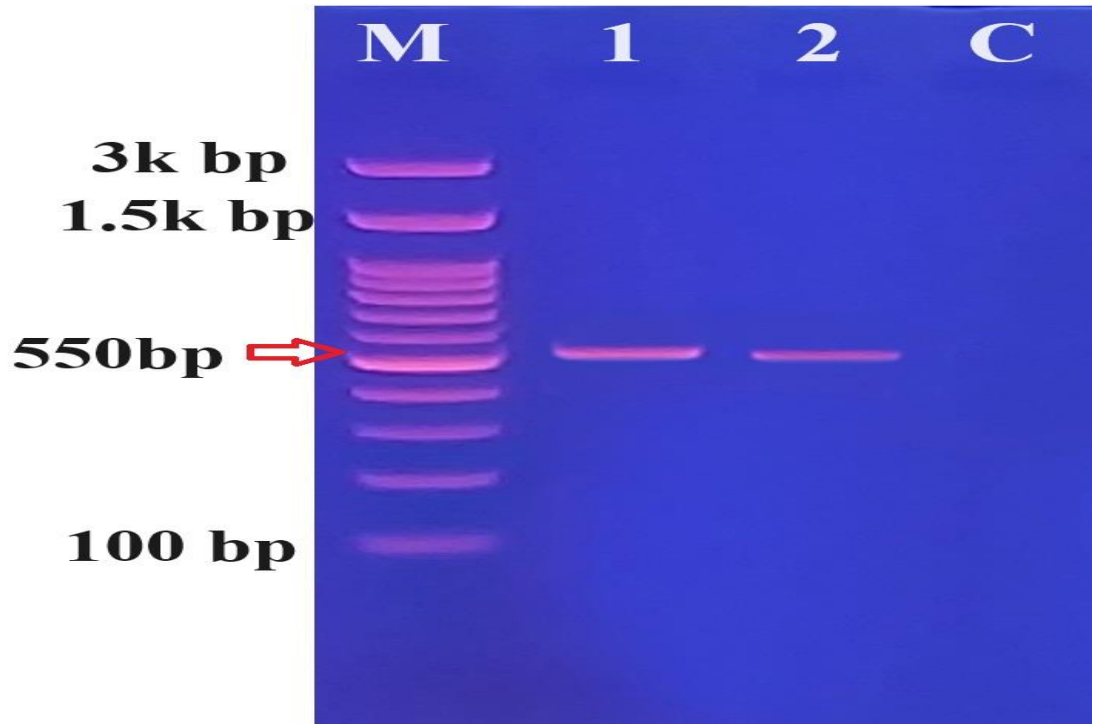

Figure 1: PCR amplification of partial *cytochrome C Oxidase I* gene from the insects.

M = Marker type mid-range DNA ladder (100-3000) bp; Lanes 1 and 2 = two replicates with 550 bp of PCR products from *Necrobia rufipes* De Geer; and C = negative control.

Sequence Analysis: The PCR product was sent for partial Sanger sequencing of the Mt-DNA COX gene using ABI 3130X genetic analyzer (Applied Biosystem) and the

sequence nucleotides were then submitted and confirmed on NCBI nucleotide database with the accession number PP175929.1. The nucleotide frequencies mt-DNA COX gene sequence of *Necrobia rufipes* were A 129 nt (30.39%), T 153nt (37.5%), C 67 nt (16,42%), and G 64 nt (15.68%), while the base compositions of the COX gene fragments produced 67.89% for A/T and 32.1% for G/C. They then underwent some bioinformatics analyzing.

Alignments and Variation Analysis: A query sequence was aligned based on the protein sequence translated by MEGA V.11 software with the five closest species (*Necrobia violacea* KU909322.1/Germany, *Necrobia rufipes* OQ842460.1/China, *Necrobia rufipes* KF956176.1/South Africa, *Necrobia rufipes* voucher KU916741.1/Germany, and *Necrobia rufipes* voucher OQ557179.1/Lebanon) in the same subfamily to reveal the similarity and genetic variations among them (see Figure 2). Alignment was done by MSA Clustal Omega (accessible at https://www.ebi.ac.uk/Tools/msa/clustalo/) while Jalview software was used to visualize and edit sequence alignments. As seen in Figure 2, the query sequence has three missed amino acids in Nos. 4, 28 and 87. Compared to the other sequences, this study's sequence was mismatched with the first sequence from Germany in the amino acid Nos. X33L, X93A, X94L and Y99S. On the other hand, the query sequence was mismatched with the 2nd and 3rd sequences in No. S94L and in only amino acid No. X106I while the 3rd sequence from South Africa and the 6th, sequence from Lebanon had one mismatched alignment in No. X32P. The mutations are indicated as "missenses mutation" because only one amino acid changed without changing the others and had normal functionality of the gene product as a COX gene. However, they are fully matched (100%) with the 5th sequence from Germany (KU916741.1) and the Percent Identity Matrix created by Clustal 2.1 (Table 4). The average base substitutions of sequences in this result have overall transitions and transversions shown in Table 5 and the frequency of each amino acid is shown in Figure 3.

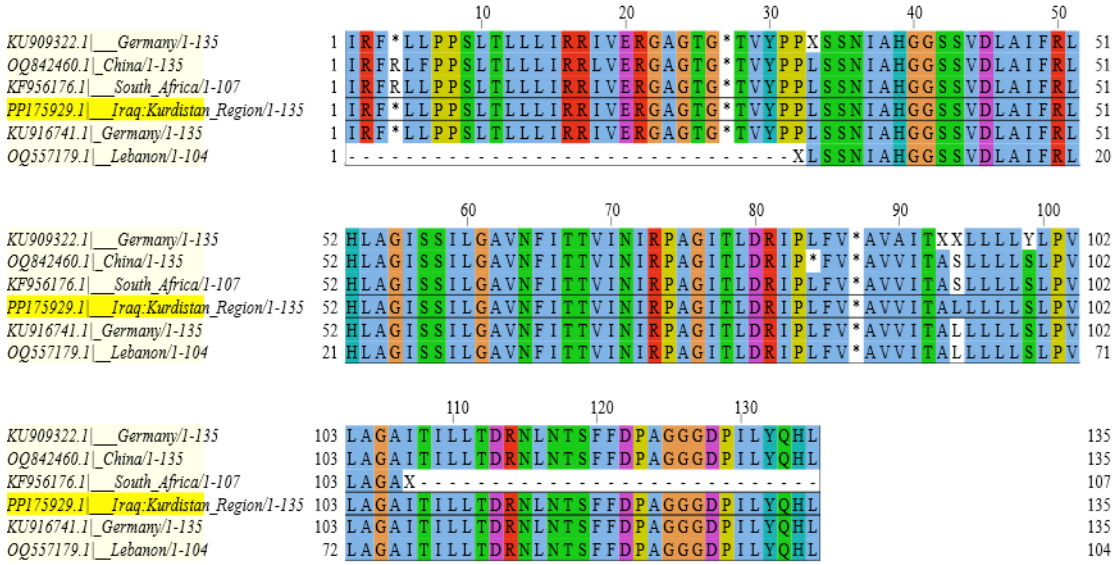

Figure 2: Clustal Omega multiple sequence alignment of the cytochrome oxidase subunit I (COX) protein sequence.

Same colors indicate perfectly matched sequences, * represents missing amino acids, different colors are a mismatch, and – represents gaps.

Table 4: Percent Identity Matrix created by Clustal2.1.

	KU909322.1 Germany	OQ842460.1 China	KF956176.1 South Africa	PP175929.1 Iraq	KU916741.1 Germany	OQ557179.1 Lebanon
KU909322.1 Germany	100.00	45.45	16.19	96.21	96.21	11.88
OQ842460.1 China	45.45	100.00	80.00	47.73	47.73	13.86
KF956176.1 South Africa	16.19	80.00	100.00	17.14	17.14	13.51
PP175929.1 Iraq_	96.21	47.73	17.14	100.00	100.00	10.89
KU916741.1 Germany	96.21	47.73	17.14	100.00	100.00	10.89
OQ557179.1 Lebanon	11.88	13.86	13.51	10.89	10.89	100.00

Accessed from: https://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-I20240619-095152-0019-68103995-p1m/pim.

Table 5: Average base substitution of the sequences (transition and transversion).

Query Sequence	Database Sequences	Transition Mutations %	Transversion Mutations %
PP175929.1 Iraq Kurdistan	KU909322.1 Germany	3.3	14.9
	OQ842460.1 China	2.6	4.6
	KF956176.1 South Africa	7.5	0
	KU916741.1 Germany	0	0
	OQ557179.1 Lebanon	3.7	0

The results from submissions to the NCBI GeneBank indicated that the highest query coverage was 100% with KU916741.1 (Germany) at 100% identity, while the lowest query coverage was 76% with OQ557179 (Lebanon). Since there were no previous submissions on *Necrobia rufipes* in Iraq and most of the Middle east countries, except Saudi Arabia's unverified sequences and one in Lebanon, the sequence submission in this study are the first on record in Iraq.

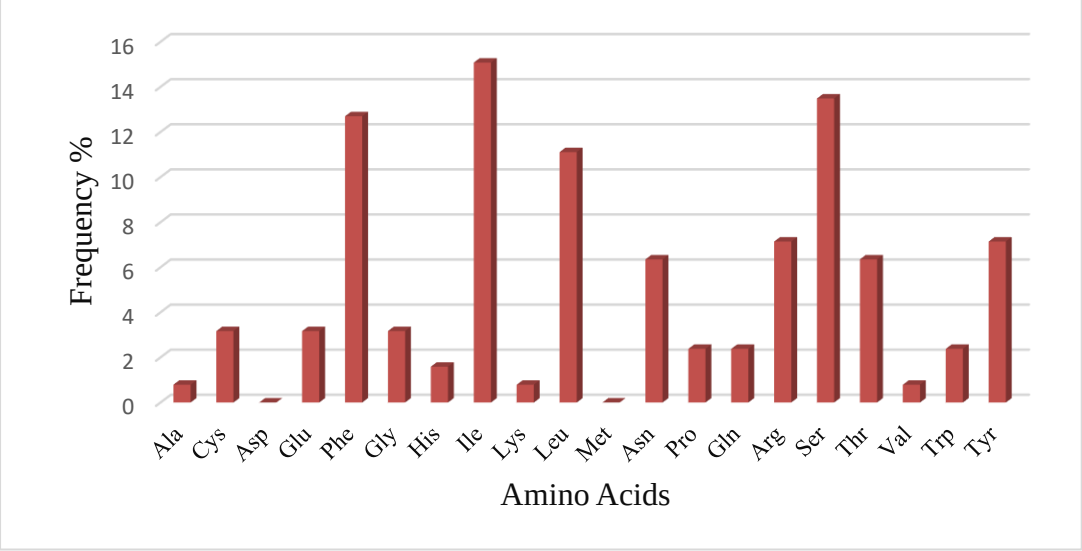

Figure 3: Amino acid frequencies of the insects.

Phylogenetic Analysis of the mt-COX gene: The bootstrap method for the test of phylogenic tree was developed with the No. bootstrap replication at 1000 via the maximum likelihood statistical method based on the Tamura-Nei model with a very strong branch swap filter in MEGA11 software (Figure 4). A segment of the mt-COX gene was utilized as an insect DNA barcode. It is known that mutation rates occur quickly and are adequate to differentiate even between closely related species (12).

To do phylogenetic analysis, species belonging to these subfamilies were used: Korynetinae (15 species, the query is included), Tillinae (5 species), Hydnocerinae (4 species), and Epiclininae (4 species) for a total of 28 species among the 4 subfamilies. The constructed tree in this study indicated the formation of some clades depending on variations in COX sequences. Maximum likelihood related tree established that the close relationships achieved from the BLASTn alignment showed that the clustered query sequence (Korynetinae subfamily) had the same recorded sequences that were analyzed within the same subfamily in other countries. From sequence divergence it was found that species fitting with corresponding genera were adjoining to one. Moreover, the query sequence in the Kurdistan region of Iraq (indicated as a red line) along with the German KU916741.1 sequence was clustered together, and both were clustered with the next clade (China OQ842460.1, Saudi Arabia MG574957.1, South Africa KF956176.1, and Lebanon OQ557179.1) with one shared ancestor. Similarly, sequences from the other researches are particularly similar and may have mixed with other subfamilies to form different clusters based on the similarities, genetic distances, and variations with each other in different countries.

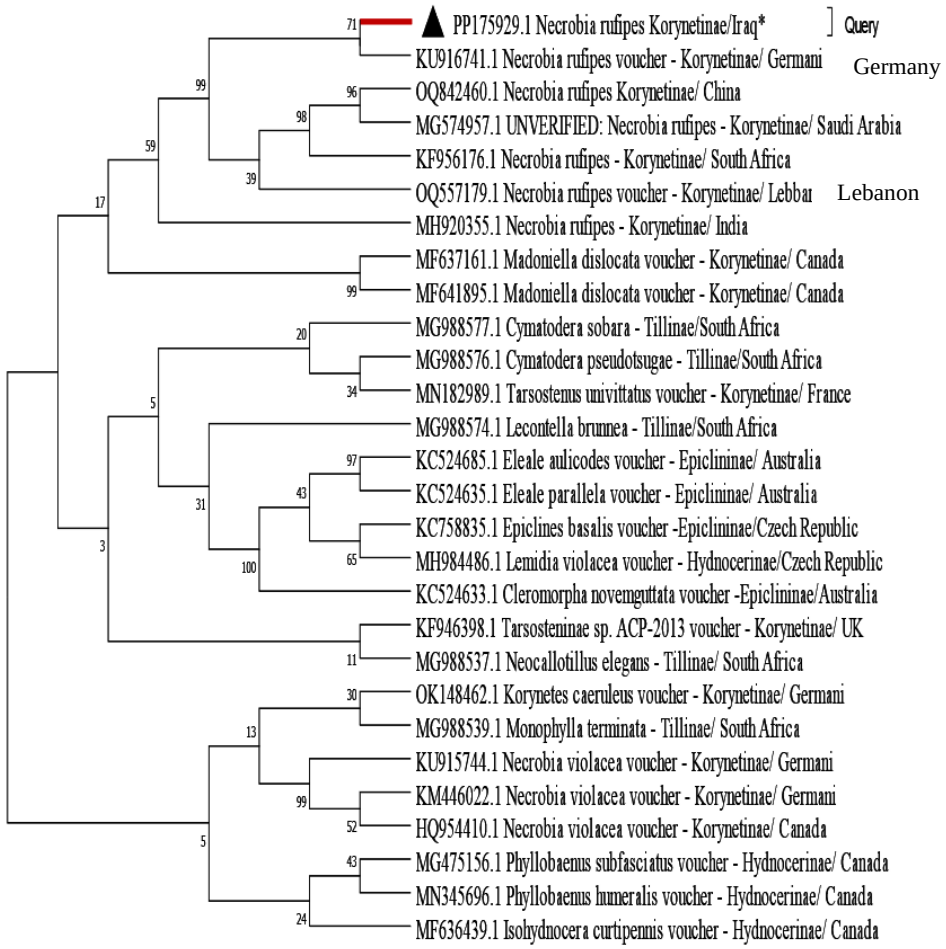

Figure 4: Phylogenic tree of *Necrobia rufipes* from Iraq's Kurdistan region (*).

Restriction Mapping and Theoretical Digestion: Restriction enzymes and their cleavage site locations have a central role in molecular studies for their ability to slice double-stranded DNA into fragments in particular sequences to yield either sticky or blunt ends. This is crucial for downstream analysis such as cloning, creating restriction mapping on the genes, and others. This study developed restriction mapping for circular and alternative linear mitochondrial COX genes based on the 1-cutter restriction enzymes, which produce multiple restriction sites on the DNA sequence. Colors and signs are illustrated in the section above the mapping as shown in Figures 5 and 6.

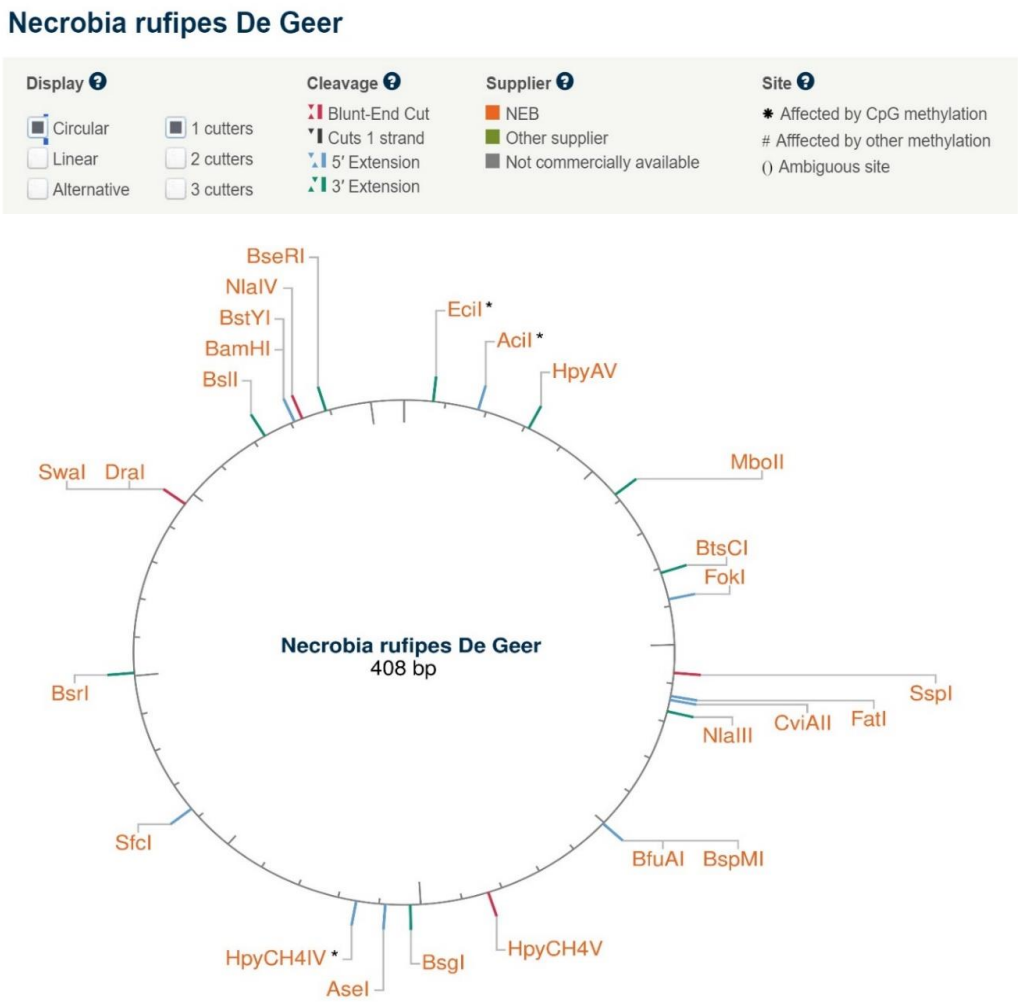

Figure 5: Circular restriction mapping for the *Necrobia rufipes* COX gene with indications.

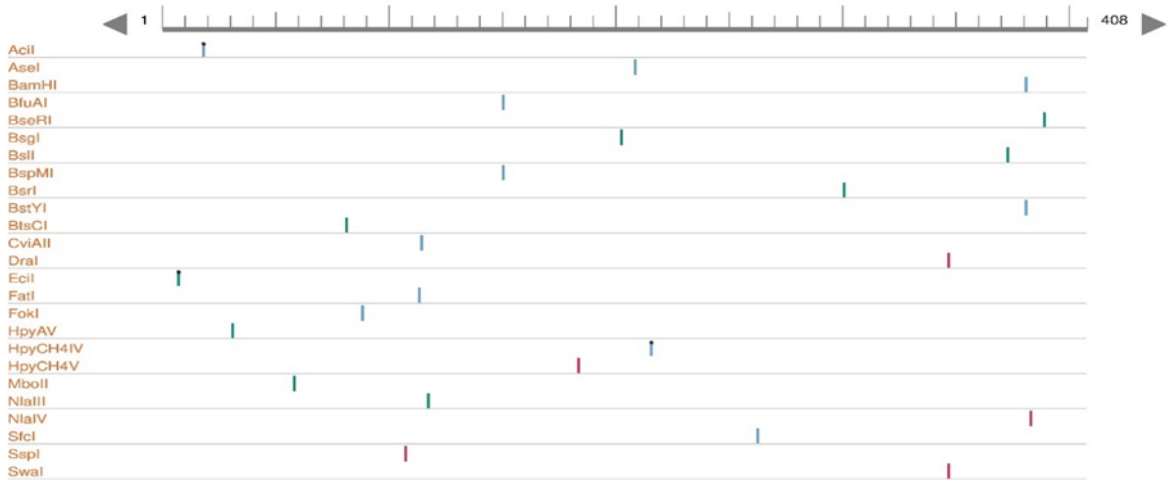

Figure 6: Alternative linear restriction mapping for the COX gene.

Colored lines indicate the cutting sites.

Then, some enzymes were selected to digest the COX gene sequence with BamHI, MboI, and ApoI endonuclease enzymes (Figure 7) which yielded some bands for each restriction enzymes. The size of the methylated and unmethylated bands are shown in

the fixed table brought from the NBE cutter V3 software (Table 6) and the AT of 67.89% and the GC of 32.1% were determined using the same software.

Table 6: Unmethylated and methylated lengths of the COX gene bands.

List: UnMethylated Lane

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	BamHI - MboI	382-381	408	Dam
2	MboI - MboI	382-121	148	Dam
3	MboI - MboI	238-336	99	CpG,Dam
4	MboI - ApoI	122-189	68	Dam
5	ApoI - MboI	190-237	48	CpG,Dam
6	ApoI - MboI	343-363	21	Dam
7	MboI - BamHI	364-381	18	Dam
8	MboI - ApoI	337-342	6	CpG,Dam

List: Methylated Lane

#	Ends	Coordinates	Length (bp)	Affected by Methylation
1	BamHI - ApoI	382-189	216	Dam
2	ApoI - ApoI	190-342	153	CpG,Dam
3	ApoI - BamHI	343-381	39	Dam

Figure 7: Virtual digestion of gel electrophoresis with BamHI, MboI, and ApoI restriction enzymes.

In this research, the first molecular analysis and application of multiple alignments, phylogenetic trees, and restriction mapping on the *Necrobia rufipes* samples from the Iraqi Kurdistan region were reported to make a comprehensive study based on the COX gene sequence, that is the residue within the mt-DNA. This study was conducted due

to disputes among researchers on this species and its genetic variations and relationships with those identified in different countries. Molecular experiments and bioinformatic databases using software (online and downloaded) were used in this study to address the issues and present accurate and reliable information.

This is the first time that the results provide partially forward sequencing for the mitochondrial COX gene of the *Necrobia rufipes* in Iraq and most countries in the Middle East, except for unverified sequences in Saudi Arabis and one in Lebanon. The COX gene was adopted for this study since it is widely used for various purposes and is usually considered a barcoding gene in insects. The obtained sequence was submitted to the GenBank and received its accession number for verification and be a database for upcoming studies on this species. Moreover, multiple sequence alignments were applied based on the downloaded reference sequences coming from the species which were reliably identified in other countries. These were utilized for search regions for similarities and in displaying the genetic variances between query and reference sequences.

In this investigation, the query sequence aligned 100% with the reference sequence KU916741.1 Germany and 92.21% with KU909322.1 Germany, while only 10.89% aligned with the OQ557179.1 Lebanon species and 17.11% for the KF956176.1 South Africa species. This lower alignment may be due to microdeletion occurring in the nucleotide numbers 1 to 31 and 107 to the end of the sequences, respectively, clearly leading to a reduction in similarity between two sequences. Then genetic variations (transition and transversion) occurred, as illustrated in Table 5. Many factors may have caused such variations and mutations but the most significant are environmental factors such as radiation, the application of insecticides to protect lands and herds, the use of various antibiotics for animals, and possibly climate change (14).

In the substitution matrix aspect, data on the transition and transversion mutation is generally utilized as a multiple substitution degrees indicator and the probability depending on the data run show a nearly similar ratio between them. As such, sufficient DNA have to be sequenced to attain a precise and accurate evaluation of the TS versus TV ratio and also the genetic variations and relatedness between taxa (22 and 25). The population of the same species in different locations may vary in their biological characteristic and genetic diversities due to geographical isolation, environmental issues, and selection pressures (9, 15 and 21). The developed COX sequences have a strong base composition bias of both AT (67.89%) and GC (32.1%) which is reliable and in line with prior studies on mitochondrial DNA sequences in several orders of insect species (28). The tendency near this bias may be due to the fact that GC bonds are stronger than AT or may be due to natural selection adaptations to natural disasters (29).

The first COX gene phylogeny for subfamilies of *Necrobia rufipes* are presented to evaluate relationships among the major grouping. Monophyly was recovered in KU916741.1 Germany while paraphyly with other groups descended from common ancestors with some in other ancestors from the initial common ancestor showing polyphyly with other species in different subfamilies. These findings are relatively consistent with Bocak (6). However, depending on our availability, relationships based on families or superfamilies within the clerid lineage could not be as fully or strongly

determined as those by (6), due to limited sampling size. Furthermore, the result of restriction mapping of the 1 cutters endonuclease revealed the different sizes of multiple fragments on the DNA segments. This study did not use 2 and 3 cutters restriction enzymes since only 3 to 4 endonucleases were incorporated into the 2 cutters whereas no restriction enzymes were seen in the 3-cutter enzymes, possibly due to the short segment of the mt-COI-DNA.

Conversely, the virtual digestion COX gene with BamHI, MboI, and ApoI showed different fragment sizes in both methylated (39,153 and 216 bp) and unmethylated (6-408 bps) DNA bands. This study collected an insufficient sample size since the tools for this in vivo assessment of the molecular characterization and bioinformatics uses of this species was done for the first time. Thus, the initial purpose was to collect basic indicators on the use of these techniques in more complex experimental designs than for existing standard morphological uses in the Iraq Kurdistan region. Among the obstacles faced by researchers in this study was the lack of public agricultural land and access to private properties for both animals and plants in Iraq and the Kurdistan region in particular. Also, no funding was received for this study and researchers had to contend with the lack of bioinformatics databases and development tools for this research.

Conclusions

This molecular investigation on detecting genetic mutations and phylogeny, and developing restriction mappings of the *Necrobia rufipes* COX gene was based on the reference collections of DNA sequences of individuals from reliably identified species and was done for the first time in Kurdistan/Iraq. Using the sequencing technique for the mt-COX gene was found to be highly effective for aligning query sequences with the closest related species in detecting regions of similarity and sequence variations such as transitions and transversions. Moreover, the phylogeny of the query species with other subfamilies of previously recorded sequences in different countries showed the suitability of this gene as the optimum variable in developing phylogenetic trees among four subfamilies in the Erbil Governorate.

In this study, the developed sequence-divergence resemblance data and phylogeny showed that the species related to matching genera were clustered together. Additionally, restriction mapping and virtual digestion are critical, reliable, and reasonable methods for detecting the palindromic sequence of each endonuclease in various molecular and biotechnology experiments. This study recommends that additional samples from other regions be investigated to develop a comprehensive phylogeny profile of family insect species.

Supplementary Materials:

No Supplementary Materials.

Author Contributions:

Kamaran M Taha: methodology, preparing the original draft, and analysis and interpretation of data; Nabeel A. Mawlood and Hero M Muhammad: conception and design, writing, review and editing; Pshtiwan A. Jalil: administrative matters. All authors have read and agreed to the published version of the manuscript.

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References

1. Abdulla Agha, W. N., Muhammad, H. M., and Taha, K. M. (2023). Phylogenetic analysis among some species of aphids (Homoptera: Aphididae) using DNA Sequencing Moleculartechnique. Tikrit Journal for Agricultural Sciences, 23(3): 71-78. https://doi.org/10.25130/tjas.23.3.8.
2. Abdul-Karim, K., E., & Z. Hussein, H. (2024). Algae (Normal and Nano): Characteristics, Economic Importance, And Role In Plant Resistance Of Disease: A Review. Anbar Journal of Agricultural Sciences, 22(1), 41–54. https://doi.org/10.32649/ajas.2024.183696.
3. Arnett Jr, R. H., Thomas, M. C., Skelley, P. E., and Frank, J. H. (2002). American beetles, volume II: Polyphaga: Scarabaeoidea through Curculionoidea. https://doi.org/10.1201/9781420041231.
4. Bartlett, J. S. (2021). A preliminary suprageneric classification for Clerinae (Coleoptera: Cleridae) based on molecular and morphological evidence, including a review of tegminal terminology. Annales Zoologici, 71(4): 737-766.
5. Bocak, L., Barton, C., Crampton-Platt, A., Chesters, D., Ahrens, D., and Vogler, A. P. (2014). Building the Coleoptera Tree-of-Life for >8000 species: Composition of public DNA data and fit with Linnaean classification. Systematic Entomology, 39(1): 97-110.https://doi.org/10.1111/syen.12037.
6. Bocakova, M., Constantin, R., and Bocak, L. (2012). Molecular phylogenetics of the melyrid lineage (Coleoptera: Cleroidea). Cladistics, 28(2): 117-129. https://doi.org/10.1111/j.1096-0031.2011.00368.x.

7. Bocakova, M., Bocak, L., Gimmel, M. L., Motyka, M., and Vogler, A. P. (2016). Aposematism and mimicry in soft-bodied beetles of the superfamily Cleroidea (Insecta). Zoologica Scripta, 45(1): 9-21. https://doi.org/10.1111/zsc.12132.
8. Farhan, K., O., W. Ibade, Kh., & A. Kream, T. (2024). Efficiency Alcoholic Extract Of Nightshade Wild And Potassium Silicate In Normal And Nano To Control Of Downy Mildew Disease In Cucumber. Anbar Journal Of Agricultural Sciences, 22(1), 706–718. https://doi.org/10.32649/ajas.2024.183806.
9. Giribet, G., and Edgecombe, G. D. (2019). The phylogeny and evolutionary history of arthropods. Current Biology, 29(12): 592-602. https://doi.org/10.1016/j.cub.2019.04.057.
10. Gunter, N. L., Leavengood, J. M., Bartlett, J. S., Chapman, E. G., and Cameron, S. L. (2013). A molecular phylogeny of the checkered beetles and a description of epiclininae: a new subfamily (Coleoptera: Cleroidea: Cleridae). Systematic Entomology, 38(3): 626-636. https://doi.org/10.1111/syen.12019.
11. Hasan, M. M., Athanassiou, C. G., Schilling, M. W., and Phillips, T. W. (2020). Biology and management of the red-legged ham beetle, Necrobia rufipes DeGeer (Coleoptera: Cleridae). Journal of Stored Products Research, 88: 101635. https://doi.org/10.1016/j.jspr.2020.101635.
12. Hebert, P. D., Ratnasingham, S., and De Waard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(suppl_1): 96-99. https://doi.org/10.1098/rsbl.2003.0025.
13. Jalalizand, A. R., Mirhendi, H., Karimi, A., Modaresi, M., and Mahmoodi, E. (2012). Morphological and molecular identification aphids of Rosae. APCBEE Procedia, 4: 12-15. https://doi.org/10.1016/j.apcbee.2012.11.003.
14. Keller, I., Bensasson, D., and Nichols, R. A. (2007). Transition-transversion bias is not universal: a counter example from grasshopper pseudogenes. PLoS genetics, 3(2): e22. https://doi.org/10.1371/journal.pgen.0030022.
15. Khidr, S. K., Hardy, I. C., Zaviezo, T., and Mayes, S. (2014). Development of microsatellite markers and detection of genetic variation between Goniozus wasp populations. Journal of Insect Science, 14(1): 43. https://doi.org/10.1093/jis/14.1.43.
16. Kim, H., Lee, W., and Lee, S. (2010). Morphometric relationship, phylogenetic correlation, and character evolution in the species-rich genus aphis (Hemiptera: Aphididae). PLoS One, 5(7): e11608. https://doi.org/10.1371/journal.pone.0011608.
17. Kolibáč, J. (1997). Classification of the subfamilies of Cleridae (Coleoptera: Cleroidea). Acta Musei Moraviae Scientiae Naturales, 81: 307-361
18. Kolibáč, J., and Huang, D. (2016). The oldest known clerid fossils from the Middle Jurassic of China, with a Review of Cleridae Systematics (Coleoptera). Systematic Entomology, 41(4): 808-823.https://doi.org/10.1111/syen.12192.
19. Kolibáč, J., Bocakova, M., Liebherr, J. K., Ramage, T., and Porch, N. (2021). Extinct and extant Pacific Trogossitidae and the evolution of Cleroidea (Coleoptera) after the Late Triassic biotic crisis. Zoological Journal of the Linnean society, 191(3): 846-882. https://doi.org/10.1093/zoolinnean/zlaa064.

20. Leschen, R. A. B. (2010). Cleroidea. Handbook of Zoology, Coleoptera, Beetles. Morphology and Systematics, 2: 237-280.
21. Lushai, G., Markovitch, O., and Loxdale, H. D. (2002). Host-based genotype variation in insects revisited. Bulletin of Entomological Research, 92(2): 159-164. https://doi.org/10.1079/BER2001138.
22. Martin, A. P., Kessing, B. D., and Palumbi, S. R. (1990). Accuracy of estimating genetic distance between species from short sequences of mitochondrial DNA. Molecular biology and evolution, 7(5): 485-488.
23. Tamura, K., Stecher, G., and Kumar, S. (2021). Mega11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution, 38(7): 3022-3027. https://doi.org/10.1093/molbev/msab120.
24. Thongprem, P., Davison, H. R., Thompson, D. J., Lorenzo-Carballa, M. O., and Hurst, G. D. D. (2021). Incidence and diversity of Torix Rickettsia–Odonata Symbioses. Microbial Ecology, 81(1): 203-212. https://doi.org/10.1007/s00248-020-01568-9.
25. Tvedte, E. S., Gasser, M., Zhao, X., Tallon, L. J., Sadzewicz, L., Bromley, R. E., ... and Hotopp, J. C. D. (2022). Accumulation of endosymbiont genomes in an insect autosome followed by endosymbiont replacement. Current Biology, 32(12): 2786-2795. https://doi.org/10.1016/j.cub.2022.05.024.
26. Valenzuela, I., Hoffmann, A. A., Malipatil, M. B., Ridland, P. M., and Weeks, A. R. (2007). Identification of aphid species (Hemiptera: Aphididae: Aphidinae) using a rapid polymerase chain reaction restriction fragment length polymorphism method based on the cytochrome oxidase subunit I gene. Australian Journal of Entomology, 46(4): 305-312. https://doi.org/10.1111/j.1440-6055.2007.00615.x.
27. Von Dohlen, C. D., Rowe, C. A., and Heie, O. E. (2006). A test of morphological hypotheses for tribal and subtribal relationships of Aphidinae (Insecta: Hemiptera: Aphididae) using DNA sequences. Molecular Phylogenetics and Evolution, 38(2): 316-329. https://doi.org/10.1016/j.ympev.2005.04.035.
28. Wang, Y. Z., Li, B. Y., Hoffmann, A. A., Cao, L. J., Gong, Y. J., Song, W., ... and Wei, S. J. (2017). Patterns of genetic variation among geographic and host-plant associated populations of the peach fruit moth Carposina sasakii (Lepidoptera: Carposinidae). BMC Evolutionary biology, 17: 1-12. https://doi.org/10.1186/s12862-017-1116-7.
29. Wang, Y., Huang, X. L., and Qiao, G. X. (2013). Comparative analysis of mitochondrial genomes of five aphid species (Hemiptera: Aphididae) and phylogenetic implications. PLoS One, 8(10): e77511. https://doi.org/10.1371/journal.pone.0077511.