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Original Research Article

Genotyping of Hymenoptera Ant Species Using DNA Sequencing in Northern Iraq

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Abstract: The current research aims to reveal the genetic variability and mutation rates in the genes of Hymenoptera species by taking several samples from different regions of northern Iraq, including Kirkuk/Wasiti and Salah al-Din/Tikrit/Alam. Samples were collected from different sites using different methods and placed in special bags. They were then transported to the laboratory, where DNA was isolated from each sample. Polymerase chain reaction (PCR) was performed to determine their sequence and record statistical analyses. The results showed that all samples belonged to the Hymenoptera species in Salah al-Din and Kirkuk governorates. The samples exhibited variation in the number and types of different genetic mutations, with a genetic concordance rate of 90% in samples collected from Salah al-Din/Tikrit/Alam governorates, while the samples from Kirkuk/Wasiti governorates exhibited a genetic variation of 91%, with changes in the nitrogenous base sequence between purines and pyrimidines. From this research, we concluded that there is significant genetic variation in the genome of the ant Hymenoptera sp. This is due to the significant influence of environmental adaptation, feeding pattern, and the stages of the ant's life cycle and its requirements, which promote biodiversity during different seasons.

Keywords: Hymenoptera Species, DNA Sequencing Technology.

1. INTRODUCTION

Ants play a vital role in the complex ecological and evolutionary systems that emerge within their ecosystems, performing many important functions, such as scavenging, predation, seed dispersal, and facilitating soil disturbance. These hardworking insects contribute to the health and stability of their environments in ways that are often overlooked. Furthermore, ants can indirectly influence plant life through their herbivores, helping to shape the vegetation dynamics in their habitats. A comprehensive understanding of the remarkable biodiversity of ants, along with the many factors that influence their dominance, distribution, and overall diversity, is essential for effective and sustainable ecosystem management practices. Furthermore, ants themselves provide highly attractive model organisms for the rigorous testing and exploration of complex ecological theories and fundamental concepts. There are currently approximately 15,000 known ant species identified globally, of which nearly 12,000 are accurately classified to the species level. This classification demonstrates their enormous diversity and significant ecological importance in maintaining healthy environments and contributing to a balanced ecosystem. Their presence and activities are critical to the health of many habitats, highlighting the need to continue researching and understanding these fascinating creatures [1]. Hymenoptera ants are primarily identified by a variety of morphological characteristics, which indicate their body structure and shape. This traditional method often requires collecting different specimens, but unfortunately, these specimens tend to deteriorate with age. Furthermore, identification can become extremely difficult when dealing with colonies that exhibit significant morphological variation [2]. This is particularly true for atypical castes and different genera within these colonies, which can complicate identification efforts. Molecular methods offer a solution to overcome these limitations and challenges through the use of molecular markers. These markers are unique to each species, yet exhibit differences between different

ant species. Subtle differences in the DNA sequences of these molecular markers can be attributed to evolutionary processes that have occurred over long periods of time. This evolutionary diversity allows researchers and entomologists to effectively distinguish between closely related species, as well as between species that may not be closely related at all. The genetic characterization of ant biodiversity is increasingly being used to study and uncover cryptic ant biodiversity, which refers to the hidden or indirectly apparent diversity within ant communities. However, the full extent of ant biodiversity in tropical regions, such as those in the Near East and the eastern Mediterranean, remains poorly understood [3, 4]. Entomological studies in northern Iraq generally suffer from the absence of reliable taxonomists for specific insect groups, and DNA barcoding studies are virtually nonexistent. On the other hand, DNA barcoding is a rapidly growing molecular method in entomology. This method aims to identify organisms with great accuracy and speed through the analysis of simple, standardized DNA sequences. It has broad applications in the study of insects [5]. DNA barcoding can help identify insects by comparing short sequences of a particular gene to a nearby database or by discovering entirely new species. It has been successfully applied to identify many ant species elsewhere in the world so far. The current study aims to determine the DNA barcode profile of Hymenoptera ant species [6].

2. MATERIALS AND METHODS

2.1. Sample Collection

Ants are one of the most ecologically distinct groups of insects. They are often one of the most abundant groups of organisms. Samples were collected from various areas in northern Iraq, including Salah al-Din/Tikrit/Alam and Kirkuk/Wasiti, between January 10, 2024, and January 15, 2024. Ant species were taken from nature using bait and soft forceps. They were preserved in the laboratory using 70% ethanol by placing them in plastic bottles with the following information written on them: collection date and region [7]. This method involves taking a number of insects ranging between 9-10 ants, depending on the size of the ant, in order to obtain the genetic material (DNA). Ant species were identified at the Research Center and Natural History Museum/University of Baghdad.

2.2. DNA Extraction

Body parts of the samples were used for DNA extraction. Genomic DNA was extracted because a large amount of body parts was required for the thoracoabdominal junction, which made it impossible to collect some data. In this case, the samples were cut into pieces, and both body and leg parts were used, then homogenized. The samples were first washed with distilled water and then stored in 90% alcohol for half an hour to ensure sterility. To store the ant material in the freezer for half an hour, the ant material was first ground with liquid nitrogen using a mortar and pestle, then boiled. After adding the proteinase K solution, homogenization continued in a water bath for 3 hours, alternating between half an hour at 60°C and half an hour without heat [8].

2.3. DNA Concentration and Purity Measurement

Nano droplet device was used to determine the concentration and purity of DNA. A single drop of extracted DNA was placed in the sample compartment of the device. The device was then switched on and the computer began measuring the DNA after the device was set to Elution Buffer (Tris-HCL, pH 8.5). The device displayed the desired measurement on the computer screen, using Nano grams/ml as the unit of measurement. The sample was then diluted to a concentration of 50 ng/ml at -80°C until it was used for molecular profiling studies. The mitochondrial COI gene was amplified, and a nucleotide sequence for this gene was generated and completed. It was then matched with the global gene bank to determine the evolutionary lineage of the ants in this research. The nucleotide sequences were designed by [9, 10].

2.4. PCR Amplification

Amplification by the polymerase chain reaction (PCR) is fundamental to genetic analysis of the ants sampled in this study. A suite of PCR protocols are outlined, with specific primers provided. These will target two loci, the mitochondrial cytochrome oxidase 1 (CO1) gene, which is used as a standard locus in insect systematics, using a PCR device to obtain the COI gene product according to the following reaction as shown in the following table:

Table 1: Shows the materials involved and their concentrations in the PCR reaction

Components	Concentration
PCR PreMix	5µl
DNA	2 µL
Reverse primer	(1 µl)
Forward primer	(1 µl)
Water	16 µl
Final volume	25µl

After introducing the mixture of materials at the specified concentrations as shown in Table (1) into a polymerase chain reaction (PCR) machine, the COI gene product was obtained according to the reaction program shown in the following table:

Table 2: Shows each stage of the PCR reaction, time, temperature and number of cycles

No.	Phase	Tm (°C)	Time	No. of cycle
1	initial denaturing	95°C	5 min.	1 cycle
2	Denaturation	95°C	20 sec	35 cycle
3	Annealing	54°C	20 sec	
4	Extension	72°C	20 sec	
5	final Extension	72°C	2 min.	1 cycle

3. Data Analysis

DNA sequences were estimated in Formicidae ant species living in a specific habitat in northern Iraq, and genetic convergence and divergence were determined from DNA sequencing results using the Geneious 2023.3 and MEGA X [11].

4. RESULTS AND DISCUSSION

4.1 External Appearance

This depends on the insect's external appearance, including its color, size, and shape. In general, an insect's body consists of three main regions: the head, the thorax, and abdomen. It is characterized by its hard, rigid external structure, making it water-resistant. This structure is composed of a substance called chitin, which gives the ant great strength. Ants are characterized by their strength compared to their very small size, as a single ant can carry a piece of food weighing ten times the weight of the ant. However, the criteria used in studying external morphology are insufficient to distinguish between species [8-12].

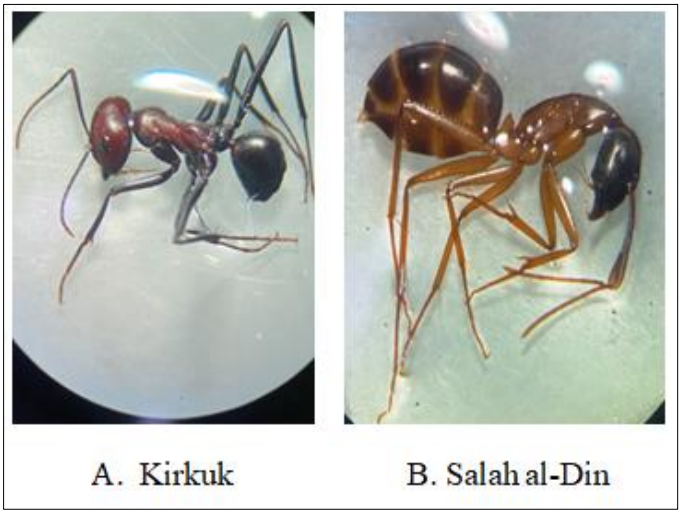

Figure 1: Shows images of the external appearance of ants for samples from two different regions.

4.2 Mutations

The first sample from Wasiti/Kirkuk region showed *Hymenoptera sp*. The number of mutations reached 38 as in table (3), It was noted that 4 of them belong to Transversion mutations, while 34 were Transition mutations. The percentage of similarity between them and the control was 90% and the comparison percentage was ID: JF866247.1.

While the second sample was collected from the Salah al-Din Tikrit/Alam and it showed us, for the same species, *Hymenoptera sp,* that the number of mutations reached 32 mutations, two of which were Transversion mutations, while the remainder were Transition mutations. The percentage of similarity between them and the control was 91%, and the comparison percentage was ID: JF866248.1.

Table 3: Shows the mutations that occurred in the genes of the two regions

No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
1	**Transversion**	**109**	**G/A**	**ID:** JF866247.1	**Hymenoptera sp. BOLD: AAQ0512 cytochrome oxidase subunit 1 (COI) gene, partial cds**	**%90**
	Transition	**118**	**C/T**			
	Transition	**133**	**T/A**			
	Transition	**142**	**C/T**			
	Transition	**157**	**C/T**			
	Transversion	**184**	**G/A**			
	Transition	**206**	**C/T**			

No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
	Transition	**217**	**A/T**			
	Transition	**220**	**C/T**			
	Transition	**235**	**C/T**			
	Transition	**238**	**C/T**			
	Transition	**242**	**C/T**			
	Transition	**259**	**T/C**			
	Transition	**263**	**T/C**			
	Transversion	**265**	**G/T**			
	Transition	**274**	**T/C**			
	Transition	**307**	**C/T**			
	Transition	**310**	**T/C**			
	Transition	**319**	**T/A**			
	Transition	**322**	**C/T**			
	Transition	**334**	**A/T**			
	Transition	**340**	**c/T**			
	Transition	**343**	**c/T**			
	Transition	**346**	**c/T**			
	Transition	**358**	**C/T**			
	Transition	**367**	**T/C**			
	Transition	**370**	**T/A**			
	Transition	**373**	**T/A**			
	Transition	**376**	**C/T**			
	Transition	**391**	**T/C**			
	Transition	**394**	**C/T**			
	Transition	**397**	**T/C**			
	Transition	**400**	**T/C**			
	Transition	**421**	**C/T**			
	Transition	**424**	**A/T**			
	Transition	**430**	**T/C**			
	Transversion	**433**	**G/A**			
	Transition	**445**	**T/C**			
2	**Transition**	**77**	**C/A**	**ID:** JF866248.1	**Hymenoptera sp. BOLD: AAQ0513 cytochrome oxidase subunit 1 (COI) gene**	**91%**
	Transition	**92**	**A/T**			
	Transition	**94**	**T/A**			
	Transition	**98**	**C/A**			
	Transversion	**109**	**G/A**			
	Transition	**121**	**C/T**			
	Transition	**133**	**T/A**			
	Transition	**141**	**C/T**			
	Transition	**156**	**C/T**			
	Transition	**196**	**t/C**			
	Transition	**199**	**t/A**			
	Transversion	**200**	**A/G**			
	Transition	**205**	**T/A**			
	Transition	**223**	**C/A**			
	Transition	**232**	**T/C**			
	Transition	**242**	**C/A**			
	Transition	**266**	**T/C**			
	Transition	**268**	**A/T**			
	Transition	**271**	**C/T**			
	Transition	**281**	**C/A**			
	Transition	**293**	**A/T**			
	Transition	**316**	**A/T**			
	Transition	**317**	**T/A**			
	Transition	**319**	**T/C**			
	Transition	**337**	**A/T**			

No.	Type of substitution	Location	Nucleotide	Sequence ID with compare	Source	Identities
	Transition	**340**	**C/T**			
	Transition	**346**	**C/T**			
	Transition	**373**	**C/T**			
	Transition	**376**	**T/C**			
	Transition	**379**	**T/A**			
	Transition	**394**	**C/T**			
	Transition	**403**	**A/T**			

Sample 1 4_1 .ab1 372
Hymenoptera sp. BOLD: AAQ0512 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
Sequence ID: JF866247.1 Length: 658 Number of Matches: 1,
Range 1: 88 to 459

Score	Expect	Identities	Gaps	Strand
477 bits(258)	4e-139	334/372(90%)	0/72(0%)	Plus/ Plus

Query 1 TTCTATTATTCTTAATGATCA**G**ACTTTTAA**C**TCTATTGTTACAAG**T**CATGCTTT**C**ATTAT 60
Sbjct 88 TTCTATTATTCTTAATGATCA**A**ACTTTTAA**T**TCTATTGTTACAAG**A**CATGCTTT**T**ATTAT 147

Query 61 AATTTTTTT**C**ATAGTTATACCTTTTATAATTGGAGG**G**TTTGGAAATTTTCTTATTCCT**C**T 120
Sbjct 148 AATTTTTTT**T**ATAGTTATACCTTTTATAATTGGAGG**A**TTTGGAAATTTTCTTATTCCT**T**T 207

Query 121 AATACTTGG**A**TG**C**CCCGATATAGCTTA**C**CC**C**CGA**C**TAAATAATATAAGATT**T**TGA**T**T**G**CT 180
Sbjct 208 AATACTTGG**T**TGTCCCGATATAGCTTA**T**CC**T**CGA**T**TAAATAATATAAGATT**C**TGA**C**T**T**CT 267

Query 181 TCCTCC**T**TCAATTTCTTTATTAATTATAAGAAACTTTAT**C**AA**T**GAAGGATC**T**GG**C**ACAGG 240
Sbjct 268 TCCTCC**C**TCAATTTCTTTATTAATTATAAGAAACTTTAT**T**AA**C**GAAGGATC**A**GG**T**ACAGG 327

Query 241 TTGAAC**A**GTATA**CCCCCCC**TTAGCAAATAA**C**TCATTTCA**T**AG**T**GG**T**CC**C**TCAATTGATCT 300
Sbjct 328 TTGAACTGTATATCCTCCTTTAGCAAATAATTCATTTCACAGAGGACCTTCAATTGATCT 387

Query 301 TAC**T**AT**C**TT**T**TC**T**TTACATATTGCAGGTATATC**C**TCAATTCT**T**GG**G**GCAATTAATTT**T**AT 360
Sbjct 388 TAC**C**ATTTT**C**TC**C**TTACATATTGCAGGTATATC**T**TC**T**ATTCT**C**GG**A**GCAATTAATTT**C**AT 447

Query 361 TTCAACAATTTT 372
Sbjct 448 TTCAACAATTTT 459

Sample 2 4_2 .ab1 360

Hymenoptera sp. BOLD: AAQ0513 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitochondrial
Sequence ID: JF866248.1 Length: 658 Number of Matches: 1,
Range 1: 51 to 410

Score	Expect	Identities	Gaps	Strand
488 bits(264)	2e-142	328/360(91%)	0/360 (0%)	Plus/ Plus

Query 1 TAAGAATAATTATTCGTCTTGAATTACGAGCTTGTGGATCAATTATACATAATGACCAGA 60
Sbjct 51 TAAGAATAATTATTCGTCTTGAATTAAGAGCTTGTGGATCATTAATAAATAATGACCAAA 110

Query 61 TTTATAATACCTTAGTTACTAGTCATGCTTTCATTATAATTTTTTTCATAGTTATACCTT 120

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|||||||||| |||||||||| ||||||| |||||||||||| ||||||||||||
Sbjct 111 TTTATAATACTTTAGTTACTAGACATGCTTTTATTATAATTTTTTTTATAGTTATACCTT 170

Query 121 TTATAATTGGAGGATTTGGAAATTTTTTTATCCCTTTAATATTAGGATCTCCCGATATAG 180
          |||||||||||||||||||||| || |||| ||||||||||||||| |||||||
Sbjct 171 TTATAATTGGAGGATTTGGAAATTTCTTAGTCCCATTAATATTAGGATCTCCAGATATAG 230

Query 181 CTTATCCTCGACTAAATAATATAAGATTTTGATTATTACCCCCTTCAATTTCTTTATTAA 240
          | |||||||| |||||||||||||||||||| | || |||||||| ||||||||
Sbjct 231 CCTATCCTCGAATAAATAATATAAGATTTTGATTACTTCCTCCTTCAATTACTTTATTAA 290

Query 241 TTATAAGAAATTTTATTAATGATGGATCTGGAACAGGATGAACTGTATACCCTCCCTTAG 300
          || |||||||||||||||||| | |||||||||||||| || ||||| ||||
Sbjct 291 TTTTAAGAAATTTTATTAATGATGGTACCGGAACAGGATGAACTGTTTATCCTCCTTTAG 350

Query 301 CCTCTAATATTTTTCATAATGGCCCTTCTGTTGATCTTACTATCTTTTCTCTACATATTG 360
          |||||||||||||||||| || || |||||||||||| ||||||| |||||||
Sbjct 351 CCTCTAATATTTTTCATAATGGTCCCTCAGTTGATCTTACTATTTTTTCTCTTCATATTG 410
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The results were read based on the DNA sequences provided by Macrogen Biotechnology in South Korea, using the BLAST search tool and website of the National Center for Biotechnology Information (NCBI)

We have seen the results of mutations that occurred in the purine bases guanine, cytosine, and adenine. In the first sample, it was noted that the number of mutations increased in the chain between (260 – 447).

While in the second sample, the number of mutations increased in the chain between (240 – 360). This is due to changes in the nitrogenous bases of DNA, as well as the effective role of the environment in causing mutations.

CONCLUSION

The gene sequences in the two regions showed significant differences compared to the standard sample. These mutations are related to climate, temperature, and the insect's diet. We conclude that each region has its own gene sequence, belonging to the same species and genus of insect, with only minor changes in its external appearance

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