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Morphological and molecular studies on tick species in Ismailia Governorate in Egypt and Al Gabal Al Akhdar in Libya

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Abstract

Background: Ticks are obligate blood-sucking ectoparasites of vertebrates that have an impact on both domestic and wild animals, especially in tropical and subtropical areas.

Aim: The objective of this study is to compare the prevalence and the tick species in both Al Gabal Al Akhder regions in northeastern Libya and Ismailia, Egypt.

Methods: Tick specimens collected from predilection sites on the hosts were identified by morphological (light microscopy) and molecular methods.

Results: In Ismailia, Egypt, 23.9% of the 230 (examined cattle and buffaloes) were infested with one species of hard ticks, *Rhipicephalus annulatus*. In the Libyan province of Al Gabal Al Akhdar, the prevalence of tick infestation in cattle, sheep, and goats, was 47% and 59%, respectively. *R. annulatus* is the identified tick species for cattle, *Hyalomma marginatum*, and *Rhipicephalus bursa*, are the identified tick species of the infested sheep and goats.

Conclusion: The 16S rDNA gene sequencing and phylogenetic analysis of sample species from Egypt and Libya proved instrumental in overcoming the difficulties associated with morphological identification techniques.

Keywords: Molecular, Microscopic, *Rhipicephalus annulatus*, *Hyalomma marginatum*, *Rhipicephalus bursa*.

Introduction

Ticks are obligate blood-sucking ectoparasites of vertebrates and have an impact on both domestic and wild animals, especially in tropical and subtropical areas (Abouelhassan *et al.*, 2019). Currently, more than 900 tick species have been described worldwide and have been divided into four important families such as Argasidae, Ixodidae, Nuttalliellidae, and Deinocrotonidae. Of these, around 700 tick species belong to the family *Ixodidae*, having several genera, for instance, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus,* which are responsible for the transmission of several pathogens (Dantas-Torres, 2018). Ticks can cause nonspecific symptoms like anemia, dermatosis, toxicosis, and paralysis, besides being important vectors for diseases such as theileriosis, anaplasmosis, babesiosis, and rickettsiosis in domestic animals (Gebre *et al.*, 2001). Haemoprotozoans, especially *Babesia*, *Theileria*, and *Trypanosomes,* have major effects on the health and production of bovines (Rajput *et al.*, 2005). These protozoa can cause substantial losses to the livestock industry throughout the world because of mortality, decreased productivity, lowered working efficiency (Uilenberg, 1995), and increased costs for control measures (Makala *et al.*, 2003).

In South Africa, the genera *Hyalomma, Boophilus*, and *Rhipicephalus* comprise the most important ixodid ticks infesting animals, specifically *Hy. anatolicum excavatum, Hy. dromedarii*, *Hy. impeltatum, Hy. marginatum marginatum*, *B. annulatus*, and *Rhipicephalus sanguineus* (Marufu, 2008). Morphological identification of tick species is not sufficient and molecular identification is needed for a correct species determination (Abouelhassan *et al.*, 2019). The objective of this study is to compare the prevalence of tick species in both the Al Gabal Al Akhder region in northeastern Libya and the Ismailia governorate.

Material and Methods

Animals and study area

A total of 440 individual tick specimens were collected in this study. Samples were collected randomly from 1,350 apparently healthy animals (cattle, buffaloes, sheep, and goats) from different localities in Egypt and Libya (Fig. 1) during the period extending from January 2020 to 2021 (Table 1).

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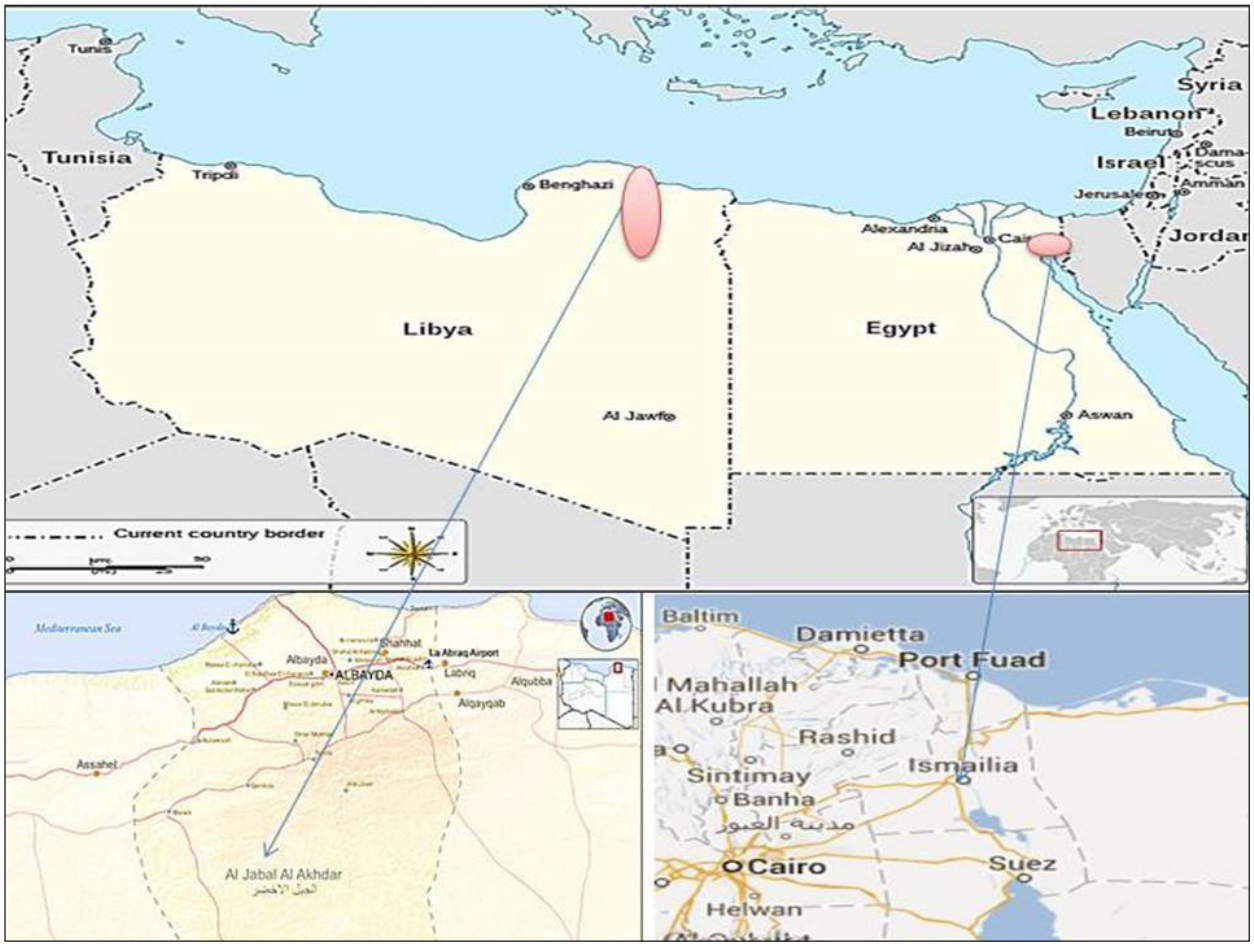

Fig. 1. Geographical map for Ismailia governorate, Egypt, and Al Gabal Al Akhdar in Libya.

Table 1. Tick specimens collected from examined animals.

Al Gabal Al Akhdar-Libya					Ismailia-Egypt		
Locality	Cattle		Sheep/Goat		Cattle/buffalo		
	No. of examined animals	No. of collected tick specimens	No. of examined animals	No. of collected tick specimens	Locality	No. of examined animals	No. of collected tick specimens
Al-Bayda	40	35	270	45	Kasassin	30	30
Shahat	30	20	180	26	Elabtal	55	20
Faydyah	20	24	150	22	Kantara	50	35
Massah	33	30	250	47	Abu atwa	60	25
Gandula	17	17	130	34	Sarabiom	35	30
Total	140	126	980	174	Total	230	140

Tick collection

Before the collection of tick samples, animals were restrained properly and their whole body was thoroughly inspected visually for the presence of ticks. Tick specimens were taken from predilection sites of the hosts, such as the ears, udder, and around the anus. The ticks collected from these sites with the help of forceps, and were transferred to bottles (containing either ethyl alcohol 70% and 10% glycerin or 70% alcohol only) and shifted to the laboratory for permanent slide preparation and identification based on the characters of the basis-capitulum, pedipalps, presence or absence of festoons, eyes, anal groove, adenal shields, coxa-1, coxa-IV, accessory adenal shields, and designs of colors present on scutum (Walker, 2003).

Table 2. Prevalence of tick infestation on cattle and buffaloes at different localities in Ismailia, Egypt.

Locality	No. of examined animals	No. of infested animals	(%)*	Tick specimens collected
Kasassin	30	12	40	30
Elabtal	55	10	18	20
Kantara	50	11	22	35
Abu atwa	60	15	25	25
Sarabiom	35	7	20	30
Total	230	55	23.9	140

Total: chi-square (χ^2) = 236.719[a]; DF = 10; *p*–value = 0.000 (highly significant).

Morphological identification

The method described by Farid *et al*. (2021) was adopted for the preparation of the tick specimens for light microscopy. Tick specimens preserved in an alcohol–glycerol mixture were taken and manually teased by forceps to remove any adherent host tissues. Blood from ticks was removed using a needle syringe. To dissolve more chitinous and other undesirable materials, the specimen was treated with 10% sodium hydroxide. After cleaning, the specimen was washed several times with water and subsequently dehydrated in a serial dilution of alcohol (25%, 50%, 75%, and 100% ethyl alcohol). To ensure better clearance, the specimen was kept in xylene for 15–30 minutes. The specimen was mounted on a glass slide using Canada balsam and covered by a cover slip, left to be dried in a hot oven at 40°C–50°C, and later examined under a light microscope (10× magnification). The specimens were morphologically identified according to Walker (2003).

Molecular identification of ticks

DNA extraction

The tick specimens preserved in 70% ethanol were processed for extraction of DNA. DNA was extracted utilizing QIAamp DNA Mini Kit (Qiagen), as per the protocol laid down by the manufacturer. Genomic DNA was stored at −20°C until use.

Molecular identification of tick samples based on both 16S rDNA genes

The tick 16S rDNA was amplified from each specimen studied using conventional PCR methodologies and primers 5′-TTGGGCAAGAAGACCCTATGAA-3′ and 5′-CCGGGTTTGAACTCAGATCAAGT-3′, previously described by Black and Piesman (1994). The thermo-cycling cycling conditions were: initial denaturation at 95°C for 5 minutes. followed by 10 cycles of 92°C for 1 minute, 48°C for 1 minute, and 72°C for 90 seconds. This step was followed by an additional 32 cycles of 92°C for 1 minute, 54°C for 35 seconds, and 72°C for 90 seconds., this was followed by a final extension at 72°C for 7 minutes. The amplification products from 16S rDNA PCR were made to electrophoretically migrate on a 1.6% agarose gel containing 0.4 µg/ml of ethidium bromide at 90 volts for 40–60 minutes.

Sequence analysis

The amplified PCR products were excised from the gel, purified, and sent for sequencing. Sanger sequencing was performed by Solgent Co. Ltd (South Korea). Sequences were then analyzed using BLAST® (Johnson *et al.*, 2008).

Phylogenetic analysis

Phylogenetic analysis and estimates of evolutionary divergence between sequences were performed based on the 16S rDNA gene sequences of several closely related tick species. They were constructed in MEGAX (Kumar *et al.*, 2018).

Ethical approval

This study was approved by the Ethics Committee of the Suez Canal University. All animal experiments were conducted following the guidelines of the Guide for the Care and Use of Laboratory Animals, Faculty of Veterinary Medicine Science, Suez Canal University, Egypt with approval number (2018123).

Results

Prevalence of tick infestation

In Ismailia, Egypt, 23.9% of the 230 animals (cattle and buffaloes) examined for tick infestations had one species of hard tick, *Rhipicephalus annulatus*. Table 2 shows the prevalence of *R. annulatus* infection in the studied animals across different locations in Ismailia province**.** In the Libyan province of Al Gabal Al Akhdar, the prevalence of tick infestation in cattle, both sheep and goats, was 47% (66/140) and 59% (580/980), respectively. *Rhipicephalus annulatus* is the identified tick species for cattle. *Hyalomma marginatum* and *Rhipicephalus bursa* are the identified tick species for the infested sheep and goats. Table 3 shows the percentage of animals with tick infection among those investigated at various locations throughout Libya's Al Gabal Al Akhdar province.

The morphology of the collected tick species using a light microscope

Hyalomma marginatum rufipes (Koch, 1844): the scutum color was dark (Fig. 1), the male had adenal and accessory adenal shields (Fig. 2), and the basis

Table 3. Prevalence of tick infestation on animals across different localities of Al Gabal Al Akhdar, Libya.

Locality	Cattle				Sheep/Goat			
	No. of examined animals	No. of infested animals	%	Tick specimens collected	No. of examined animals	No. of infested animals	%	Tick specimens collected
Al- Bayda	40	12	30	35	270	150	56	45
Shahat	30	15	50	20	180	33	19	26
Faydyah	20	18	90	24	150	100	67	22
Massah	33	20	61	30	250	185	74	47
Gandula	17	09	53	17	130	110	85	34
Total	140	66	47	126	980	580	59	174

Total: chi-square (χ^2) = 1,162.719; DF = 10; *p*–value = 0.000 (highly significant).

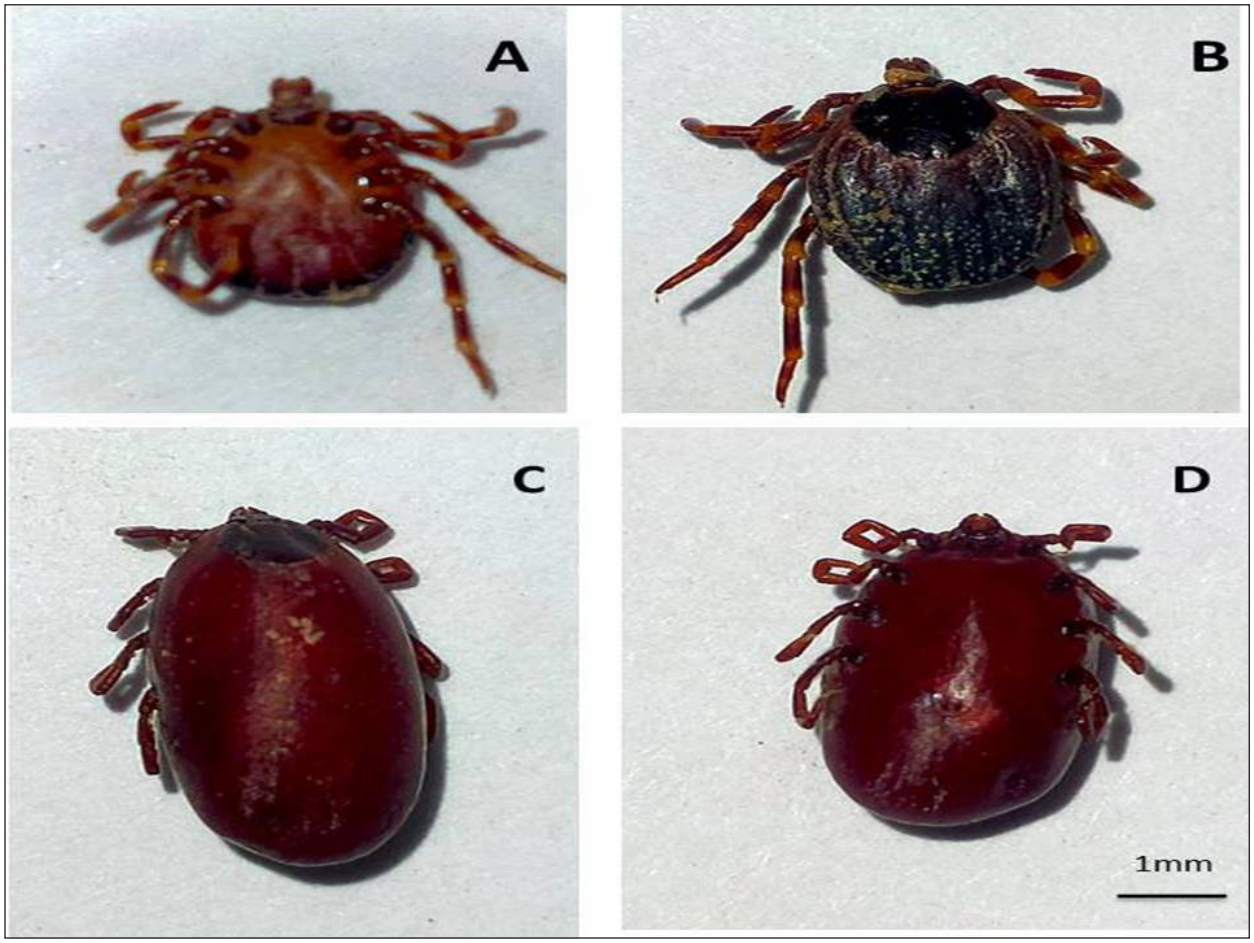

Fig. 2. (A): Ventral view of *H. marginatum* male. (B): Dorsal view of *H. marginatum*, male. (C): Dorsal view of *H. marginatum*, engorged female. (D): Ventral view of *H. marginatum*, engorged female.

capitulum had medium angular lateral margins. Palp articles 2 were longer than articles 1 and 3 (Fig. 3), and festoons were absent in the male and un-engorged female (Fig. 3). The spiracular plate was large and posterior to the legs (Fig. 4). The genital aperture anterior groove was deep, and the genital aperture posterior lips had a V shape (Fig. 2).

Rhipicephalus annulatus (Say, 1821): was characterized by a weakly indistinct anal groove. The male had adenal and accessory adenal shields without a caudal process (Fig. 4). The basis capitulum was hexagonal in shape and laterally produced. Palpi were very short, consisting of four segments. Mouth parts were as long as the basis capitulum. The second segment of the

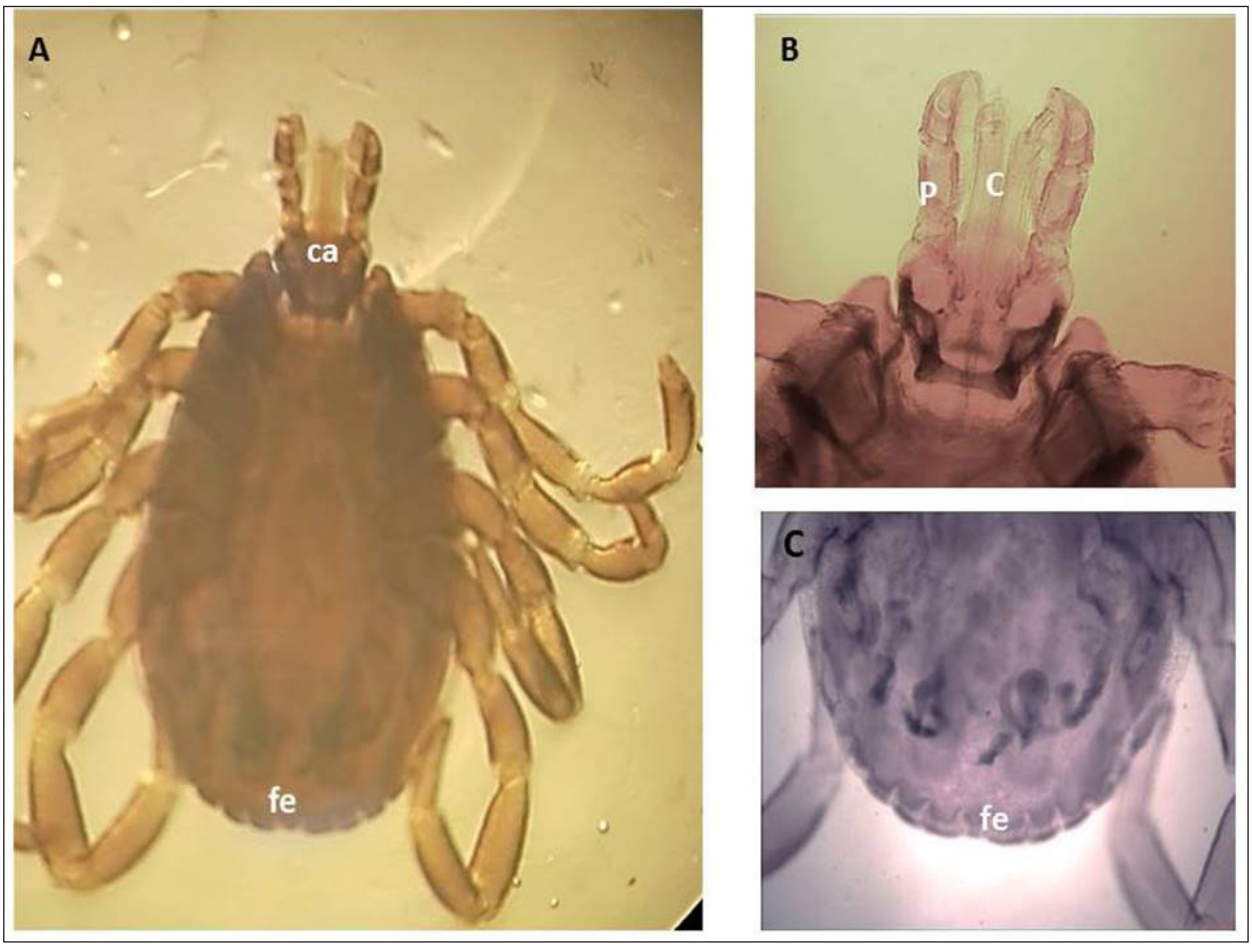

Fig. 3. LM of permanent slide of *H. marginatum*, male. (A): Dorsal view. (B): Anterior part. (C): Posterior part. (Sp): Spicular plate; (fe): festoon; (ca): capituli; (c): Chelicera; (P): palpai.

palpi was as long as it was wide and was not produced laterally. Caudal appendages and festoons were absent (Fig. 4). The spiracular plate had an oval shape and was made up of stigma and peritreme. Coxa 2, 3, and 4 had no external spurs, while coxa 1 was broad with internal and external spurs. The genital aperture posterior lips had a broad U shape (Fig. 5).

Rhipicephalus bursa **(**Canestrini and Fanzago, 1878): was characterized by dark-colored scutum (Fig. 6). The male had broad adenal and small accessory shields (Fig. 6). The basis capitulum lateral angles were sharply produced. Palp pedicles were short and festoons were present (Fig. 6). The spiracular plate was comma shaped and consisted of a stigma, peritreme, and a tail. In Coxa 1 anterior spurs were visible dorsally, and the genital aperture posterior lips had a narrow V shape (Fig. 7).

Molecular identification of tick species

A total of 100 tick samples were analyzed (Fig. 8) which were collected from Egypt and Libya. They were identified based on the16S rDNA PCR products as *H. marginatum*, *R. bursa*, and *R. annulatus*, the GenBank accession numbers are OM661198, OM661199, OM661200, and OM661201.

Phylogenetic analysis

The phylogenetic analysis based on the 16S rDNA gene was performed using MEGA X10.1 software and the trees were constructed using neighbor-joining (NJ) methods (Fig. 9). There is a low degree of sequence variations was observed within most of the species of *R. annulatus* from Egypt, Sudan, and Cameroon since they all clustered into one original branch, and low variation between the present *R. annulatus* samples from Egypt (Fig.10).

While *H. marginatum* sequence of our study had a close relationship and clustered with the same species from Turkey (Figs. 9 and 10).

Discussion

In Ismailia, Egypt, the overall prevalence of tick infestation was 23.9% in cattle and buffaloes, whereas in Al Gabal Al Akhdar, Libya, the overall prevalence of tick infestation was 47% in cattle, sheep, and goats. The difference in tick distribution may be attributed to the difference in environmental conditions, host density, host susceptibility, grazing habits, and pasture herd management (Pegram *et al.*, 1981).

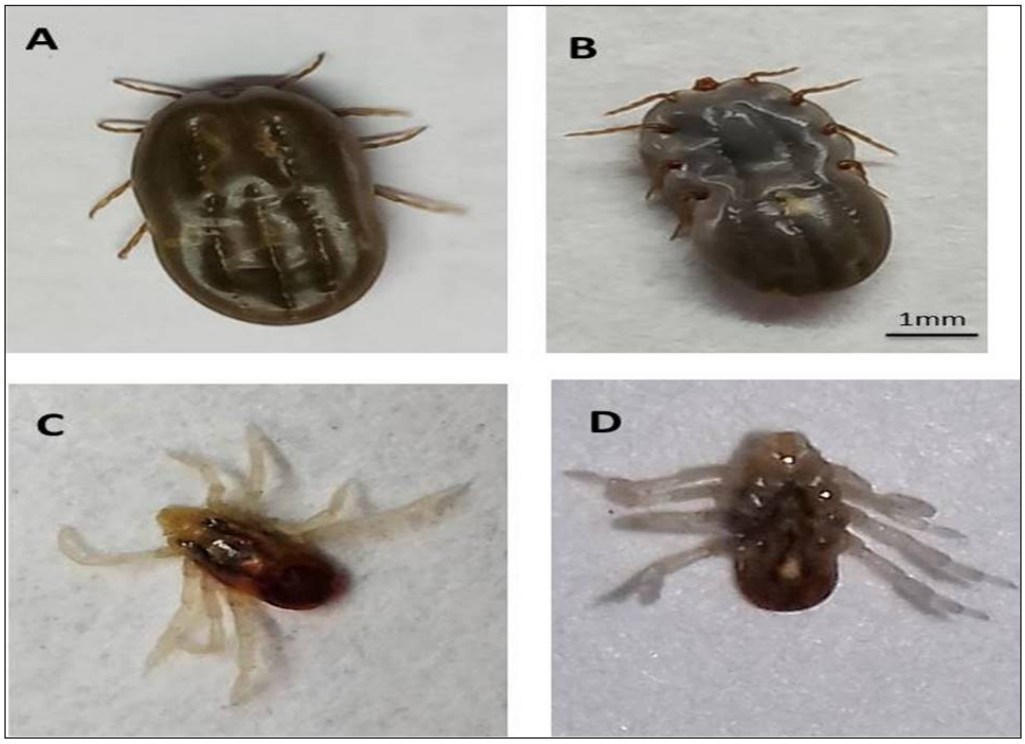

Fig. 4. (A): Dorsal view of *R. annulatus*, engorged female. (B): Ventral view of *R. annulatus*, engorged female. (C): Dorsal view of *R. annulatus*, male. (D): Ventral view of *R. annulatus*, male.

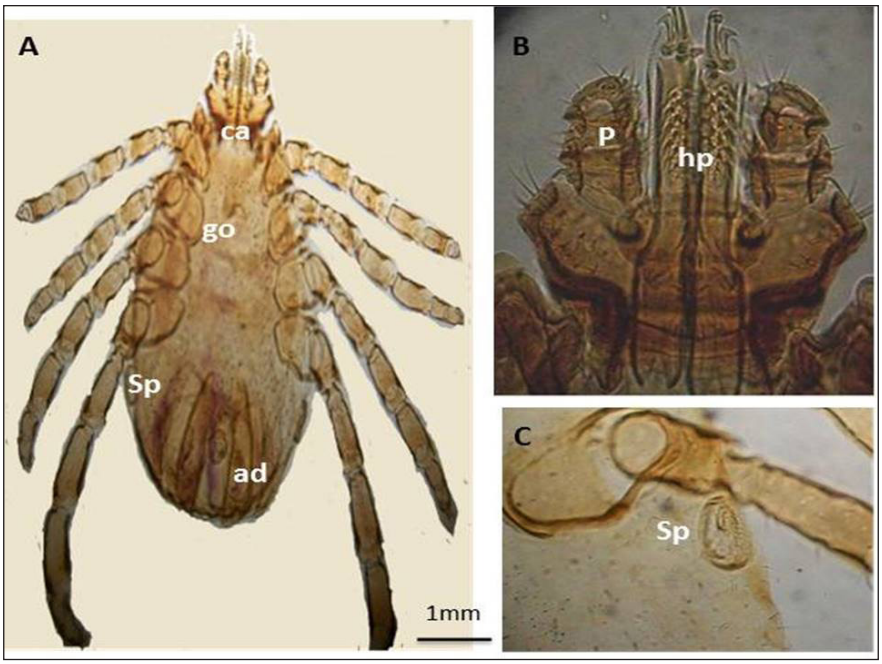

Fig. 5. LM of permanent slide of *R. annulatus* male. (A): Dorsal view. (B): Anterior part. (C): Specular plate. (Sp): Spicular plate; (go): genital opening; (ca): capituli; (hp): hypostome; (P): palpai; (ad): adenal shield.

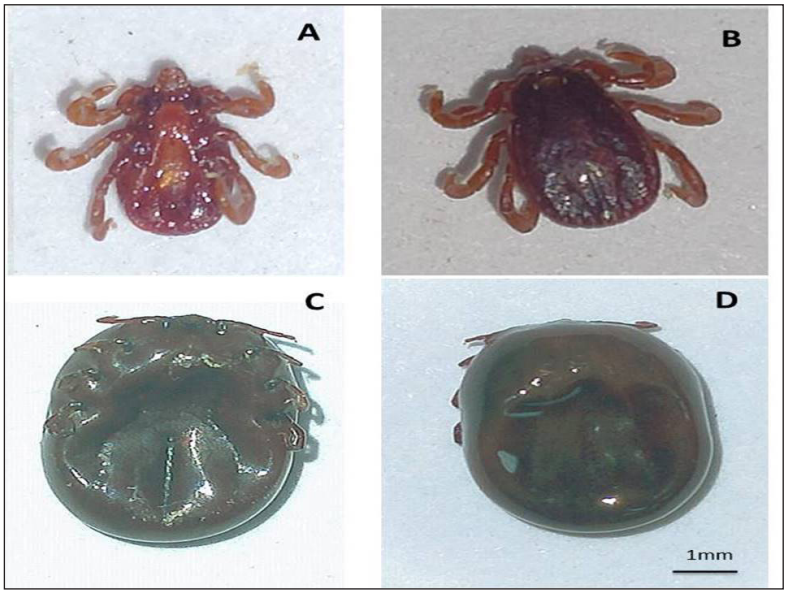

Fig. 6. (A): Ventral view of *R. bursa*, male. (B): Dorsal view of *R. bursa*, male. (C): Ventral view of *R. bursa*, engorged female. (D): Dorsal view of *R. bursa*, engorged female.

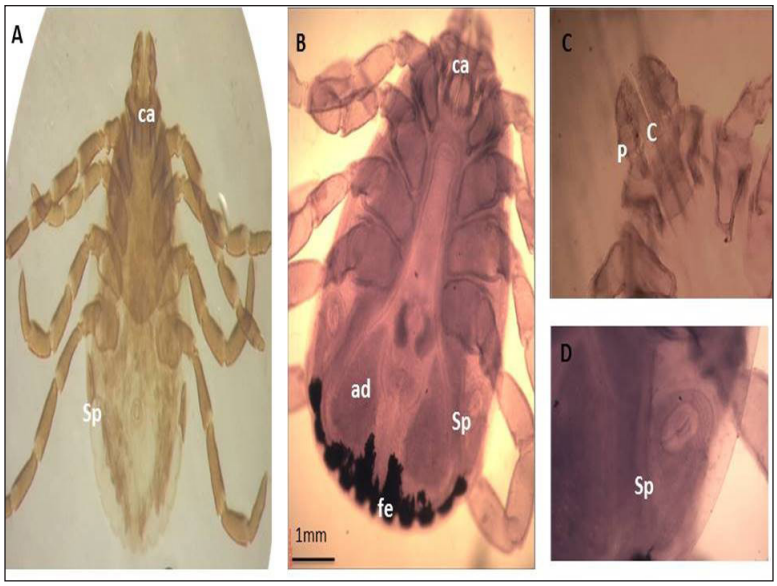

Fig. 7. LM of permanent slide. (A): Ventral view of *R. bursa* female. (B): Ventral view of *R. bursa*, male. (C): Anterior part. (D): Specular plate. (Sp): Spicular plate; (ca): capituli; (C): chelicera; (P): palpai; (ad): adenal shield.

In addition, the highest prevalence of tick infestation was observed in sheep and goats (59%). This may be attributed to the fact that tick infestation is facilitated by a large livestock population and herd size, as ticks can easily gain access to hosts and complete their life cycle quickly. Poor veterinary service and herders' disregard for animal management practices may also contribute to tick infestation (Alessandra and Santo, 2012).

The difference in the prevalence of tick species may be due to the different environmental conditions and nature of the land as well as the management style and season of sample collection. It is reported that tick activity can be influenced by rainfall, altitude, season, and atmospheric relative humidity (Pegram *et al.*, 1981). The collected ticks from cattle, sheep, and goats were identified as *Hyalomma* sp. and *Rhipicephalus* sp. at Al Gabal Al Akhdar in Libya.

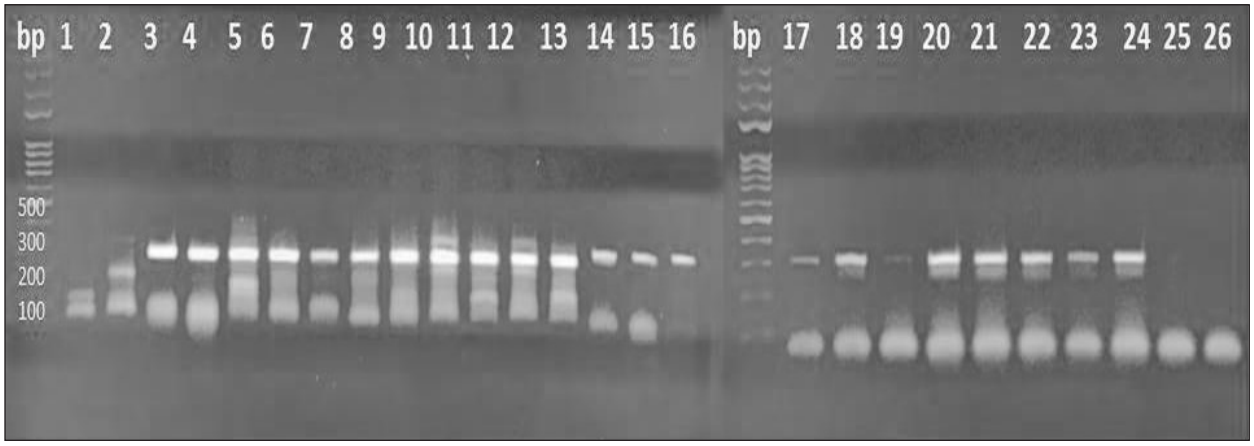

Fig. 8. PCR amplification utilizing tick samples of different species using: 16S rDNA Gene, DNA ladder is located on the left sides of the gel, fragment sizes are represented in base pairs (bp), 1: 25 tick samples, and 26: negative control.

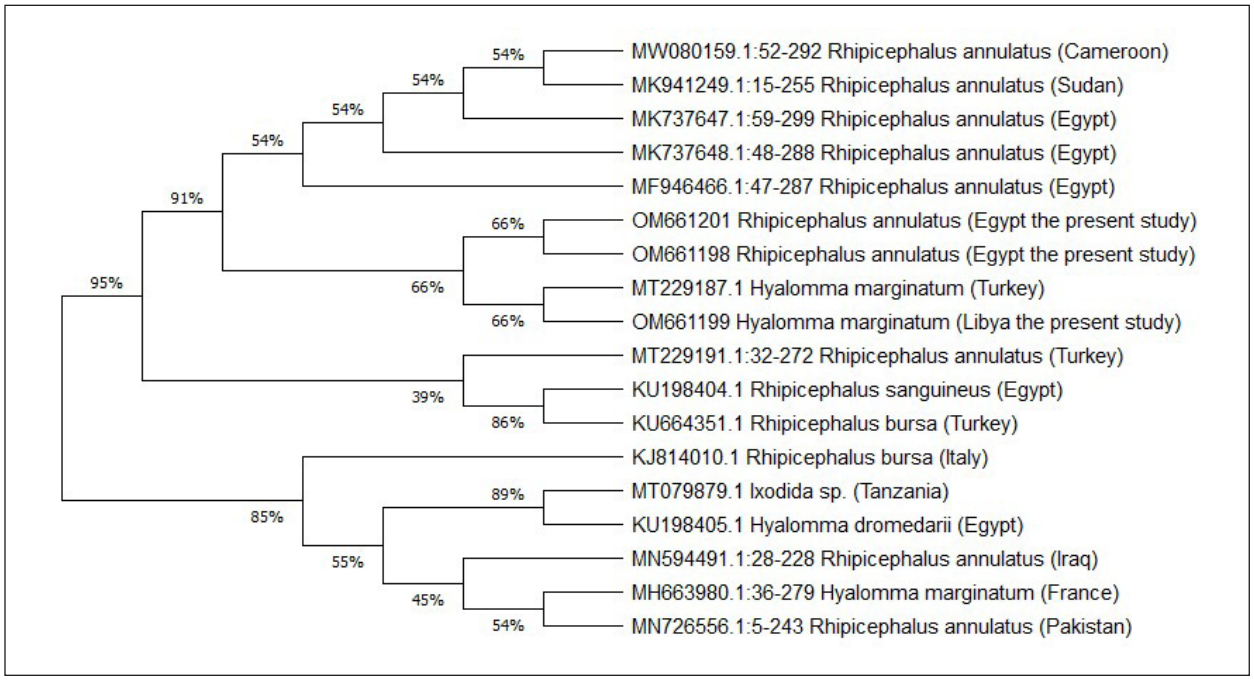

Fig. 9. The phylogenetic analysis was constructed using NJ methods, to construct the tick phylogenetic tree of some of hard tick species sequences from the Genbank, and our sequences samples are included based on 16S r DNA sequences.

The higher prevalence of *R. sanguineus* in this study may be attributed, in our opinion, to high temperatures in these localities, as they are the greatest and most important factor for the development of this tick species (Dantas-Torres *et al.*, 2010). Furthermore, there is no significant difference between the infestation rate and the changes in localities of *R. sanguineus*. This may be due to its adaptation to the environment in each locality. This corroborates well with Dantas-Torres *et al*. (2010), who stated that the wide geographical distribution of the brown dog tick; *R. sanguineus* was related to the cosmopolitan distribution of its primary host and to its adaptability to different environments, under variable climatic conditions.

The current investigations seek to resolve the issues and challenges connected with morphological identification by applying molecular approaches of identification based on DNA sequences to corroborate morphological identification and solve the problems and difficulties associated with morphological identification. For instance, certain tick species are categorized as species complexes, such as the *R. sanguineus* group, which has a total of 17 different tick species (Dantas-Torres *et al.*, 2013). As a result, the amplification and sequencing of

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	OM661201_Rhipicephalus_annulatus_(Egypt_the_present_study)		27.8	35.4	40.2	36.2	48.2	63.5	0.0	23.0	57.0	36.7	35.9	29.3	29.3	29.6	29.6	29.3	30.8	31.3
2	OM661200_Rhipicephalus_annulatus_(Egypt_the_present_study)			31.7	38.3	37.2	23.3	30.6	27.8	53.5	49.6	60.7	35.6	37.4	37.4	37.4	37.4	37.4	30.3	31.6
3	MT079879.1_Ixodida_sp._(Tanzania)				37.3	39.7	32.5	58.3	35.3	40.5	23.6	35.1	58.6	62.2	62.2	62.2	62.2	62.2	38.9	37.2
4	KJ814010.1_Rhipicephalus_bursa_(Italy)					31.8	23.8	39.6	40.2	35.3	40.3	51.1	62.0	30.8	30.8	30.8	30.8	30.8	39.1	56.4
5	MT229187.1_Hyalomma_marginatum_(Turkey)						40.4	38.9	36.1	34.4	17.2	56.9	59.7	65.3	65.3	65.3	65.3	65.3	23.2	28.5
6	KU198405.1_Hyalomma_dromedarii_(Egypt)							35.5	48.2	52.8	39.2	34.9	40.5	39.1	39.1	39.1	39.1	39.1	36.5	57.8
7	MH663980.1:36-279_Hyalomma_marginatum_(France)								63.8	53.2	36.5	24.4	26.5	53.5	53.5	53.6	53.6	53.5	38.3	36.4
8	OM661198_Rhipicephalus_annulatus_(Egypt_the_present_study)									23.1	56.7	36.7	35.9	29.3	29.3	29.6	29.6	29.3	30.9	31.3
9	KU198404.1_Rhipicephalus_sanguineus_(Egypt)										56.0	49.8	50.6	18.2	18.2	18.2	18.2	18.2	21.0	48.6
10	MT229191.1:32-272_Rhipicephalus_annulatus_(Turkey)											65.1	53.4	63.3	63.3	63.3	63.3	63.3	29.4	32.7
11	MN726556.1:5-243_Rhipicephalus_annulatus_(Pakistan)												23.3	39.4	39.4	39.4	39.4	39.4	58.4	38.2
12	MN594491.1:28-228_Rhipicephalus_annulatus_(Iraq)													58.3	58.3	58.6	58.6	58.3	57.9	36.8
13	MW080159.1:52-292_Rhipicephalus_annulatus_(Cameroon)														0.0	0.0	0.0	0.0	43.5	52.1
14	MK941249.1:15-255_Rhipicephalus_annulatus_(Sudan)															0.0	0.0	0.0	43.5	52.1
15	MK737648.1:48-288_Rhipicephalus_annulatus_(Egypt)																0.0	0.0	43.5	52.1
16	MK737647.1:59-299_Rhipicephalus_annulatus_(Egypt)																	0.0	43.5	52.1
17	MF946466.1:47-287_Rhipicephalus_annulatus_(Egypt)																		43.5	52.1
18	KU664351.1_Rhipicephalus_bursa_(Turkey)																			48.5
19	OM661199_Hyalomma_marginatum_(Libya_the_present_study)																			

Fig. 10. Estimates of evolutionary divergence between this study tick sequences and sequences of tick spp. 16S r DNA.

a 16S rDNA fragment served as the study technique of tick genetic identification.

On the other hand, concerning phylogeny, mtDNA sequences are a good phylogenetic marker for groups of organisms that have diverged relatively, based on their higher rate of base substitution than most nuclear markers (Parola and Raoult, 2001). Even though Black and Piesman (1994) reported that errors in the construction of phylogenies can happen by mistake when the mitochondrial gene transfers to the nucleus as mitochondrial pseudogenes (called numts), which are the problem of the mitochondrial gene.

Conclusion

In this study, the 16S rDNA gene sequencing and phylogenetic analysis of sample species from Egypt and Libya proved instrumental in overcoming the difficulties associated with morphological identification techniques. Therefore, using mitochondrial genes is a good marker in tick species identification. The changes in environmental conditions affect tick development. Therefore, this idea will help in devising a better strategy for the control of ticks among livestock by providing useful insights into the epidemiology of these parasites.

Conflict of interest

The authors have no conflicts of interest relevant to this article.

Availability of data and material

All data generated or analyzed during this study are included in this article.

Funding

No funding was received for this study.

Authors' contribution

All authors contributed to the preparation of the manuscript.

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