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التوصيف المورفولوجي والجزيئي للنوع *Cybister tripunctatus* (Olivier, 1795) (Coleoptera: Dytiscidae) في محافظة ذي قار، العراق: دراسة تصنيفية.

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المستخلص:

تناولت الدراسة الحالية التعرف المورفولوجي على نوع من الخنافس المائية. جُمعت العينات من بيئات مائية مختلفة في محافظة ذي قار، وتبيّن أنها تعود إلى النوع *Cybister tripunctatus*، وهو من الخنافس المائية المفترسة الواسعة الانتشار ينتمي للعائلة Dytiscidae في رتبة غمدية الاجنحة Coleoptera. وأكد التحليل الجزيئي هذا التشخيص، إذ أظهرت تسلسلات جين mtCOX1 المستخلصة من الحمض النووي المضخّم بتقنية PCR تطابقًا بنسبة ٩٨% مع السجلات الموجودة مسبقا في قاعدة بيانات بنك الجينات GenBank وقد أُودِعت هذه التسلسلات برقم انضمام جديد في بنك الجينات ليكون مرجعًا مستقبليًا. وتمثل هذه الدراسة الأولى من نوعها في محافظة ذي قار التي تدمج بين المنهجين المورفولوجي والجزيئي لتحديد هذا النوع.

الكلمات المفتاحية: المظهري، التعريف، *Cybister*، الخنافس الغواصة

Morphological and molecular characterization of *Cybister tripunctatus* (Olivier, 1795) (Coleoptera: Dytiscidae) from Thi Qar Governorate, Iraq: A taxonomic study

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Abstract:

The current study involved the morphological identification of an aquatic beetle species (Dytiscidae: Coleoptera) collected from various aquatic habitats in Thi Qar Governorate. The specimens were identified as *Cybister tripunctatus*, a widely distributed predatory aquatic beetle. Molecular analysis further confirmed this identification, with mtCOX1 gene sequences from PCR-amplified DNA showing a 98% match with GenBank entries. These sequences were submitted to GenBank under a new accession number to serve as a future reference. This represents the first study in Thi Qar Governorate to integrate both morphological and molecular approaches for identifying this species.

Key words: Morphological, Characterization, Cybister, Dytiscidae.

1. Introduction:

Aquatic beetles, belonging to the order Coleoptera, represent a broad group of insects that have adapted to aquatic environments worldwide. Their presence and diversity in freshwater ecosystems have fascinated researchers and naturalists for centuries. Numerous scientific studies have delved into the ecology, behavior, and evolutionary adaptations of aquatic beetles (Sharma *et al*. 2019). The Dytiscidae (diving beetles), or predatory diving beetles, or water striders, are among the largest families in the order Coleoptera. They are aquatic insects of varying sizes, ranging in length from 1 to 50 mm (Ribera *et al*. 2008). The genus *Cybister* (Curtis, 1827) is a large genus of the family Dytiscidae, comprising approximately 106 known species or subspecies worldwide (Nilsson & Hájek, 2022). These beetles are characterized by their large size, some reaching 45 mm, and their dark green to black color. Some species have yellow margins on the elytra or anterior part of the pronotum. *Cybister* species often inhabit stagnant waters rich in dense submerging vegetation, and their abundance is particularly high in Afro-tropical and eastern regions. One species of this genus, *Cybister tripunctatus*, is a predatory diving beetle, and is distributed across a wide range of regions worldwide (Miller & Bergsten, 2016). The high phenotypic

similarity among species of the genus *Cybister* has led to numerous taxonomic challenges. Sharp (1882) attempted to divide it into six groups based on morphological features, and Brinck (1945) divided it into seven subgenera, before Vazirani (1969) pointed out problems with this classification through a study of reproductive characters. Later, Miller *et al*. (2007) re-evaluated the structure of the genus and proposed four subgenera using morphological criteria supported by DNA analysis. With the recent increase in interest in biodiversity, recent studies have emphasized the need to combine phenotypic diagnosis with molecular analysis techniques, such as DNA barcoding, to accurately verify taxonomic identity and reduce errors resulting from morphological similarity between species (Megna *et al*., 2021; Ribera *et al*., 2023). Therefore, combining phenotypic description and molecular analysis is an important and essential step to confirm the identity of Cybister species and ensure the reliability of taxonomic study results, especially in complex aquatic environments that represent a haven for high and yet fully unexplored diversity. This study aims to conduct a detailed morphological description of the external characteristics of the complete stage of Cybister tripunctatus for a number of specimens found in different aquatic habitats in Thi Qar Governorate, while confirming the taxonomic identity using molecular fingerprinting to verify the compatibility of traditional phenotypic diagnosis with the results obtained from genetic analysis, which contributes to improving the accuracy of classification and revealing any potential genetic diversity within the studied samples.

2. Materials and methods: .

The current study was conducted in Thi Qar Governorate, one of the southern governorates of Iraq, located 366 km south of the capital, Baghdad. Samples were collected using sieves and aquatic insect catch nets (dip nets) prepared for this purpose. The captured insects, which were to be morphologically identified, were preserved in bottles containing 70% ethyl alcohol, while a portion of the samples that were to be molecularly identified were frozen. Local taxonomic keys were used to aid morphological identification (Abdul Karim, 1978; Alaq, 2017). DNA was extracted from insect samples and isolated using the gSYNC™ DNA Extraction Kit

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provided by the Taiwanese company Geneaid, according to the manufacturer's protocol for isolating DNA from tissues based on the method of Sambrook *et al*. (1989). PCR technique was used to amplify the Mitochondrial Cytochrome Oxidase subunit1 (mtCOX1) gene extracted from insect tissues. The primers specific to the target gene (mtCOX1) were used as shown in the table (1).

Table (1) Primer and base pair sequence of mtCOX1 gene.

Length	Primer	Gene sequence	Gene	Source
25	LCO1490	5'-GGTCAACAAATCATAAAGATATTGG -'3	mtCOX1	Folmer *et al*., 1994)
26	HCO2198	5'-TAAACTTCAGGGTGACCAAAAAATCA-'3	mtCOX1	Folmer *et al*., 1994)

3. Results:

3.1 Morphological Description of Cybister *tripunctatus* (Olivier, 1795)

Number of examined samples: 32 (13 males and 19 females). The body shape is elongated, oval, flattened, and streamlined, resembling a boat, allowing it to move efficiently through the water. Typical lengths for fully grown specimens range from 24 to 28 mm (Plate 1).

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Image (1) External appearance of the species *Cybister tripunctatus*

The head: is dark green, large, flattened and strongly rigid. The dorsal side of the head consists of strong upper jaws, prepared for gnawing(biting) and predation. Clypeus, which is a hard piece covering the mouth and forming part of the external shape of the face and can be clearly seen. It is followed at the top of the head by the forehead(vertex), and there is a distinctive transverse groove extending over the dorsal surface of the head between the clypeus and the forehead, called the front-clypeal suture. There are four pits in the front of the forehead, one pair of which is parallel to the frontal suture and the other pair is higher, closer to the middle of the forehead. On either side of the forehead are the compound eyes, which are prominent, large, and black in color with dark green to yellow colors, so that under examination they appear mosaic (variegated). The antennae are of the filiform type, appear yellow under examination and consist of 11 segments, the first of which, the basal segment (scape), is the longest, while the second segment, called the pedicel, is the shortest. The rest of the segments are approximately equal in size. Two pairs of pulps can be seen on the ventral side of the head, a pair of four-segmented maxillary pulps that are clearly yellow, and a pair of three-segmented labial pulps that are pale yellow. At the posterior end of the head is the nuchal opening (Plate 2, Plate 3).

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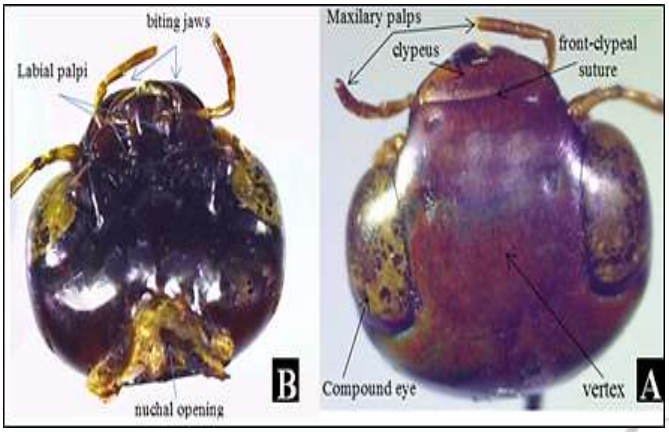

Plate (2) the head in the species *Cybister tripunctatus* A- Dorsal side B- Ventral side

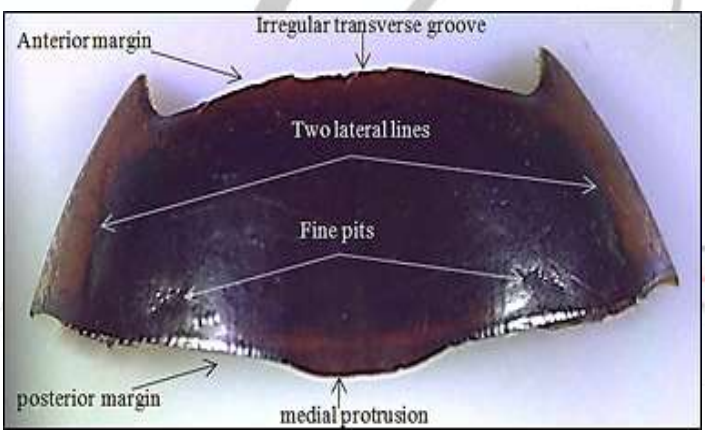

Plate (3) Antenna of the species *Cybister tripunctatus*

The thorax: is well developed and divided into three segments: the prothorax, the mesothorax, and the metathorax, each bearing a pair of jointed legs used for swimming. Dorsal surface of the prothorax is covered by the pronotum, a large transverse dorsal plate, twice as wide as it is long. Its upper surface is smooth in the middle, and its anterior corners are narrowly tapered. The anterior lateral margins are bright yellow, while the remaining

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surface is dark green. The anterior margin of the dorsal plate is arched(convex) forward and has an irregular transverse groove in its middle, opposite a protrusion at the posterior margin parallel to the elytra, and on either side of this protrusion are a group of fine pits. There are also two lateral lines in depressions near the yellow lateral margins (Plate 4).

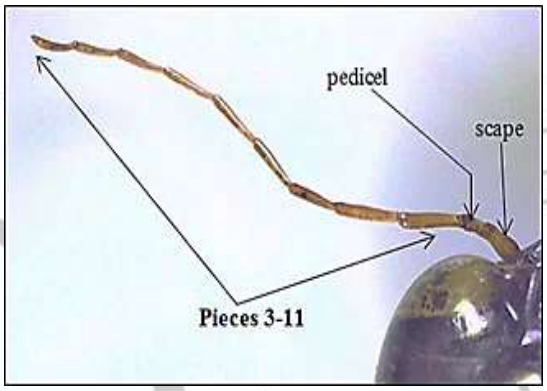

Plate (4) Pronotum in the species *Cybister tripunctatus*

Pronotum is separated from the lateral plate (pleuron) by a notch called the notopleuron which consists of two regular plates; the anterior episternum and the posterior epimeron. The anterior episternum is small and triangular in shape, while the epimeron extends behind the ilium on each side and does not reach the medial part of the anterior episternum, called the prosternal process, thus keeping the axial cavities open for ease of movement. The upper surface of the prothorax is characterized by the presence of an internal longitudinal ridge on each side with a distinct median line. As for the ventral side, the prosternum is characterized by being solid and narrow, extending over the anterior edge of the ventral side of the prothorax, and bearing a prominent median posterior piece, which is the prosternal process (or what is called the sternal process), the posterior part of which is smooth and spear-shaped (Plate 5).

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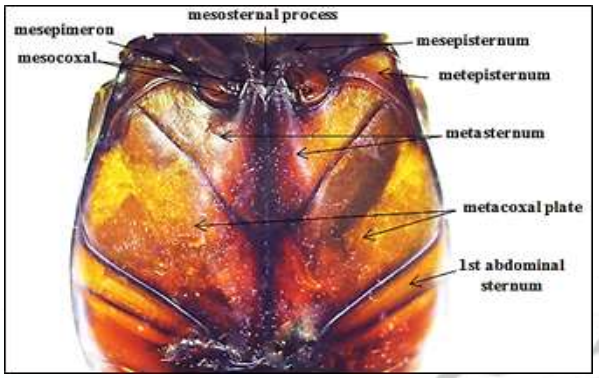

Plate (5) Prosternum in the species *Cybister tripunctatus*

Mesothorax and Metathorax are directly connected to each other, middle one being relatively small and on its dorsal side there are wing projections (anterior pair elytra) on the sides followed by sclerotic plates of targum which is divided into three sections starting from the front with prescutellum and on the sides the scutum then the median scutillum, and the dorsal targum is surrounded by the axillary membranes. Dorsal side of posterior thoracic segment is large and the parts of targum are more clearly visible and they are in the same previous arrangement as the middle thoracic segment in terms of wing projections (posterior pair membranous) and the sections of dorsal targum with a difference in size. The first pair of spiracles is located in the area of the membrane separating the second and third thoracic targum (Plate 6).

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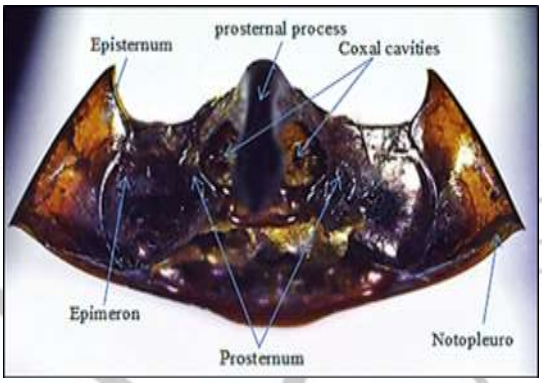

Plate (6) Targa of the second and third thoracic segments in *Cybister tripunctatus*

The mesosternum of the second thoracic segment is a sclerotic plate consisting of a raised and prominent middle part called the mesosternal process extending posteriorly. On either side of the mesosternum is a plate called the mesepisternum. The mesocoxal cavities are surrounded and closed by a triangular plate called the mesepimeron which reaches the midcoxa on both sides. Metasternum of the third thoracic segment consists of a large, broad, centrally located sclerotic plate called the posterior sternum with a small anterior medial process that overlaps the prominent posterior end of the midcoxa. The anterior edge of the posterior sternum wraps around the bases of the midcoxa, and ends open posteriorly to facilitate movement. Above it and on both sides, there is a triangular plate called the metepisternum, and below it there is a large heart-shaped plate called the metacoxal plate. In the middle of the posterior end of that plate and on both sides of its midline there are protrusions which are the points of articulation of the metacoxal processes with the trochanter (Plate 7).

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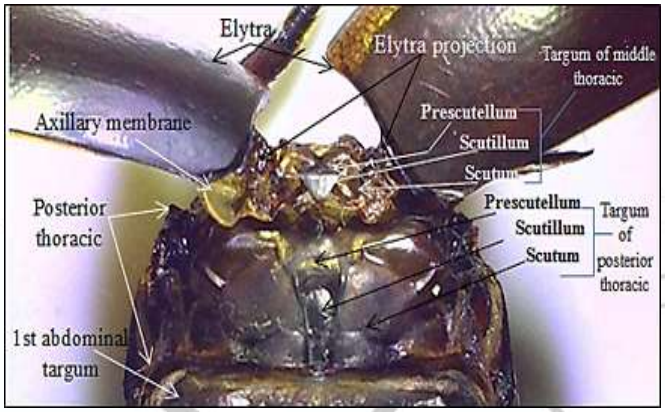

Plate (7) Sternites of the second and third thoracic in *Cybister tripunctatus*

Legs: reddish-brown to black, and are equipped with soft, dense hairs that aid in propulsion through the water. Forelegs consist of oval coxa, trochanter triangular in shape and connects the coxa to the femur, which is thicker than the rest of the segments, its length is approximately twice its width, and it is provided with small spines and downy hairs that spread on the outer edge of it more than its inner edge. Then, tibia, which is relatively thin and elongated, its length is approximately three times its width, and its outer side is grainy and provided with thick structures resembling blades or spurs located near its ends, and two equal sharp terminal spines at its end near the tarsus. The last part is tarsus, which consists of five small segments that differ in shape in males from females. In the male, the first three segments swell and expand to form a disc-like structure and are densely covered with fine hairs that act as suckers that help in holding onto the female during the mating. The last two segments of the tarsus are connected to these disc-like structures, the terminal of which ends in two small claws. While the tarsus segments on the female's front leg consist of five approximately equal segments containing hairs on the sides and small sharp spines protruding on

the inner surface of those segments. The terminal segment of the tarsus ends with two claws of equal length (Plate 8). Middle leg consists of the same segments as the foreleg and is similar in shape. The basal part of the tarsus is the longest, while the other four segments are approximately equal. The inner surface of the metatarsus is equipped with stiff spines and dense bristles on both surfaces, which aid swimming. The metatarsus ends in two claws of equal length (Plate 9). Hind leg differs from the fore and middle leg in size and shape, as it is noted that it is approximately twice as long and the coxa is in the form of a large oval plate located below the metasternum and merges with it and the opposite coxa plate to form the two coxa plates together in a heart shape. The trochanter is relatively small in shape, while the femur is approximately three times its width in length and its surface is smooth on both sides and is not equipped with hairs, spines or any other appendages. The tibia is thick and contains at its end a row of small comb teeth and in the area where it connects to the tarsus there are two long, thick spines of equal length. As for the tarsus, it consists of 5 pieces, the first of which is the longest and largest piece, then the remaining four pieces gradually decrease in size until the ends with the smallest end piece which ends with a single apical claw. The inner edge of the tarsus pieces is equipped with dense and long hairs as is the outer edge, but the hairs in the latter are shorter and less dense (Plate10).

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Plate (8) Foreleg in *Cybister tripunctatus* A- in male B- in female

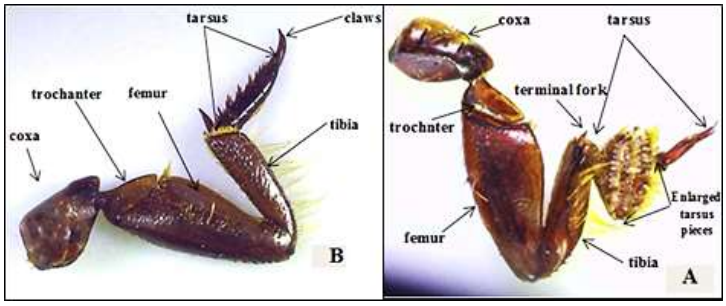


Plate (10) Hind leg in *Cybister tripunctatus*

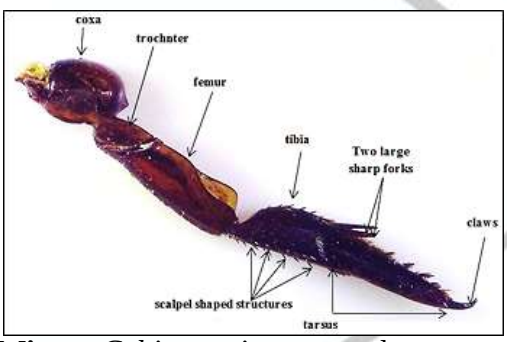
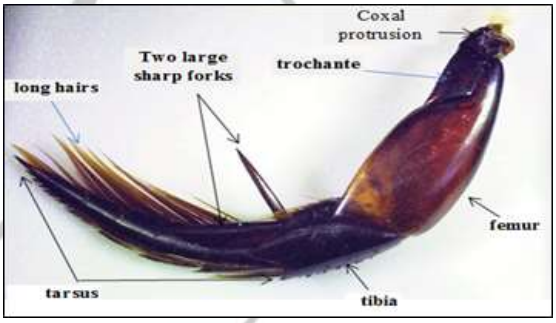


Plate (9) Middle leg in *Cybister tripunctatu s*



Wings: *Cybister tripunctatus* has two pairs of wings, the front ones or so-called elytra are strong and stiff and act as protective covers for the hind wings and the abdominal segments that lie beneath them and meet in a straight line in the middle of the back and open upwards when the beetle is in flight. The elytra are dark green (olive) with a slight metallic sheen and have distinctive bright yellow edges on the side. They are broad at the base and narrow at the top, 17-18 mm long and 8 mm wide. Their dorsal surface is smooth and soft with two lines of dots starting near the basal edge of the wing and extending to its narrow end. The ventral side contains circular pits and has a grainy texture. Plate11.

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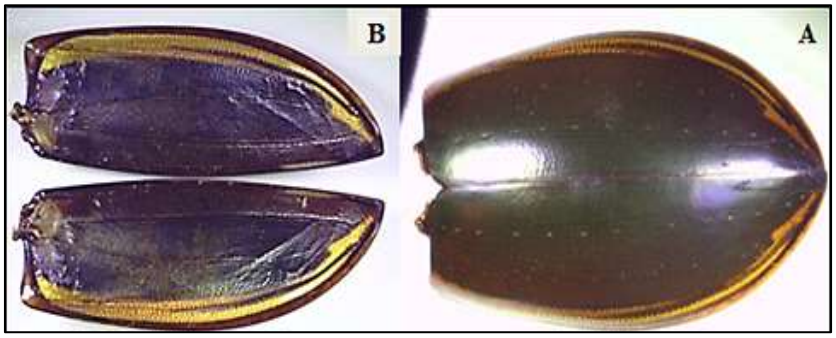

Plate (11) Elytra in *Cybister tripunctatus* A – Dorsal view B – Ventral view

Hind wing is membranous, folded at rest under the elytra and opened in flight. It is triangular in shape, with a narrow apical edge and a broad base. Its length is 24 mm and its width is 12 mm. It shows a simplified veining system, where the costal vein (C), the subcostal vein (Sc) and the radial vein(R) can be seen extending along the outer edge of the wing. The costal vein (C) and directly below it the subcostal vein (Sc) extend from the base of the wing and run along the anterior edge to reach approximately before the middle after the first third of the outer edge of the wing, where it is difficult to separate them. The radial vein (R) appears clearly below the subcostal vein and continues beyond the middle to connect to the median vein(M) by a transverse vein (r-m) below which there is an open wing cell towards the edge and next to it a thick, opaque membranous fold. Under the median vein lies the Cubitus vein (Cu), which is thick and convex and extends from the base of the wing, penetrating its median space and branching into two veins: (Cu1), at the end of which there is an elongated, closed oval cell, and (Cu2), which in turn divides into two branches: (1stCu2) and (2ndCu2). Then there are the posterior (Anal) veins (A), which are four veins (A1, A2, A3, A4). The first posterior vein A1 is weak and is connected to the Cubitus vein by a transverse vein, cu-a1, near the base of the wing, while the second posterior vein A2 divides into two veins: 1stA2, which merges with the first posterior vein A1, and 2ndA2. The third posterior vein A3 is thick and continues towards the inner edge of the wing, and finally the fourth posterior vein A4, which is weak and is located on the inner edge parallel to the base of the wing. Plate 12.

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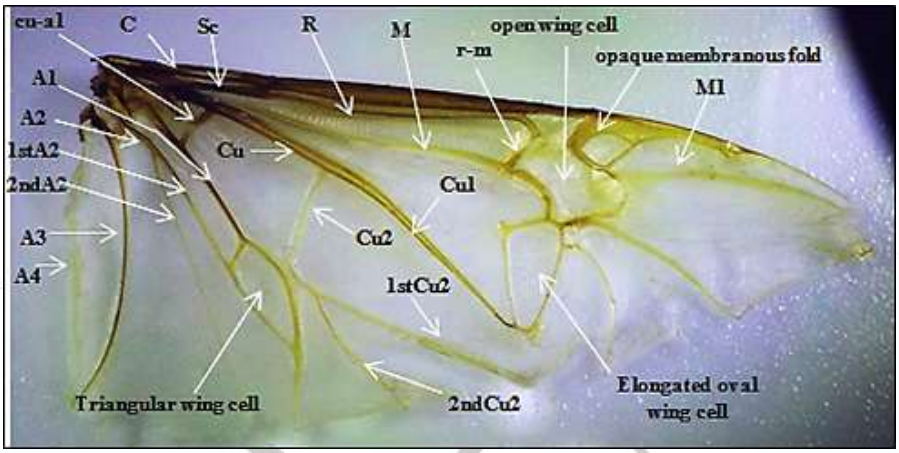

Plate (12) Hind wing (membranous) in *Cybister tripunctatus*.

Abdomen: divided into eight segments decreasing in size posteriorly, covered by the elytra when the beetle is at rest. Eight abdominal targum segments can be distinguished, the first six are broad and membranous in appearance and almost equal, while the seventh and eighth are triangular and darker in color. The last is narrow and small, called the sacral plate, and has rectangular plates. All dorsal targum segments are surrounded by the pleural membrane. Eight spiracles can be seen on each side of the abdominal segments (Plate 13). Ventrally, six smooth, reddish-brown sternal plates can be distinguished. The first sternum, which is fully visible from the front, is divided by the metacoxae. The second sternum is clearly visible and incompletely divided by the metacoxal cavity. The third, fourth and fifth are equal and similar in shape. The sixth is narrow and contains two small triangular plates (Plate14).

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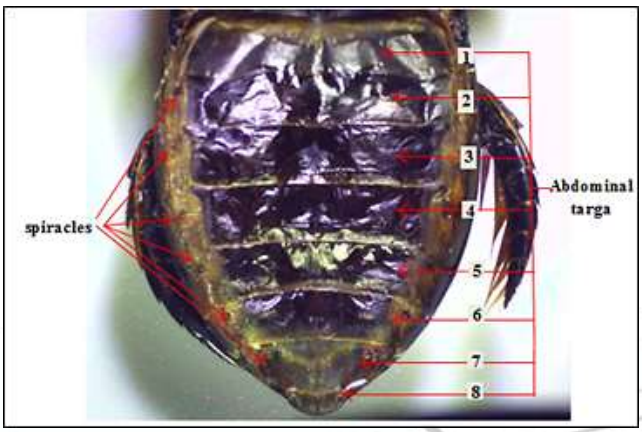

Plate (13) Targets of the abdominal segments in *Cybister tripunctatus*

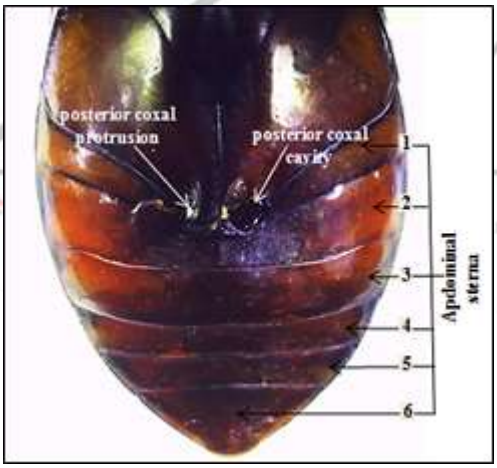

Plate (14) Sternites of the abdominal segments in *Cybister tripunctatus*

3.2 Molecular diagnosis:

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Polymerase chain reaction (PCR) results of DNA samples extracted from insect models showed bright bands appearing at 700 base pairs of the molecular analysis indicator (DNA ladder) carried with the samples using 1.5% agar gel. The results of the samples sent to Macrogen Company for sequencing analysis using the Genetic Analyzer showed a match rate of 98% compared to the reference sample previously registered in the gene bank. The sequencing results of the mtCOX1 gene for this type were documented as a source for the first time in a molecular diagnostic study in Thi Qar Governorate and were registered with a new accession number (Table 2).

Table 2: Matching percentages of the target gene segments mtCOX1 and the accession number of the registered species

N.	species	Reference sample accession number	GenBank accession number	Matching percentage
1	*Cybister tripunctatus*	KX360227.1 India	LC872749 Iraq	98 %

Figure 1 Comparison of the mtCOX1 gene sequences of the current study specimen *Cybister tripunctatus* with the source sequences recorded in GenBank.

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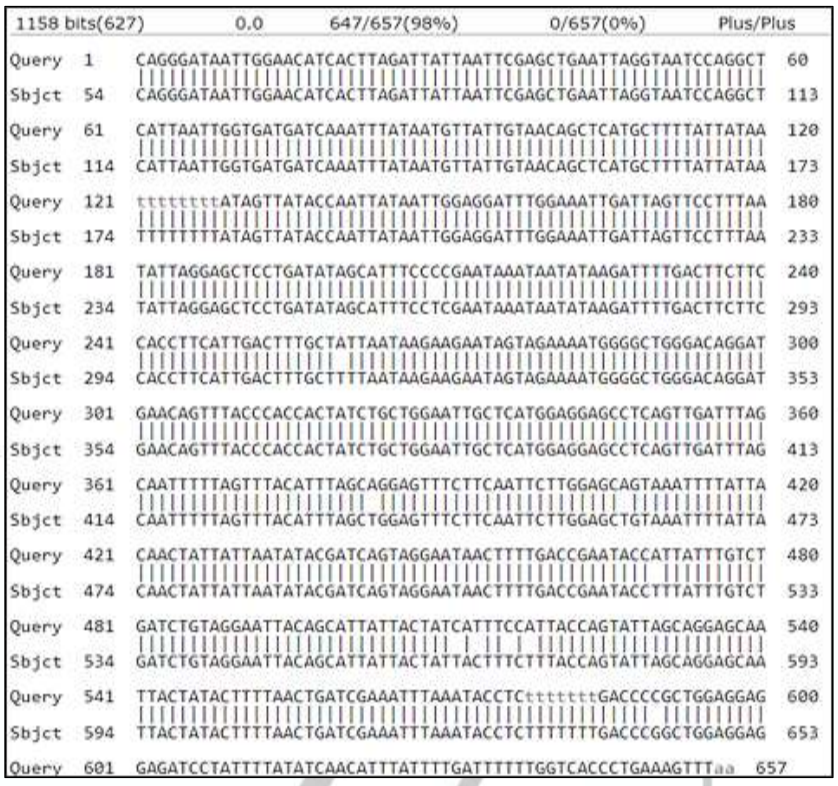

4. Discussion:

The morphological diagnosis of this species is consistent with what was reported by Alak (2017) and Jiang, *et al*. (2023), who stated that the body shape is oval, flattened, elongated, and streamlined. The body length of the adult ranges from 24–28 mm. The dorsal surface of the head, dorsal plate, and sheath are dark green. The legs are large and flattened, with long, special bristles on the hindleg, which aid in swimming. *C. tripunctatus* lives primarily in calm ponds and lakes, as well as slow-flowing freshwater rivers. It prefers habitats rich in aquatic vegetation, which provides cover to protect it from predators and aids in catching its prey. It is typically found in clear or moderately turbid waters with sufficient dissolved oxygen and it avoids fast-flowing waterways or heavily polluted environments (Balke, 2005; Nilsson & Hájek, 2022). *C. tripunctatus* is widespread and has been documented in southern Europe (Spain, Portugal, France, Italy, the Balkans), North Africa (Morocco, Algeria, Tunisia, Egypt), South Asia (India, Pakistan), Central Asia such as Uzbekistan, Kazakhstan (Nilsson & Hájek, 2022), and countries

from the Middle East where it has been recorded in Turkey, the Levant, Iraq, Iran (GBIF.org, 2024). It has also been documented in some coastal areas of the Arabian Peninsula (Balfour-Browne, 1944).

The results of the molecular analysis in the current study showed that the DNA sequence extracted from the sample was 98% identical to a published sequence for a sample from India, which strengthens the reliability of the taxonomic identification based on phenotypic diagnosis. This high congruity indicates that the two samples belong to the same species, with the possibility of minor differences that may reflect geographic variation within the natural range of this species. Molecular analysis is a vital tool for studying insect biodiversity, especially species that are difficult to distinguish based on morphological characteristics alone. Studies have shown that techniques such as DNA sequencing of the cytochrome oxidase subunit 1 gene (the gene barcode) enable researchers to accurately identify species even within groups that are highly morphologically similar. This approach has helped solve many of the problems facing traditional taxonomy. (Hebert *et al*., 2003). Molecular analysis is particularly important when studying aquatic insects such as the Dytiscidae beetles, due to the significant overlap in phenotypic characteristics between species due to adaptation to their surrounding environment, making isolation difficult. Molecular validation helps strengthen taxonomic identification and prevents errors resulting from phenotypic similarities or phenotypic differences resulting from environmental factors. (Hajibabaei *et al*., 2007). Moreover, the use of molecular tools can reveal previously undescribed species (cryptic species), contributing to the enrichment of local and global biodiversity inventories. This advantage has made molecular analysis a pillar of modern systematics research. (Pons *et al*., 2006). Therefore, it has become necessary to support morphological diagnosis with molecular analysis to ensure more accurate and impartial species identification and reduce bias resulting from examiner experience or sample variation. Most recent studies recommend combining morphological and molecular evidence to obtain comprehensive and reliable results. (Schmidt *et al*., 2015).

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