A Comparative Molecular Study Between Some Genera Of The Ground Beetles Carabidae Family Spread In Diyala Governorate - Iraq Using Sequencing Of Cytb Gene

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Abstract
The study aims using the nucleotide sequence of the cytochrome b gene to classify some genera Distichus planus , Harpalus rufipes, Chlaenius nigricornis and Brachinus bayardi of the ground beetles family and to find the degree of genetic affinity between them and determine the common ancestry of these genera by phylogenetic tree. When comparing the four genera by amplifying the Cytb gene using the polymerase chain reaction (PCR), the results showed that the molecular weight of the resulting bundles is 480bp, The nucleotide sequence of the gene showed the presence of genetic variation between the four genera in the form of substitution mutations for some nitrogen bases along the nucleotide sequence of the gene on the one hand, and with the GenBank samples: Abax parallelepipedus, Calleida angusticollis, Blackburnia rupicola, Harplus sinicus, Progonus iridipennis and Halipuls flavicollis on the other hand. The phylogenetic tree based on the nucleotide sequence of the gene, when comparing the four genera with each other, showed that the most recent common ancestor or the so-called common ancestor of the tree is the genus Harpalus rufipes, from which the other three genera descended, and the genetic distance or time period of evolution between it and each of the genera C. nigricornis, D. planus and B. bayardi 0.16765, 0.01285 and 0.04470 respectively, While the results of the phylogenetic tree when comparing the four genera under study with gene bank genera showed that the most recent common ancestor or common ancestor is both the genus H. rufipes from the genera of the current study and the genus A. parallelepipedus from the gene bank and from them the other genera descended, as the tree split into three subgroups, the first group included the genera H. flavicollis and B. bayardi, and the genetic distance or time period of genesis between these two genera and between the genera of the common ancestor or ancestor was 0.00688, while the second subgroup of the tree included the two genera C. nigricornis, D. planus and reached The genetic distance or period of evolution between these two genera and between the genera of the common ancestor or ancestor 0.00442, the third subgroup of the tree included the genera C. angusticollis, B. rupicola, H. sinicus, P. iridipennis and the genetic distance or time period of evolution between these genera and between the genera of the common ancestor or ancestor 0.06208.

Introduction

Beetles constitute about 40% of insects, as they are the most described species. The known species are estimated between 5-8 million species. To this day, new species are still discovered from time to time, as well as the species found in fossils [1]. Beetles are found in most environments, from fields to forests and deserts, although some species are associated with specific ecosystems such as meadows

or crop fields, and they can be used as biomarkers to assess land changes between different ecosystems [2]. Ground beetles are a diverse group of insects with numbers of more than 40,000 species all over the world, 2,000 species of which are in North America, where the sizes of adult beetles range from 2 mm to more than 35 mm [3], Its nocturnal species are black or brown in color, located under an earthen mass, a rock, or tree trunks. Ground beetles can be distinguished from other types of other beetles by the speed of their movement during the day, and they tend to be brightly colored or embossed and have long legs, which allows them to move quickly To catch prey and to avoid other predators [4], Ground beetles are one of the most important components of natural and human ecosystems, and their importance comes from their diverse biology and presence in most environments, and their importance comes from the primary role in organizing the numbers of insects, mollusks and other invertebrates [5]. Some ground beetles are opportunistic species that consume a variety of foods; however, it has been noted that the majority of species are primarily predators and feed on other insects [6]. Most species locate food by random search, although some of the day-active species hunt by sight. It has been observed that females tend to follow a more varied diet than males, due to an increase in the size and number of eggs [3]. Also, some of the ground beetles feed on stored materials such as rice and wheat, and some are predators that devour and nibble trees and invertebrates, and thus they are considered a domestic and agricultural pest that gnaws furniture from the inside and destroys crops [7], Including the species Distichus planus and the species Harpalus rufipes, which is a common seed predator and is widespread as it was introduced to North America in 1937 [8], It is characterized by an elongated oval body and reddish legs, varying from 1-25 cm in length]8[, They are highly mobile beetles and are more active at night, and this type of ground beetles stores seeds in burrows under plant residues, how much they are more active in areas with vegetation cover (Daria and Viktor, 2016), It is characterized by the diversity of its life cycle in the fall season and causes damage to crops of wheat, millet, barley, oats, and to a lesser extent on leguminous plants such as peas, beans and industrial crops such as beets, potatoes and sunflowers [3]. While Chlaenius is a large and diverse genus of ground beetles found in Europe, the Near East, North Africa, near the North Pole and throughout the world, there are about 1000 species of them, including the type Chalenius nigricornis found in the eastern regions and the African tropics [9], While the species Brachinus bayarii is known as the slanderer beetles because of its strange defense system, it can release chemical sprays in the form of rapid pulsed bursts, and the ejection is accompanied by a popping sound as the slinging beetles produce and store two types of chemical compounds in a separate tank at the back end of the abdomen represented by hydroquinone and peroxide Hydrogen, when the insect is threatened, the beetle constricts the muscles that push the two reactants through valved tubes into a mixing chamber containing water and a mixture of catalytic enzymes When mixed, the reactants undergo a violent chemical reaction, which raises the temperature to nearly the boiling point of water [10]. The larvae of this species are external parasites and their adult body size depends on the size of their hosts. They are widely varied in geographical areas except for mainland Australia [11]. Molecular taxonomic studies began in 1970 when rRNA was used to classify bacteria [12]. During the past 25 years, molecular methods have been widely used to classify different organisms [13]. It is possible that the complete classification of the animal kingdom includes at least 10 million species divided into more than one million genera. Due to this great diversity in living organisms, researchers have resorted to using molecular methods to classify organisms because they are more accurate and depend on DNA data. [14]. The use of DNA data in classifying living organisms has led to a great deal of controversy among researchers, but there is a general opinion that genetic clues are very useful in identifying the different stages of the evolution of organisms and diagnosing preserved samples, which may not be suitable for phonotypical study because they may be damaged [15]. DNA data provide a character system universal to all life stages with the potential to overcome the problems of working with different semaphoronts. A DNA-based approach has already been used to associate different developmental stages in order to identify agricultural pests and invasive species, forensically important insects [16], larval parasitoids and endangered species in their early life stages [17]. Initial attempts have also been made to survey larval or mixed larval and adult assemblages with DNA methods [18]. The increasing taxonomic content of DNA databases and rapid sequencing technology now permit tree construction at ever larger scales, However, traditional phylogenetic methodologies struggle to accommodate these huge data sets, whilst newly developed techniques, more capable of coping with largescale analyses, have not become generally established.

In the last decade, technical progresses in molecular biology have allowed evolutionary biologists to collect large DNA sequence data sets in a reasonably short amount of time. This has opened the way for extensive studies on the pattern of evolution of several mitochondrial and nuclear genes and for using DNA sequences to reconstruct phylogenetic relationships at different taxonomic levels [19]. Molecular markers can be divided into DNA markers and protein markers. DNA markers have been widely used due to the disadvantages of allozymes and isozymes which can be referred as protein markers. The thousands of protein-coding genes in the eukaryotic nuclear genome present the richest untapped source of genetic data for phylogenetic research. These genes show a number of favorable properties for phylogenetic analysis, Species identification represents a pivotal component for biodiversity studies and conservation planning, but represents a challenge for many taxa when using morphological traits only (e.g. the correct identification of juveniles or larval stages).
Given the importance of ground beetles from an economic and environmental point of view, the current research aims to use the Cytb sequencing to distinguish among Distichus planus, Harpalus rufipes, Chlaenius nigricornis and Brachinus bayardi because it is a technique that depends on DNA data and is able to separate species of common origin .

Materials and Methods

Specimen Collection

This research was carried out in the Molecular Genetics Laboratory, College of Education for Pure Sciences, University of Diyala, Iraq. Insect specimens were collected from different areas in Diyala Governorate, Iraq. A total of 40 specimens were collected, comprising 10 samples for each species, using light traps and bait traps. The specimens were preserved in 70% ethanol and transported to the laboratory for DNA extraction.

DNA Extraction

Genomic DNA was extracted using a Genomic DNA Mini Kit (Bioneer, South Korea) according to the manufacturer's instructions. The extracted DNA was stored at 4°C. DNA quality and concentration were assessed using a spectrophotometer. DNA concentration was determined by measuring optical density (OD) at 260 nm, as DNA exhibits maximum ultraviolet absorption at this wavelength [20].

Cytb Gene Amplification

The cytochrome b (Cytb) gene was amplified using the following primers:
CytbF (5'-TATGTACTACCATGAGGACAAATATC-3') and
CytbR (5'-ATTACACCTCCTAATTTATTAGGAAT-3').
The PCR reaction was performed in a total volume of 25 µL, consisting of:
5 µL Master Mix
1.5 µL forward primer
1.5 µL reverse primer
5 µL DNA template
12 µL deionized water
PCR conditions were as follows:
Initial denaturation at 94°C for 5 min
35 cycles of:
Denaturation at 94°C for 45 s
Annealing at 53°C for 45 s
Extension at 72°C for 1 min
Final extension at 72°C for 10 min
PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide and visualized under UV light using a gel documentation system. Ten PCR products from each species were sent to Macrogen (South Korea) for sequencing.
Cytb Gene Sequencing and Phylogenetic Analysis

The nucleotide sequences of the Cytb gene were aligned using BioEdit. Identical sequences were grouped into haplotypes. Phylogenetic analyses were conducted using the neighbor-joining and maximum likelihood methods implemented in MEGA [21].

Results

Figure 1. Cytb amplification product of some genera ground beetles family Carabidae carried over on agarose gel at a concentration of 1% for 1.5 hours after staining with silver stain and photographed under UV light. S1: Distichus planus , S: Harpalus rufipes, S3: Chlaenius nigricornis and S4: Brachinus .

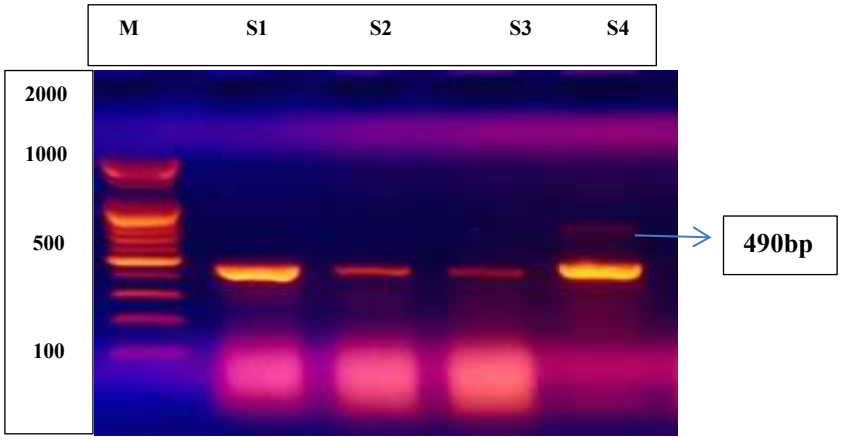

Figure 2. Comparison of the nitrogen base alignment fora part of Cytb gene between the samples of the current research and the samples of the gene bank. (Current research samples are : Distichus planus, Harpalus rufipes, Chlaenius nigricornis and Brachinus bayardi , Gene bank samples are : Abax parallelepipedus, Calleida angusticollis, Blackburnia rupicola, Harplus sinicus, Progonus iridipennis and Halipuls flavicollis) .

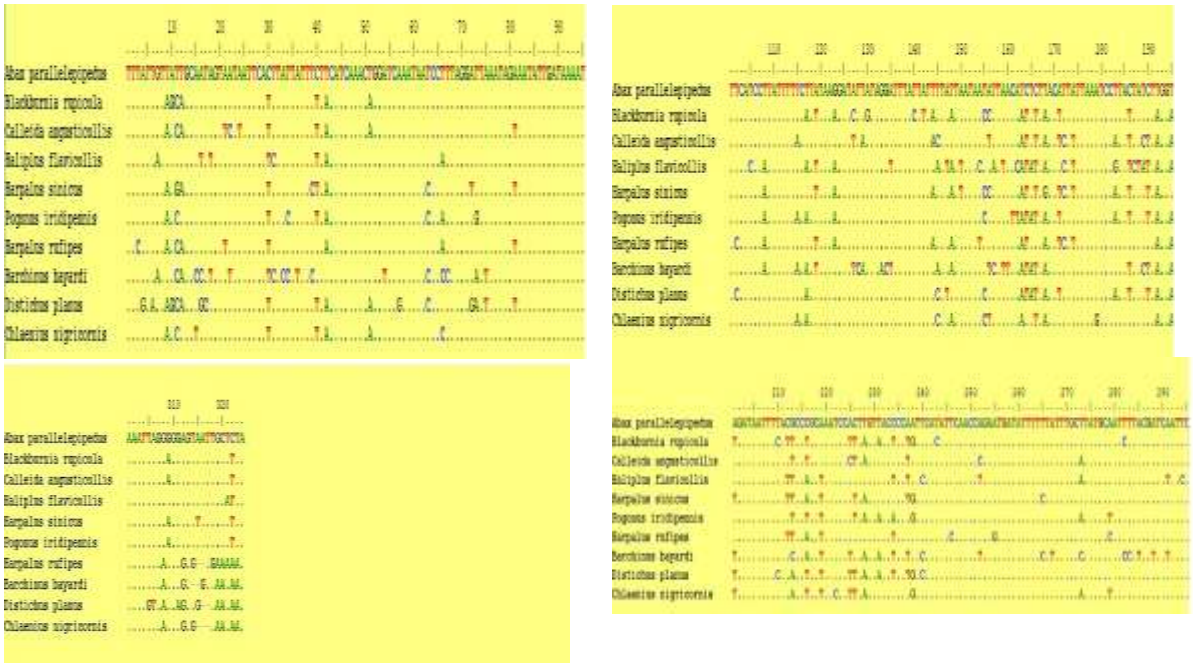

Figure 3. Phylogenetic tree of current research samples and gene bank samples based on the nucleotide sequence of Cytb gene. (Current research samples are : Distichus planus, Harpalus rufipes, Chlaenius nigricornis and Brachinus bayardi , Gene bank samples are : Abax parallelepipedus, Calleida angusticollis, Blackburnia rupicola, Harplus sinicus, Progonus iridipennis and Halipuls flavicollis) .

Table 1. Genetic distances or periods of evolution between the samples of the current research and the samples of the gene bank

Cycle	OUT / NODE	JOINS OUT / NODE
7	4 (0.09260)	8 (0.15520)
6	9 (0.08344)	10 (0.04896)
5	1 (0.07272)	7 (0.07807)
4	Node 1 (0.00721)	4 (0.03134)
3	1 (0.00461)	9 (0.01898)
2	Node 1 (0.00669)	6 (0.05128)
1	2 (0.05285)	5 (0.05695)
Last cycle	Node 1(0.00688)	2 (0.00442) JOIN OUT 3 0.06208

Figure 4. Comparison t of the nitrogenous bases alignment for a part of Cytb gene between the samples of current research .

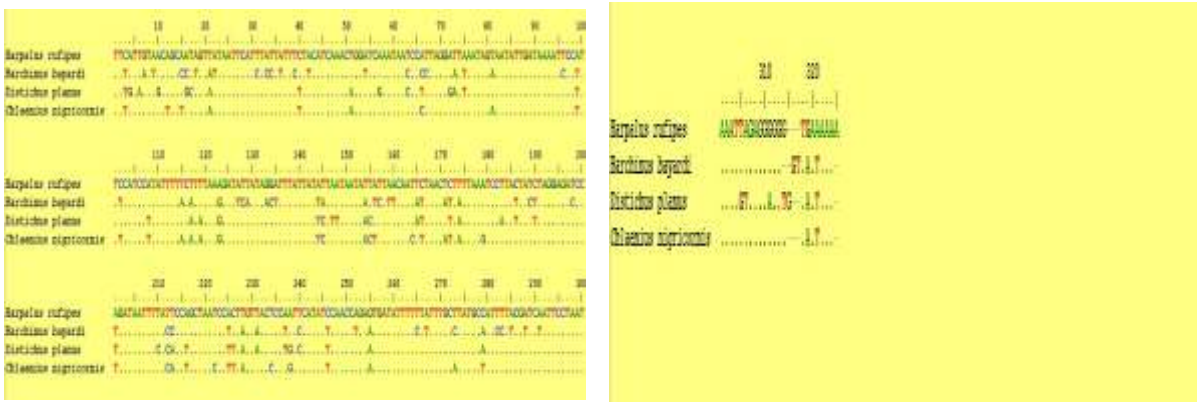

Figure 5. Phylogenetic tree of current research samples based on the nucleotide sequence of Cytb gene.

Table 2. Genetic distances or periods of evolution between the samples of the current research

Cycle	OUT / NODE	JOINS OUT / NODE
1	2 (0.16765)	3 (0.07945)
Last cycle	1(0.10600)	Node 2 (0.01285) JOIN OUT 4(0.04470)

Discussion

The amplification of the cytochrome b (Cytb) gene in the samples of the current study (Distichus planus, Harpalus rufipes, Chlaenius nigricornis, and Brachinus bayardi) produced a fragment of approximately 490 base pairs (bp) for all species (Figure 1). This fragment size is consistent with previous studies that used mitochondrial Cytb as a molecular marker in insects [22,23].

The nucleotide sequence analysis of the Cytb gene revealed the presence of genetic variation among the four species in the form of substitution mutations at several nucleotide positions along the gene sequence. Such variations are commonly observed in mitochondrial genes due to their relatively high mutation rates [24,25]. Additionally, sequence comparisons with GenBank data, including Abax parallelepipedus, Calleida angusticollis, Blackburnia rupicola, Harpalus sinicus, Progonus iridipennis, and Harpalus flavicollis, showed further genetic divergence (Figures 2 and 4) [26,27].

Phylogenetic analysis based on Cytb gene sequences demonstrated that Harpalus rufipes clustered closely with the other studied species. However, phylogenetic trees represent inferred relationships rather than direct ancestor–descendant lineages; therefore, no extant species can be considered a true common ancestor [28,29]. The genetic distances between H. rufipes and the other species were 0.16765, 0.01285, and 0.04470 for Chlaenius nigricornis, Distichus planus, and Brachinus bayardi, respectively (Figure 5, Table 2). These values fall within the range typically reported for interspecific divergence in insects using mitochondrial genes [30].

When comparing the studied species with GenBank sequences, the phylogenetic tree showed clustering into three main groups. The first group included Harpalus flavicollis and Brachinus bayardi, with a genetic distance of 0.00688. The second group included Chlaenius nigricornis and Distichus planus, with a genetic distance of 0.00442. The third group included Calleida angusticollis, Blackburnia rupicola, Harpalus sinicus, and Progonus iridipennis, with a genetic distance of 0.06208 (Figure 3, Table 1). These clustering patterns are consistent with phylogenetic studies that group closely related taxa based on mitochondrial DNA similarity [31,32].

The Cytb gene sequences obtained in this study were submitted to the NCBI GenBank database under accession numbers OM964854.1, OM964853.1, OM964852.1, OM964851.1, OM964850.1, OM964849.1, OM964848.1, and OM964847.1.

Conclusion

Ground beetles are similar in many phonotypical characteristics, including size, shape, type of antennae, their life cycle and season of activity, which in turn leads to the correctness of distinguishing between genera and species. Phenotypic classification is inaccurate compared to molecular classification because many phenotypic traits are affected by environmental conditions,

while genetic traits are fixed because they depend on DNA data. The nucleotide sequence of the Cytb gene showed the common ancestry Harpalus rufipes, from which other genera Distichus planus, Chlaenius nigricornis and Brachinus bayardi descended.

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