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# Molecular Identification and Phylogenetic-tree Analysis of Hard Ticks from Long Eared Hedgehog *Hemiechinus auritus* (Gmelin, 1770) in Iraq

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**Abstract**

A total of 77 (35 female and 42 male) hard ticks samples were isolated from 22 long-eared hedgehog during January 2021 to May 2022. All animals were infested with one or two species of hard ticks with 100% infestation rate With the density of infestation mean of 3.5. The areas of collection were Baghdad, Wasit, Karbala and Al-Anbar provinces. Morphological study revealed that both species belonged to one genus of hard ticks: *Rhipicephalus sanguineus* (Neumann, 1904) 50.64% and *Rhipicephalus turanicus* (Morel, 1969) 49.35%. The molecular investigation of current study revealed that the sensitive and specific PCR assay allowing rapid and reliable identification of *Rhipicephalus* sp. by the fragment size amplified, was 390-400 bp 12S ribosomal RNA gene in *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* isolate samples from the under-study animals. The accession numbers in NCBI-Genbank are ON211060 and ON211307 respectively.

Phylogenetic tree analysis was based on small subunit ribosomal RNA gene partial sequence in local *Rhipicephalus turanicus* Iraqi isolate that was used for genetic relationship analysis. The local *Rhipicephalus turanicus* Iraqi isolate showed close relationship to NCBI-BLAST *Rhipicephalus turanicus* from Iran, Turkmenistan, Italy, Greece, Saudi Arabia and the Chinese isolates with total genetic changes at 0.01%. Whereas Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local *Rhipicephalus sanguineus* Iraqi isolate showed clear genetic difference to NCBI-BLAST *Rhipicephalus sanguineus* isolates from Argentina, Italy, Brazil, France, Portugal, USA and Spain at total genetic changes of 0.005-0.0020%.

**Keyword:** Hard Ticks, Iraq, Long-eared hedgehog, Molecular, Phylogenetic-tree.

# التحليل الجزيئي وشجرة النشوء والتطور للقراد الصلب من القنفذ طويل الأذن في العراق

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**الخلاصة**

تم عزل 77 عينة (35 أنثى و 42 ذكر) من القراد الصلب من 22 قنفذ طويل الأذن للفترة من شهر كانون الثاني 2021الى شهرايار 2022 . جميع الحيوانات مصابة بنوع او نوعين من القراد الصلب ، وكانت معدلات الإصابة 100٪ وكثافة الإصابة 3.5 في القنفذ طويل الأذن. كشفت الدراسة المظهرية أن نوعين ينتميان إلى جنس واحد من القراد الصلب: *Rhipicephalus sanguineus* 50.64٪ و *Rhipicephalus*

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*turanicus* 49.35 ٪ في القنفذ طويل الأذن. كشفت الدراسة الجزيئية للدراسة الحالية لمقايسة PCR الحساسة والخاصة التي تسمح بتحديد سريع وموثوق لـ .sp *Rhipicephalus* وفقًا لحجم الجزء الذي تم تضخيمه، أن الجين RNA الريبوزومي S12 من 400 −390 زوج قاعدي في عينات *Rhipicephalus turanicus* و *Rhipicephalus sanguineus* من قنفذ طويل الأذن ؛ أرقام الادخال في بنك الجينات هي: ON211060 و ON211307 على التوالي.

تم تحليل شجرة النشوء والتطور على أساس التسلسل الجزئي لجين الحمض النووي الريبي الريبوسومي في العزلة العراقية التي تستخدم لتحليل العلاقة الجينية. تم العثور على عزلة *Rhipicephalus turanicus* العراق المحلية قريبة التطابق مع عزلة *Rhipicephalus turanicus* في إيران،تركمانستان، إيطاليا، اليونان، المملكة العربية السعودية والصين عند إجمالي تغيرات جينية (0.01٪). في حين أن تحليل شجرة النشوء والتطور على أساس التسلسل الجزئي لجين الحمض النووي الريبي الريبوسومي الصغير في عزلة العراق المحلية ل *Rhipicephalus sanguineus* أظهر اختلافًا جينيًا واضحًا عن العزلات لـ*Rhipicephalus sanguineus* في كل من: الأرجنتين،إيطاليا، البرازيل، فرنسا ،البرتغال،الولايات المتحدة الأمريكية وإسبانيا عند إجمالي تغيرات جينية (0.0020−0.005٪).

## Introduction

Long-eared hedgehog *Hemiechinus auritus* (Gmelin, 1770) belongs to the Chordata phylum, Mammalia class, Eulipotyphla order and family Eerinaceidae [1]. The Erinaceidae family contains fifteen genera and species are found in Africa and Asia, including sections of Indo-Malaysia [2]. Hedgehogs are considered useful animals for natural ecosystems and are, therefore, protected because they eat different organisms [3]. These animals can act as a reservoir for zoonotic infections, including acquiring some infections through tick or flea bites. They play a role in the endemic cycle of these pathogens. Hedgehogs are recognized to be a possible host for several *Anaplasma phagocytophilum* types, as well as tick–borne encephalitis virus (TBEV) [4]. Hedgehogs have been increasingly popular among pet owners in recent years, while offering both benefits and risks to their owners. Hedgehog ectoparasites have been the subject of some research [5].

The aim of this study was to shed light on the biological diversity of the hard tick isolated from wild animals (long-eared hedgehog) and its direct relationship with the spread of these species in the Iraqi environment and other related diseases.

## Materials and Methods

**Collection of Samples:** A total of 77 (35 female and 42 male) hard tick samples were isolated from 22 long-eared hedgehog *Hemiechinus auritus* (Gmelin, 1770) that were trapped and examined to detect the hard ticks during January 2021 to May 2022. The samples were collected from Baghdad, Wasit, Karbala and Al-Anbar provinces.

The subject animals were infested with hard ticks in the ears, femoral and udders. All collected hard ticks were kept in 70% alcohol for diagnosis in the Iraq Natural History Research Center and Museum (INHM) by dissecting microscopically and were later on photographed by digital camera. The taxonomy and scientific nomenclature were confirmed by key [6, 7].

### PCR Technique

PCR technique was performed for the detection of *Rhipicephalus* sp. based small subunit ribosomal RNA mitochondrial gene. This method was carried out according to method described by [8] as following steps:

**Tissue DNA Extraction:** Genomic DNA from insect tissue samples were extracted by using gSYAN DNA mini kit extraction kit (Tissue protocol) Geneaid. USA, and according to company instructions.

**Genomic DNA Estimation**: The extracted genomic DNA was tested using a nanodrop spectrophotometer (THERMO. USA) which checks and measures DNA purity by reading the absorbance at 260 /280 nm.

**Primers**

Small subunit ribosomal RNA mitochondrial gene PCR primers were based on *Rhipicephalus* sp. Table 1 shows the primers provided by the Macrogen company in Korea [8].

**Table 1:** Primers used in this study with their sequence and PCR size of *Rhipicephalus* sp

| Primers | Sequence (5'-3') | | PCR Product Size |
|---|---|---|---|
| 12S ribosomal RNA gene | F | AAACTAGGATTAGATACCCTATTATTTTAG | 390- 400 bp |
| | R | CTATGTAACGACTTATCTTAATAAAGAGTG | |

**PCR Master Mix Preparation**

The PCR master mix was made with (Maxime PCR PreMix Kit) by following the company's instructions as shown in Table 2.

**Table 2:** PCR Master Mix (Standard).

| Master Mix of PCR | Volume |
|---|---|
| Template DNA (5-50 ng) | 5µL |
| Forward of Primer 10pmol | 1µL |
| Reverse of Primer 10pmol | 1µL |
| (PCR water) | 13 µL |
| Total Volume | 20µL |

Following that, these PCR master mix components listed in the table above were placed in a standard Maxime PCR PreMix that contained all other PCR reaction components, such as Taq DNA polymerase, pH: 9.0, KCl, Tris-HCl, dNTPs, MgCl2, stabilizer and tracking dye. The PCR tubes were then placed in an ExiSpin vortex centrifuge and spun at 3000rpm for 3 minutes. The samples were then placed in a PCR thermocycle, (Table 3).

**Table 3:** the PCR program

| Step of PCR | Temperature. | Time | Repeat. |
|---|---|---|---|
| Initial of Denaturation | 95ºC | 5min. | 1 |
| Denaturation | 95 ºC | 30 sec. | 35 cycles |
| Annealing | 58 ºC | 30 sec | |
| Extension | 72 ºC | 2 min. | |
| Final of Extension | 72 ºC | 5 min. | 1 |
| Hold | 4 ºC | Forever | - |

**Analysis of PCR Products**

1.5% agarose gel was prepared by dissolving 1X TBE in a water bath at 100°C for 15 minutes, then cooling to 50°C. 3µl of ethidium bromide stain was added to the agarose gel solution in increments. After placing the comb in the proper position, the agarose gel solution was poured into a tray and allowed to solidify for 15 minutes at room temperature before gently removing the comb from the tray.

The gel tray was then placed in the electrophoresis chamber before adding 1X TBE buffer. Then 10 µl of PCR product was added into each comb well and 3µl of (100bp Ladder) in first well. Later 100 volt and (80 mA) electric current was run for 1.5 hour to visualize PCR products by using UV Transilluminator.

The PCR products of the small subunit ribosomal RNA mitochondrial gene were sent to Macrogen Company in Korea in for DNA sequencing of Iraqi isolates of *R. turanicus* and *R. sanguineus*.

**Sequencing DNA and Phylogenetic Analysis**

Multiple sequence alignment analysis based on ClustalW alignment analysis and Molecular Evolutionary Genetics Analysis version 6.0 (Mega 6.0), were used in the DNA sequencing analysis. The evolutionary distances were calculated using the phylogenetic tree UPGMA method and the Maximum Composite Likelihood method

.

Using NCBI BLAST, homology sequence identity and mutation analysis were determined. Finally, local isolates were submitted to the NCBI-Genbank database for the purpose of obtaining Genbank accession numbers

.

**Results and Discussion**

A total of 77 (35 female &42 male) hard ticks samples were isolated from 22 long-eared hedgehog that were infested with one or two species of hard ticks. Infestation rate was 100% and the density was 3.5 in long-eared hedgehog. (Table 4).

**Table 4:** Rates and the density of infestation hard ticks long-eared hedgehog in Iraq

| Host | No. of Exam Samples | No. of Infest Samples | % | No. of ticks | Infestation density |
|---|---|---|---|---|---|
| **Long-eared Hedgehog** | 22 | 22 | 100 | 77 | 3. 5 |

**Morphology Study**

The results of current study revealed that two of the species belonged to one genus of hard ticks: *Rhipicephalus sanguineus* (Neumann, 1904) 50.64%, and *Rhipicephalus turanicus* (Morel, 1969) 49.35%, as shown in Table 5 and Figures 1 &2.

**Table 5:** Distribution of hard tick species in long-eared hedgehog (22 samples) in Iraq

| Species | ♂ | ♀ | Total | % |
|---|---|---|---|---|
| ***R. turanicus*** | 23 | 15 | 38 | 49.35 |
| ***R. sanguineus*** | 19 | 20 | 39 | 50.64 |
| **Total** | 42 | 35 | 77 | 100 |

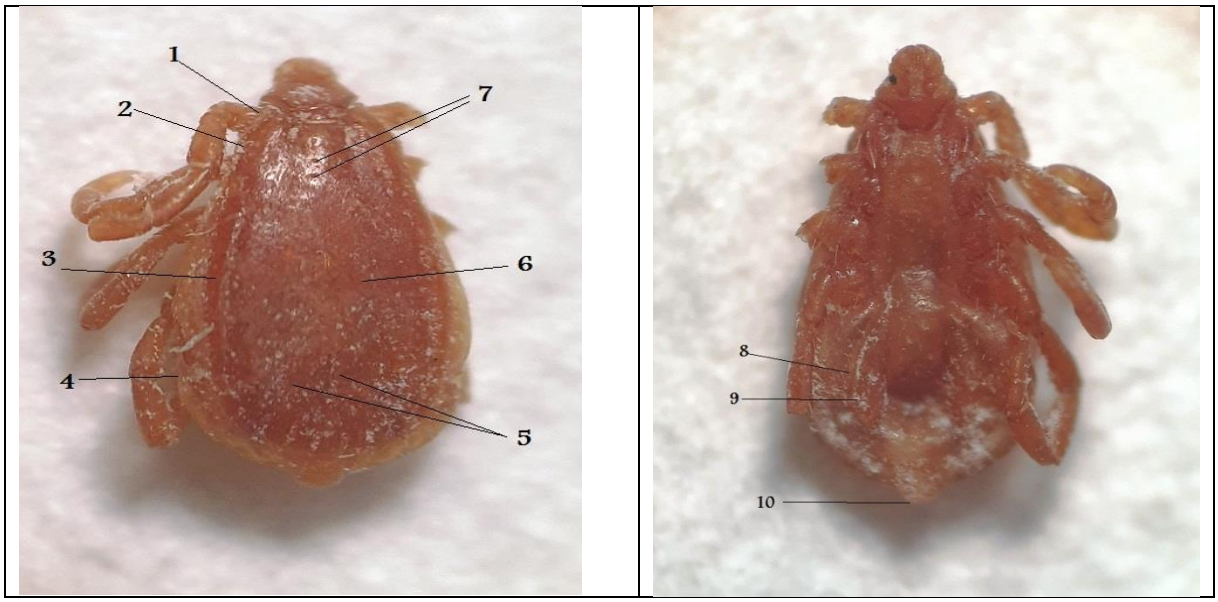


**Figure 1:** Dorsal and ventral side of *Rhipicephalus turanicus* male in long eared hedgehog in Iraq.

1. The anterior spurs of coxa are not visible dorsally. 2. The eyes are completely flat. 3. A lateral groove is a unique groove. The texture of the lateral grooves is smooth. 4. Setae are sparse in spiracle areas. The tails of the spiral plate are broad, the same width as the neighboring festoon.) 5. The grooves in the back are distinct (deep depressions with wrinkled texture). 6. Conscutum has a dark color. 7. The interstitial punctuation is tiny to medium and the spread of interstitial punctuation is sparse. 8. Adjacent adanal plates are quite large. 9. The form of the adanal plates is narrow and trapezoid. 10. In fed males, the caudal appendage is wide and protruding as a distinct bulge, as shown in dorsal and ventral view.

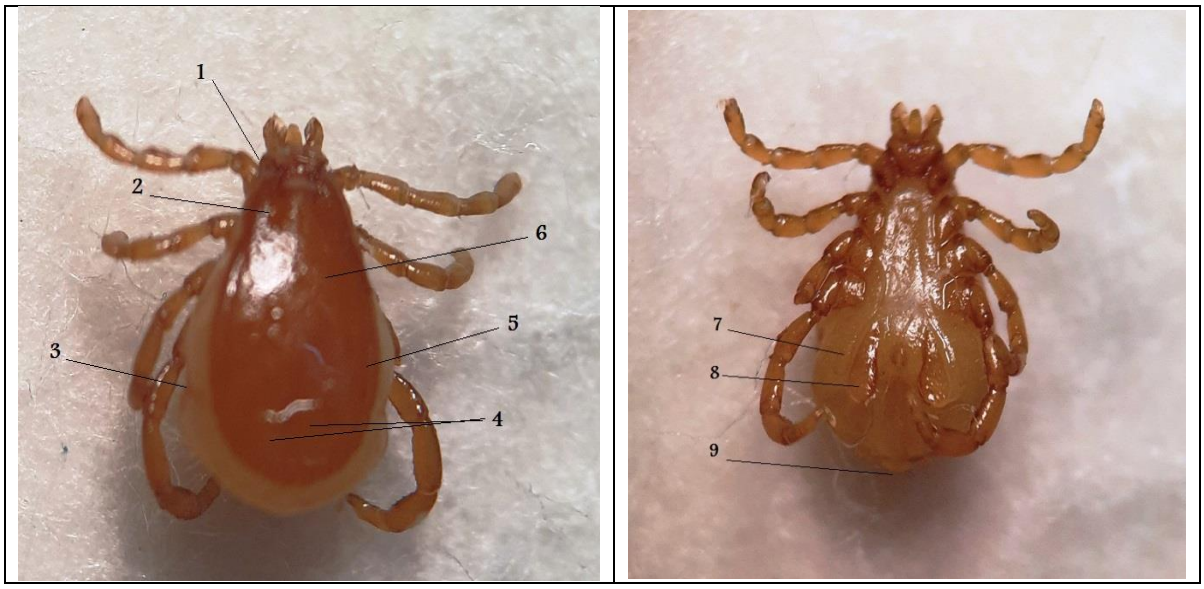


**Figure 2:** Dorsal and ventral side of *Rhipicephalus sanguineus* male in long eared hedgehog in Iraq.

1. The anterior spurs of coxa 1 are not visible dorsally. 2. Indistinct setiferous punctuations. 3.Setae are sparse in spiracle areas. 4. The grooves in the back are distinct (deep and wide with

wrinkled texture). 5. The type of lateral grooves is distinct with smooth texture . 6. The hue of conscutum is light. 7. Adanal accessory plates are big. 8. The form of the adanal plates is narrow and trapezoid but tend to appear broad and curved. 9. No information on the caudal appendage in fed males.

The current study recorded two species of hard ticks in Iraq; *Rhipicephalus turanicus* was found in long-eared hedgehogs, which agrees with the findings reported in [9, 10, 11, 12] in Iraq. And it has been documented in Iran by several researches [13, 14, 15]. While, Desoky [16] found it in the same animals in Egypt.

Long-eared hedgehog has recorded to be the host for the species *Rhipicephalus sanguineus* which agrees with [17] in Ninevah, Iraq. This record adds to the hard tick infestation in both domestic and wild animals, thus agreeing with [18] who recorded it in Saudi Arabia in the same animals.

**Molecular Study**:

The current study revealed a sensitive and specific PCR assay allowing rapid and reliable identification of *Rhipicephalus* sp. by the fragment size amplified that was 390 - 400 bp 12S ribosomal RNA gene in *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* isolate samples from long-eared hedgehogs, as shown in Figure 3. Multiple sequence alignment of the small subunit ribosomal RNA gene in a local *R. turanicus* Iraqi isolate was inserted in NCBI-Genbank with Accession Number: ON211060.

The multiple alignment analysis created using the ClustalW alignment tool in MEGA 6.0 (Diagram 1). Multiple sequence alignment of the small subunit ribosomal RNA gene in a local *R. sanguineus* Iraqi isolate was inserted in NCBI-Genbank with accession number :ON211307. The multiple alignment analysis was constructed using Clustal Walignment tool in MEGA 6.0 version as shown in Figure 3.

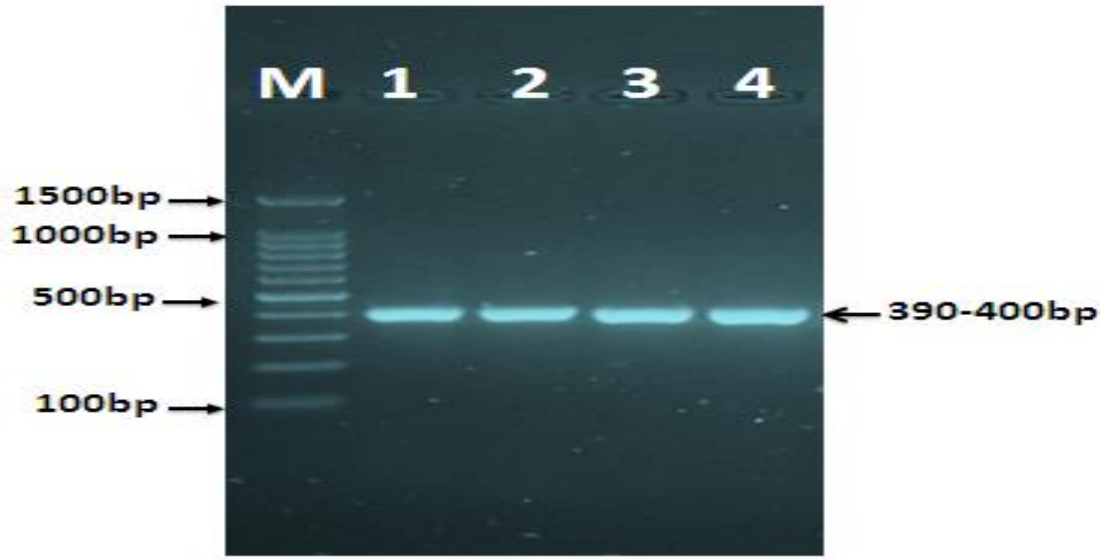


**Figure 3:** The PCR product analysis of the 12S ribosomal RNA gene in *R. turanicus* and *R. sanguineus* isolate samples are visible on an agarose gel electrophoresis image. M stands for marker DNA ladder (1500-100bp). At (400bp) PCR product size, lane (1-2) duplicate positive DNA for *R. turanicus* isolation sample. At (390bp) PCR product size, lane (3-4) triplicate positive DNA for *R. sanguineus* isolation sample.

**Analysis of Phylogenetic Trees**

The genetic relationship analysis was performed using a phylogenetic tree based on the partial sequencing of the small subunit ribosomal RNA gene in a local *R. turanicus* Iraq isolate. The unweighted pair group method with arithmetic mean (UPGMA tree) was used to create the phylogenetic tree (MEGA 6.0 version). *R. turanicus* isolates from Iraq were shown to be closely related to NCBI-BLAST *R. turanicus* isolates from Iranian, Turkmenistani, Italian, Greek and Saudi Arabian, and Chinese isolates at total genetic changes (0.01%) (Figure 4 and Table 6).

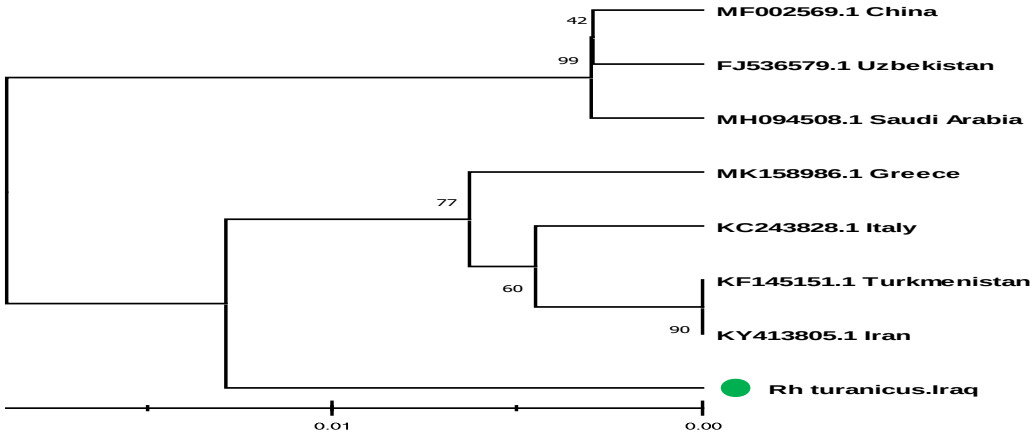


**Figure 4:** The genetic relationship analysis was performed using a phylogenetic tree based on the partial sequencing of the small subunit ribosomal RNA gene in a local *R. turanicus* Iraqi isolate. The unweighted pair group method with arithmetic aean (UPGMA tree) was used to create the phylogenetic tree (MEGA 6.0 version). At total genetic alterations, the local *R. turanicus* Iraqi isolate was shown to be closely linked to NCBI-BLAST *R. turanicus* Iranian, Turkmenistani, Italian and Greek isolates (0.01 percent).

**Table 6:** Shows the percentage identity of NCBI-BLAST homology sequences between local *R. turanicus* Iraqi isolates and NCBI-BLAST submitted nation related *R. turanicus* isolates.

| NCBI-*Rhipicephalus turanicus* isolate | Genbank Accession number | NCBI-BLAST Homology Sequence identity (%) |
|---|---|---|
| *R. turanicus* (Iran isolate) | KY413805.1 | 99.16% |
| *R. turanicus* (Turkmenistan isolate) | KF145151.1 | 99.13% |
| *R. turanicus* (Italy isolate) | KC243828.1 | 99.11% |
| *R. turanicus* (Greece isolate) | MK158986.1 | 99.09% |
| *R. turanicus* (Saudi Arabian Isolate) | MH094508.1 | 98.82% |
| *R. turanicus* (Uzbekistani Isolate) | FJ536579.1 | 98.60% |
| *R. turanicus* (Chinese Isolate) | MF002569.1 | 98.54% |

Phylogenetic tree analysis was based on the partial sequence of the small subunit ribosomal RNA gene in the native *Rhipicephalus sanguineus* for genetic related analysis. Phylogenetic trees were constructed using the unweighted pair method of arithmetic mean (UPGMA tree) in MEGA version 6.0. Local *R. sanguineus* Iraqi isolates showed significant genetic differences from NCBI-BLAST *R. sanguineus* national isolates: overall genetic variation (0.005-0.0020%) in Argentina, Italy, Brazil, France, Portugal, USA and Spain (Figure 5 and Table 7).

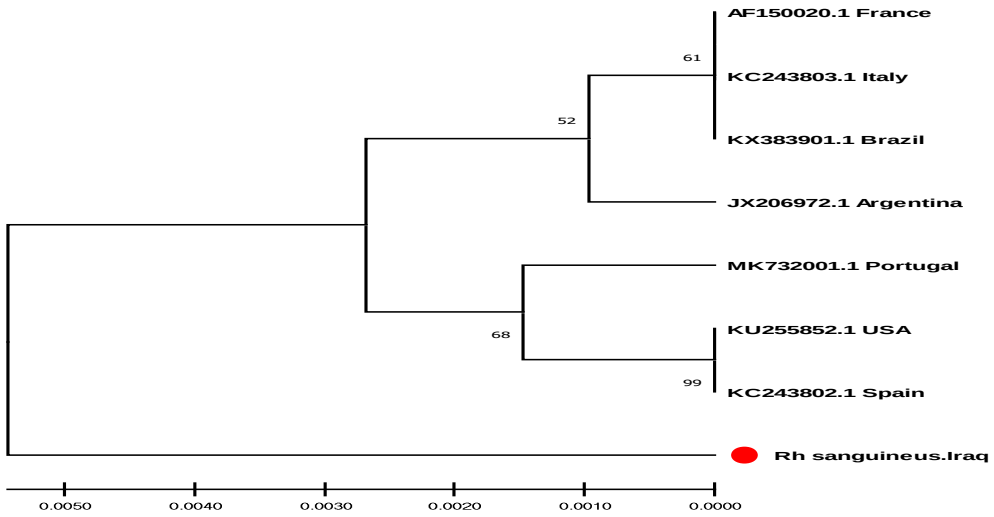


**Figure 5:** The phylogenetic tree analysis was based on the partial sequence of the small subunit ribosomal RNA gene in the native *R. sanguineus* for genetic relatedness analysis. Phylogenetic trees were constructed using the unweighted pair method of arithmetic mean (UPGMA tree) in MEGA version 6.0. The local isolates *R. sanguineus* from Iraq and the NCBI-BLAST *Rhipicephalus* country isolates exhibited significant genetic differences (0.005-0.0020%) in overall genetic variation.

**Table 7:** The NCBI- BLAST Homology Sequence identity (%) between original *R. sanguineus* Iraqi insulate and NCBI- BLAST submitted country related *R. sanguineus* isolates.

| NCBI-*Rhipicephalus sanguineus* Isolate | Genbank Accession Number | NCBI-BLAST Homology Sequence Identity (%) |
|---|---|---|
| ***R. sanguineus* (Argentinan Isolate)** | JX206972.1 | 98.21% |
| ***R. sanguineus* (Italian Isolate)** | KC243803.1 | 97.67% |
| ***R. sanguineus* (Brazilian Isolate)** | KX383901.1 | 97.38% |
| ***R. sanguineus* (French Isolate)** | AF150020.1 | 98.17% |
| ***R. sanguineus* (Portugal isolate)** | MK732001.1 | 97.00% |
| ***R. sanguineus* (USA Isolate)** | KU255852.1 | 97.01% |
| ***R. sanguineus* (Spanish Isolate)** | KC243802.1 | 97.72% |

## Conclusion

The current study aimed to shed light on the biological diversity of the hard tick isolated from wild animals (long-eared hedgehog). All the collected animals were infested with one or two species of hard ticks.

The morphological and the molecular study revealed two species that belonged to one genus of hard ticks: *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* in the long-eared hedgehog.

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