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Molecular Identification and phylogenetic analysis of *Rhipicephalus* hard-tick species from different Palestinian districts

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Thesis Approval

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Dedication

I dedicate my work to those dearest to me, my family especially my mother, sisters and brothers for their support and advice. To the spirit of my father.

Thank you all

Ra´ida Salim Ihmeidan Taqatqa

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of it) has not been submitted for a higher degree to any other university or Institution.

Signed: Ra'ida

Ra'ida Salim Ihmeidan Taqatqa

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Abstract

Ticks are obligate blood-sucking hematophagous ectoparasites of terrestrial vertebrates, including amphibians, reptiles, birds, and mammals. Two general families of ticks are recognized: *Argasid* (soft ticks) and *Ixodid* (hard ticks).The family *Ixodidae* of hard ticks is divided into two groups based on morphological features: the Metastriata and the Prostriata. In the *Ixodidae* family the genera *Ixodes*, *Amblyomma*, *Dermacentor*, and *Rhipicephalus* are considered medically important. Among *Rhipicephalus* ticks, *R. sanguineus, R. turanicus* and *R. bursa*, are the most common species in Palestine. All three *Rhipicephalus* species are medically important vectors; therefore their accurate identification is necessary. Morphological identification of these species is difficult, especially when the specimens are damaged or engorged with blood or in on immature stage.

The objectives of this study are to identify the most common hard-ticks species in Palestine, to establish a molecular approach for discrimination between hard ticks species that infest sheep, and dogs and to study the genetic variation within each species in comparison to local and foreign hard ticks species. By identification of hard tick species, the potential risk to animals as well as humans may be evaluated and thus more adequately controlled.

A total of 351 hard ticks (*Ixodidae*) were collected from sheep, goats and dogs during March to October 2014. Ticks were identified based on morphological features into two main genus; *Rhipicephalus* (97.4%) and *Haemaphysalis* (2.6%). The ticks were further identified down to the species level as following: *R. sanguineus* (79.2%), *R. turanicus* (9.7%), *R. bursa* (3.4%), *H. alderi* (0.9%) and *H. parva* (1.6%). All tick samples were identified by polymerase chain reaction (PCR) targeting the *COX-1* gene followed by RFLP using *AluI* restriction enzyme. A highly significant correlation was observed between RFLP and microscopy identification (p= 0.01). Phylogenetic analysis based on *COX-1* genetic sequences showed four main clusters, *R. sanguineus*-like cluster, *R. turanicus* -like cluster G1 and G2, and R. *bursa*-like cluster.

This study is the first of its kind to identify the hard tick species, using *COX-1* gene followed by RFLP as genetic marker. Distinction between the closely related *Rhipicephalus* species: *R. bursa, R. turanicus* and *R. sanguineus* was successfully accomplished.

Table of contents

Lists of Tables

List of Figure

List of Appendixes

Table of Abbreviations

Abbreviation	Full Word
A	*Argasida*
H	*Haemaphysalis*
Hy	*Hyalomma*
I	*lxodes*
R	*Rhipicephalus*
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal Ribonucleic acid
ITS-1	Intergenic transcribed spacer subunit 1
ITS-2	Intergenic transcribed spacer subunit 2
COX-1	Cytochrome c oxidase subunit I
ALU1 restriction enzymes.	A restriction enzyme that cut the *Alu* family of DNA repeats in *AluI* restriction site (AGCT)
µL	Microliter
µM	Micromolar
mL	Milliliter
EDTA	Ethylene diamin tetraacetic acid
TAE	Tris acetate EDTA
V	Volt
Bp	Base pair
F	Forward

R	Reverse
G	Gram
°C	Celsius or centigrade degree
BLAST	Basic local alignment search tool
BioEdit	Biological sequence alignment editor

Chapter 1

Introduction

Ticks are obligate blood-sucking hematophagous ectoparasites of terrestrial vertebrates, including amphibians, reptiles, birds, and mammals (de la Fuente et al., 2008). Ticks are considered as the second most important transmitters of different pathogens. In addition the ability of ticks to injure their hosts through direct action or by vectoring disease organisms grant them considerable importance in medical and veterinary transmitting sciences (Mangold et al., 1998; Barker et al., 2004).

1.1 Tick Taxonomy

Ticks are separated based on having a scutum (shield) into three major families:

The *Argasidae* (soft ticks), the *Ixodidae* (hard ticks) and the *Nuttalliellidae*. About 80% (683/ 867) of currently known tick species are *Ixodid* ticks (hard ticks), with the exception of one species in the family *Nuttalliellidae*. The remainder are *Argasid* ticks (soft ticks, 183 species) (Camicas et al.,1998). Based on biological and morphological characteristics, *Argasidae* and *Ixodidae* can be distinguished (Mans et al., 2012). The hard ticks of all life stages have a sclerotized scutum while soft ticks do not possess one (Fig. 1.1). The hard ticks feed for long periods (several days to weeks) and ingest more than 100 folds their body mass of blood. Soft ticks can engorge more than ten times of their body mass just in minutes to hours. This refers to their leathery integument which can rapidly expand (Coons et al., 1986). Soft ticks use their coxal organs to secrete blood meal-derived water back into the host while hard ticks do that via their salivary glands (Sonenshine, 1991; Mans et al., 2004).

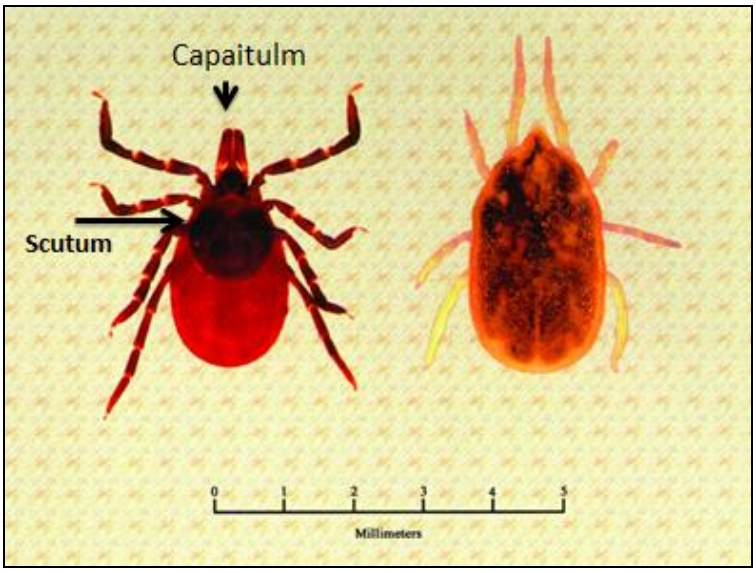


Figure 1.1 Dorsal view of a female *Ixodes scapularis* (family *Ixodidae*, hard ticks) (left), and a female *Ornithodoros hermsi* (family Argasidae, soft ticks), (right) (Schwan et al., 2002).

Worldwide, there are approximately 200 *Argasid* species divided into four genera: *Argas, Carios, Ornithodoros* and *Otobius*. The most diverse genus of the *Argasidae* family is the *Ornithodoros*. Most species of this genus are classified only from the larval stage so the keys for specific diagnosis refer to larval stage (Kohls et al., 1965; Kohls et al., 1969; Jongejan and Uilenberg, 2004).

Soft ticks usually have several stages. The female feeds many times and after each meal they produce a small batch of eggs. *Argas miniatus* is vastly allocated in the Neotropical region. Both *A. persicus* and *A. reflexus* are found in central Asia and southern Europe and they commonly feed on birds. *A. monolakensis* is an important *Argasid* tick of birds that can also feed on humans in Western USA (Schwan et al., 1992). Some of human diseases are transmitted by *Argasid* ticks especially by *Ornithodoros* which may transmit *Borrelia* species *spirochaetes* responsible for relapsing fevers in humans, the infections may be confused with malaria (Jongejan and Uilenberg, 2004).

Nuttalliella has features similar to both hard and soft ticks that appear in certain developmental stages (Fig. 1.2) (Fig. 1.3) (Fig. 1.4). For example, *Nuttalliella* nymphal and adult stages have leathery cuticle and engorge rapidly (Mans et al., 2011). Moreover, larvae have a sclerotized scutum, while nymphs and adults possess a semi-sclerotized pseudo-scutum (Mans et al., 2011; Latif et al., 2012). However, it can be distinguished from others families by its ball and socket leg joints in nymphal and adult stages and blood

meal-derived water is secreted within the Malpighian tubules (Mans et al., 2011; Keirans et al., 1976), Therefore, classification of this family according to their morphological features still problematic (Barker et al., 2004).

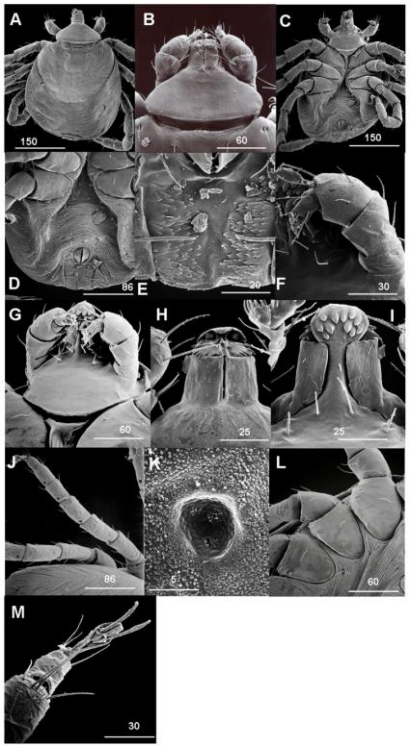


Figure 1.2: *Nuttalliella namaqua* larva. A) Dorsal integument. B) Dorsal basis capituli. C) Ventral integument. D) Posterior venter. E) Anal plate. F) Palps. G) Ventral basis capituli. H) Hypostome dorsal. I) Hypostome ventral. J) Pores in origin of femur, metatarsus, tibia and tarsus. K) Leg pore structure. L) Coxae. M) Haller's organ and claws. Scale bars are indicated in μm (Latif et al., 2012).

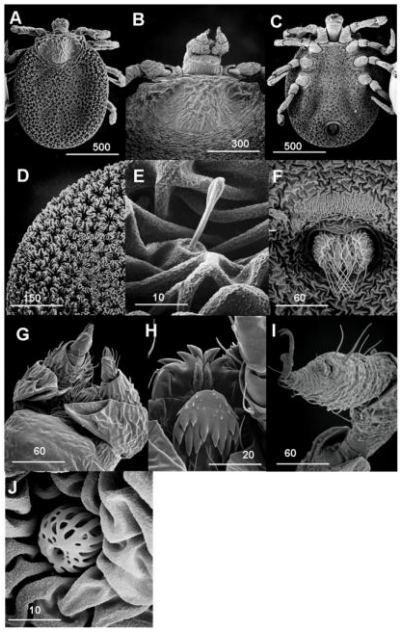


Figure 1.3: *Nuttalliella namaqua* nymph. A) Dorsal integument. B) Dorsal basis capitulum. C) Scanning electron micrograph of ventral body integument. D) Integument. E) Setae in rosette pits. F) Anal pore. G) Palps. H) Hypostome ventral. I) Haller's organ and claws. J) Spiracle plate. Scale bars are indicated in µm". (Latif et al., 2012).

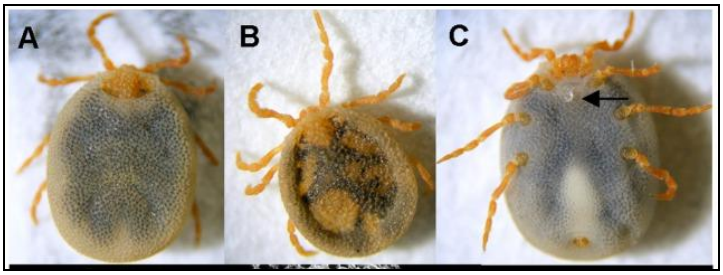


Figure 1.4: *Nuttalliella namaqua* male. A) Dorsal view *Nuttalliella namaqua* female. B) Dorsal view of *Nuttalliella namaqua* male. C) Ventral view of *Nuttalliella namaqua* female, arrow show spermatophore deposited in genital pore. Scale bars are indicated in µm (Latif et al., 2012).

The family *Ixodidae* or hard ticks (Arachnida: Acari: Parasitiformes) includes 694 species subdivided into two main morphological and phylogenetic groups: the Prostriata and the Metastriata (Sonenshine, 1991). The Prostriata are represented by the one subfamily *Ixodinae* that includes a single genus, *Ixodes*. Whereas, the Metastriata group consists of five subfamilies: *Haemaphysalinae, Amblyomminae, Hyalomminae, Bothriocrotoninae, and Ripicephalinae,* (Black and Piesman, 1994; Klompen et al. 2002). *Ripicephalinae* includes different species such as*: R sanguinus, R. bursa, and R. turanicus* (Dantas-Torres,

2013; Morel and Vassiliades, 1962). In the Metastriata, seven genera out of the thirteen described genera have species that are involved in disease transmission (Hoogstraal and Wassef 1986). All *Ixodidae* are hematophagous and obligate ectoparasites and it is considered the most important tick family of medical and veterinary importance because it is important group of pathogens vectors in the phylum Arthropod, being comparable only to mosquitoes *Culicidae* family (Hoogstraal, 1985; Barker et al., 2004). They transmit and maintain different pathogens affecting humans and domestic animals including different species of bacteria (*Rickettsia, Ehrlichia, Borrelia*), viruses, helminthes, and *protista* (*Babesia* and *Theileria*), (Jongejan and Uilenberg, 2004; Mihalca et al., 2011; Dantas-Torres, 2013; Sonenshine, 1993).

A tick species is considered a vector for a particular pathogen just if it:

1. Can feed on an infected vertebrate host,
2. Is able to get the pathogen during the blood meal,
3. Can keep the pathogen during one or more life stages, and
4. When feeding again, can pass it on to other hosts (Kahl et al., 2002).

1.2 Life Cycle

Ixodidae during their life cycle may have one, two, or three-host species. Larvae and nymphs must feed once to engorgement and then molt. One- host ticks molt twice on the same host, from larva to nymph then from nymph to adult. While two-host ticks molt once from the larval to the nymph stage on the host, the engorged nymph drops off, molts off the host and the resulting adult must find a second host (which may or may not be of the same species as the first one). Three-host ticks do not molt on the host, the engorged larva drops off, molts to a nymph, which must find a second host animal to engorge and drop off again, then molts to the adult stage and attach to a third host animal. Usually *Ixodidae* adults mate on the host, then the female feeds to engorgement, drops off, lays a large batch of eggs and dies; however the male might remain on the host for several months. The egg batches of one-host ticks contain on average far less eggs than that of three-host ticks, as the latter have to find a new host three times in their life cycle, and the former only once. Two-host ticks are considered less risk than three-host species but more than one-host ticks, and their egg batches are intermediate in size (Fig. 1.5) (Jongejan and Uilenberg, 2004).

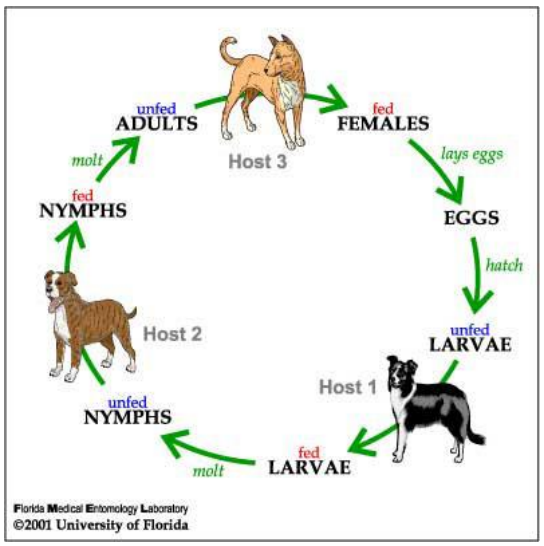


Figure 1.5: Life cycle of *Rhipicephalus sanguineus* Latreille. Drawing by James Newman and Leah LeFevre, University of Florida.
http://entnemdept.ufl.edu/creatures/urban/medical/brown_dog_tick.htm

Objectives

To our knowledge, this is the first study in Palestine to identify the hard tick species, using *COX-1* gene. Therefore, the main objectives of this study are:

1- To identify the most common hard-tick species collected from different hosts in different geographical areas in Palestine.

2- To establish a molecular approach for the discrimination between hard tick species *R. sanguineus* , *R. turanicus* and *R. bursa*.

3- To study the genetic variation within each species in comparison to local and international hard tick species.

The Significant of the study

By identification hard tick species, the potential risk to humans as well as animals and may be evaluated and thus more adequately controlled. On the other hand, classification of ticks depending on their morphological features is time consuming, not accurate and difficult to distinguish between very close species, so the results of the present study may be helpful to overcome these problems.

1.3Literature Review

1.3.1 The most common hard ticks (*Ixodid*) in Palestine: Characteristics and geographic distribution:

The *Ixodidae* is split into five subfamilies *(Ixodinae, Amblyomminae, Haemaphysalinae, Hyalomminae, and Ripicephalinae*) (Wilamowski et al., 1999).

1.3.2 *Haemaphysalis*:

The *Haemaphysalis* genus (*Ixodid* family, the Metastriata group, *Haemaphysalinae* subfamily), which contains 168 species can be differentiated from other genera by the characteristic lateral projection of palpal article beyond the margins of the basis capituli. All *Haemaphysalis* species are three-host ticks and eyeless, a few species of them have favorable domestic livestock which found on livestock in Europe, Asia and to a certain degree, Australia. For instance, *H. longicornis*, an East Asian species that favorable cattle and other domestic animals has been introduced into New Caledonia Australia, and New Zealand, while *H. bispinosa* were obtained from cattle in the Indian subcontinent. In Europe *H. punctata* is common on ruminants, *H. parva* was also found in Jerusalem and different localities throughout Palestine (Jongejan and Uilenberg, 2004; Wilamowski et al., 1999; Ereqat et al., 2016).

1.3.3 *Hyalomma*:

Hyalomma (*Ixodid* family, the Metastriata group, *Hyalomminae* subfamily) consists of 30 species with medium size to large ticks, characterized by eyes typically in sockets and long hypostomes. Most of this genus follows a three-host life cycle but some species undergo either a two-host or a three-host cycle, depending on the host species, other *Hyalomma* species like *H. scupense* is a one-host tick. *Hyalomma* differ from most other *ixodid* ticks, as they can wait on the vegetation for a host to pass, adult *Hyalomma* actively run out from their resting sites when a host access. *Hyalomma* species parasitize domestic and wild mammals and birds; they are abundant in semi-arid places (Jongejan and Uilenberg, 2004).

Hy. marginatum is widely spread in North Africa and Asia, where it is found in Algeria, Armenia, Azerbaijan, Egypt, Ethiopia, Georgia, Iran, Iraq, Israel, Morocco, Sudan, Syria, Tunisia and Turkey (Hoogstraal, 1979; Latif and walker, 2004; Estrada-Pena et al., 2010; Bouattour et al., 1999). Furthermore, the following *Hyalomma* species were reported in Palestine, *Hy. detritum , Hy. dromedarii* and *Hy. Impeltatum* (Ereqat et al., 2016).

1.3.4 *Ixodes*:

The largest genus of hard ticks is *Ixodes* (*Ixodid* family, the Prostriata subfamaily *Ixodinae*) with 241 species. They have a three-host life cycle and a lot of species live in burrows or nests. They are characterized by the anal groove curving anteriorly to the anus, a scutum lacking ornament and lack of eyes. The genus is widely distributed throughout wooded or herb environments, but relatively few *lxodes* species parasitize larger mammals. The most important species in North America is *I. scapularis*, whereas *I. ricinus* and *I. persulcatus* are the most common *Ixodes* in Europe and Asia. In Palestine, *I. redikorzevi* and *I. scapularis* nymphs were reported, the latter species brought from New Jersey (Wilamowski et al., 1999). The indiscriminate feeding behavior of these species on a variety of hosts, makes them important vectors of a large number of zoonotic tick-borne diseases (Jongejan and Uilenberg, 2004; Wilamowski et al., 1999).

1.3.5 *Rhipicephalus*:

The genus *Rhipicephalus* (Acari: *Ixodidae*, the Metastriata group, subfamily *Ripicephalinae*) comprises 84 species (Apanaskevich et al., 2013; Horak et al., 2013). These small to medium-sized ticks characterized by short, broad palps which are in the ornate and have festoons and eyes. They are three-host ticks, but some species have a two-host cycle. Identification of *Rhipicephalus* ticks based on morphological features is difficult and the reader should refer to a recent revision of the entire genus (Walker et al., 2000). *R. sanguinus, R. bursa,* and *RR. turanicus* are the most common *Rhipicephalus* tick species in the Mediterranean countries including Palestine, (Morel and Vassiliades, 1962; Gilot et al., 1992; Mumcuoglu et al., 1993; Guberman et al., 1996). Some *Rhipicephalus* species transmit different pathogens such as *Rickettsia rickettsii, Rickettsia conorii* and *Ehrlichia canis* (Dantas - Torres et al., 2012).

1.3.5.A *Rhipicephalus sanguineus*:

The most widespread tick in the world is *R. sanguineus* (the brown dog tick), even considering that many ticks currently identified *as R. sanguineus* may actually represent other closely related species like *R. turanicus*. This tick is a parasite of dogs that also can occasionally parasitize other hosts, including humans. In addition, *R. sanguineus* considered as a vector of too many disease pathogens, examples: *Rickettsia rickettsia*, *Rickettsia conorii*, *Ehrlichia canis* and *Coxiella burnetii* (Dantas-Torres F, 2012; Guberman et al., 1996).

R. sanguineus is one of the most studied ticks because of its veterinary and public health relevance. *R. sanguineus* have three development stages in their life: larva, nymph and adult stages (Fig. 1.6) (Dantas-Torres, 2010).

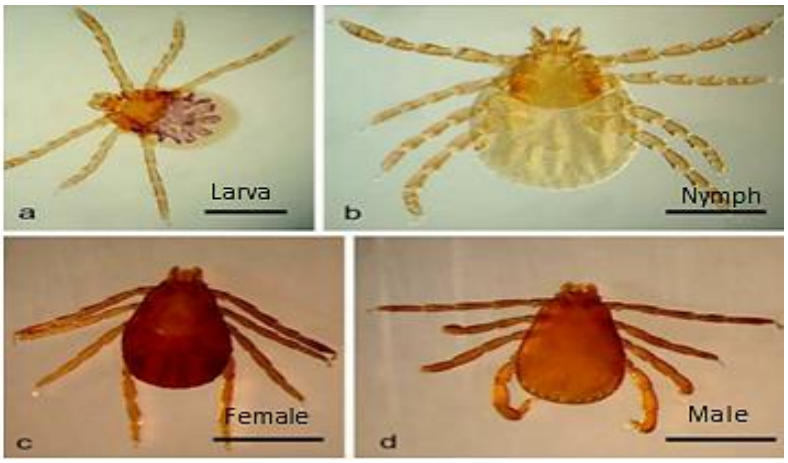


Figure 1.6: Immature and adult stages of *R. sanguineus*. A: larva B: nymph. C: female . D: male (Dantas-Torres, 2010).

Biology of *R. sanguineus*

Ethology

From an ethological standpoint, *R. sanguineus* is adapted to indoor living (an endophilic), all developmental stages feed on the same host species (monotropic), and each life stage requires a new host to feed on three-host tick species. Although *R. sanguineus* is highly endophilic, it can survive in outdoor environments, mainly in refuges such as limestone

walls. Moreover, the monotropic. This species can feed on other hosts including humans, which do not belong to its 'natural trophic chain' indicating that *R. sanguineus* is being able to adopt different strategies for survival, as needed (Dantas-Torres et al., 2010).

Attachment, feeding

When *R. sanguineus* attach to the host, it uses its chelicerae to bore the host's skin, then inserts its hypostome and chelicerae into the host's epidermis and reaching the upper layers of dermis and then it secretes a cement-like substance, which forms a cone on the surface of epidermis which then extends up to the stratum corneum (Szabó MP et al., 1999). As a result, capillary and small blood vessels are ruptured and bleeding occurs, creating a feeding pool (Mans et al., 2004), from where the tick sucks blood and other fluids (telmophagy).

The feeding period of *R. sanguineus* change depending on tick developmental stage and host, for example the feeding period of larvae is two days while females need several weeks for feeding. In addition, engorgement of females take long time on rabbits than on dogs (Koch, 1982; Troughton et al., 2007). *R. sanguineus* ticks can attach to any part of the the dog's body, but their preferred attachment sites are: the head (particularly on ears), interdigital spaces, inguinal region, back and axilla (Koch et al., 1982) (Fig. 1.7). *Rhipicephalus* ticks have short hypostome and attach superficially in comparison with others ticks but they can attach hard to the host's skin (Fig. 1.8).

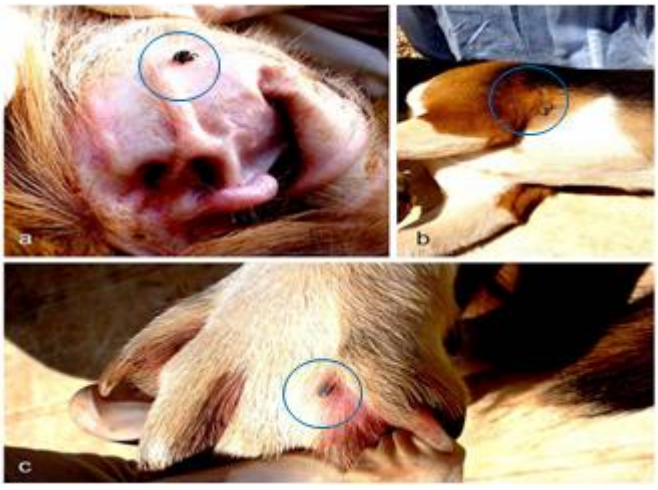


Figure 1.7: Attachment sites of *R. sanguineus* to their animal host' skin. A: adult ticks attached to the ear of a dog. B: two ticks on the axilla of a dog. C: An engorged nymph on the interdigital region of a dog. (Dantas-Torres et al., 2010)

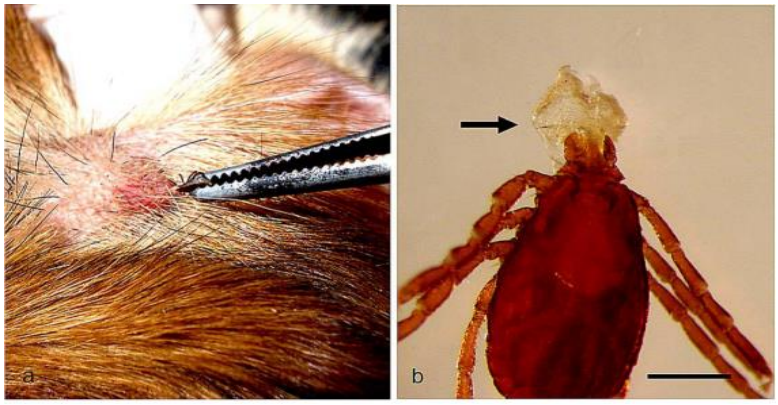


Figure 1.8: Attachment of *R. sanguineus*. A: A male firmly attached to the dog's skin. B: A female exhibiting a piece of a dog's skin. (Dantas-Torres et al., 2010).

The brown dog tick and tick borne diseases:

R. sanguineus have been reported to transmit several pathogens such as, *Babesia canis and Ehrlichia canis* which infects dogs, *R. sanguineus* transmits two life-threatening *rickettsial* diseases to humans: Mediterranean spotted fever (MSF) caused by *Rickettsia conorii* in the old world, and Rocky Mountain spotted fever (RMSF) caused by *Rickettsia rickettsii* in USA (Parola et al., 2005). *R. sanguineus* also transmits *Rickettsia massiliae* (Vitale et al., 2006). In 2003, 22 *R. sanguineus* found attached to an alcoholic homeless person who died by MSF in Marseille (Parola et al., 2005). Morover, several cases of MSF were recognized in Oran, Algeria, in 1993 peaked in 2005 (Mouffok et al., 2006).

1.3.5.B *Rhipicephalus turanicus*:

R. turanicus is a three-host tick. The adult ticks are usually abundant from late Spring to Summer, they commonly infest cattle, dogs, sheep in Mediterranean region, the infestation predominately occurs on sheep and human (Estrada-Pena et al., 2004; Inna Ioffe et al., 1997).

Description

The length of unfed ticks changed from 3.2mm to 4.8mm. The color of relatively mid brown and the slender legs had pulvilli. In dorsal view, mouthparts were noticed in anterior part. *R. turanicus* had a short palps and hypostome. Moreover, basis capituli clearly appeared hexagonal shape and the length of palps was almost equal to basis capituli. The

scutum usually brown and grooves with smooth texture. Eyes and festoons are present (Fig. 1.9) (Youssefi et al., 2011).

Figure 1.9: Dorsal view of the entire body male of *R. turanicus* (Youssefi et al., 2011).

The spiracles plates are present in the rearward part of fourth legs, the entrance and tail are slightly broad, same the adjacent festoon (Fig. 1.10).

Figure 1.10: Spiracle plates of *R. turanicus* (male) (Youssefi et al., 2011).

The posterior margin of scutum in females appeared meandrous shape. Anal shields were observed just in males, they seemed narrow and taper with the same size and anal groove was located in posterior position (Fig. 1.11). The genital aperture posterior lips made a narrow U shape (Youssefi et al., 2011).

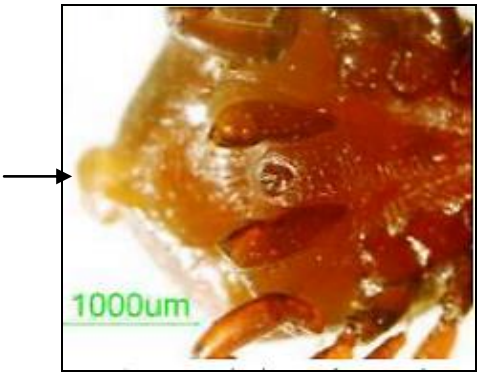


Figure 1.11: Ventral view of posterior portion of *R. turanicus* showing the anal plates (Youssefi et al., 2011).

Identification of ticks becomes crucial not only due to their economic impact in livestock, but also due to their impact in human health, to which they have become a threat (George et al., 2004). The accurate identification of ticks at any developmental stages by morphology is not always possible because of bad preservation of the specimens that can prevent correct identification based on morphological features (Chitimia et al., 2009). In addition, detailed morphological descriptions of the immature stages remain very difficult because they are very similar in appearance (George et al., 2004).

To overcome these disadvantages many approaches using different genetic makers have been evaluated for identification and phylogenetic studies of ticks (McLain et al., 1995; Poucher et al., 1999; Zahler et al., 1997; Fukunaga et al., 2000; Hilnka et al., 2002; Shaw et al., 2002; Marrelli et al., 2007). Some of these studies depend on the genetic variation at the nucleotide level which provides the highest resolution available for systematic studies and phylogenetic analyses. Other studies were performed such as: mitochondrial *rDNA* sequences (*28S* and *18S* nuclear *rDNA* genes and *12S* mitochondrial *rDNA* gene (Black et al., 1994; Norris et al., 1997).

Similarly, *16S rDNA* has been used to assess the phylogenetic relationships of various economically important tick species (Black and Piesman, 1994; Caporale et al., 1995; Chao et al., 2009; Norris et al., 1996). The first (*ITS-1)* and the second internal transcribed spacers (*ITS-2*) of ribosomal DNA (*rDNA*) showed low interspecies variation but their considerable interspecies variation was useful genetic marker for defining species and for inferring their phylogenies (Barker et al., 1998). Highly conserved *rDNAs* flanked region in both *ITS* regions relatively make it easy to be amplified by polymerase chain reaction (Murrell et al., 2001).

The *ITS-2* of *rDNA* has been sequenced extensively in ticks to study closely related species (Barker, 1998; McLain et al., 1995; Zahler et al., 1995; Fukunaga et al., 2000; Murrell et al., 2001) and other ectoparasites. None of the previous studies was able to differentiate between the most common species of *Rhipicephalus* ticks, for that it is important to

introduce a new DNA marker to distinguish these species for epidemiological and medical purposes.

Chapter 2

Materials and Methods

2.1 Samples collection

This descriptive study was used to investigate the presence of three species of *Rhipicephalus* ticks (*R. sanguineus*, *R. bursa* and *R. turanicus*) to be characterized *by* molecular genetic marker in Palestine. A total of 351 hard tick species were collected at random between March 2014 to October 2014 from different mammalian hosts (62 sheep, two goats, and 41 dogs), which were residing in different regions in Palestine: Ramallah, Tubas, Jenin, Nablus and Jericho. One to five hard ticks were collected from each infested host, the tick samples were then transferred into sterile microfuge tubes (1.5 ml) (SARSTEDT, Nümbrecht, Germany) containing 70% ethanol to Al-Quds Nutrition and Health Research Institute (ANAHRI) laboratory - Al-Quds University and stored at −20˚C for future use.

2.2 Ticks Identification

Using stereomicroscope, ticks were classified according to published identification taxonomic and structural differences keys by sex and species. The genus, species, gender, and developmental stage were determined (Walker et al., 2000; Estrada-Pena, 2004).

Rhipicephalus genus was identified depending on the main morphological features. It has hexagonal basis capitulim and divided coxa in ventral view of the tick. Developmental stage was determined depending on the number of legs. Adult ticks have eight legs, whereas larva has six legs. Then the ticks have been identified to the species level on the basis of their morphological characteristics such as basis capatiuli lateral angle which are blunt in *R. turnicus*, while its sharp in *R. sanguinus*, there are many details that must be determined to facilitate the classification. (Fig. 2.1, 2.2, 2.3) show some features of *Rhipicephalus* ticks.

Discrimination between *R. sanguineus* and *R. turanicus* was based on the shape of the adanal plates in males sharp external angle in *R. sanguineus* and a blunter angle in *R. turanicus*, and on the genital pore and the spiracle shape in females (Walker et al., 2000; Estrada-Pena, 2004). Identification of *R. bursa* was based on different

characteristics as follow: shape of the genital pore in females narrow V shape comparison to narrow U-shape in *R. turanicus*, and broad U-shape in *R. sanguineus*, shape and ciliae around the spiracle plates, shape of adanal plates in male. Identification of *R. bursa* nymphs was based on the hexagonal shape of the capitulum, compared to the triangular shape of the capituli of nymphs from other *Rhipicephalus* species (Walker et al., 2000).

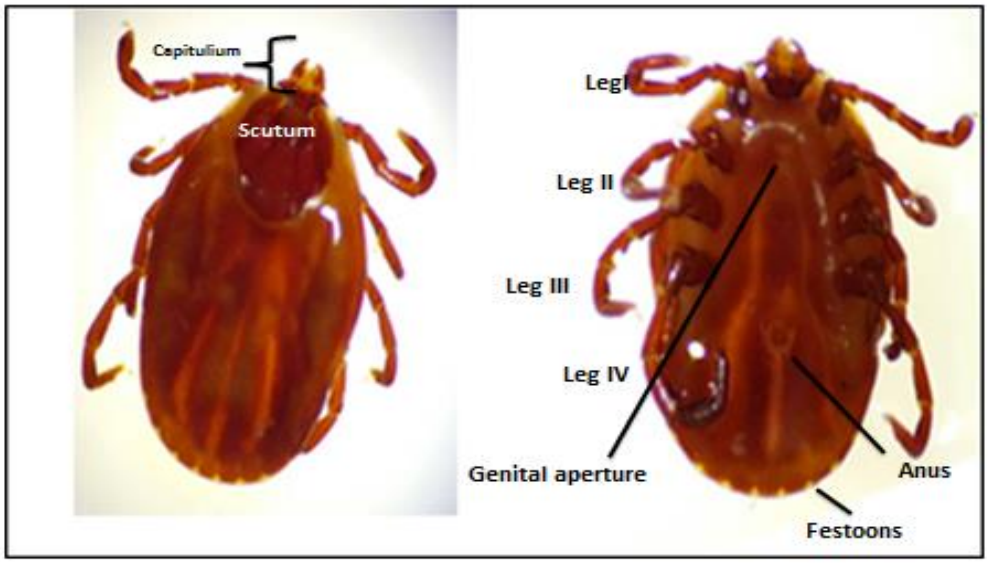


Figure 2.1: Some morphological features of female *Rhipicephalus* tick: Dorsal view (left) and ventral view (right). The arrow indicates the scutum which covered part of dorsal view and other features (photo taken by Raida Taqatqa).

Figure 2.2: Dorsal view of male *Rhipicephalus* tick: the arrow show the hexagonal basis capitulim (photo taken by Raida Taqatqa).

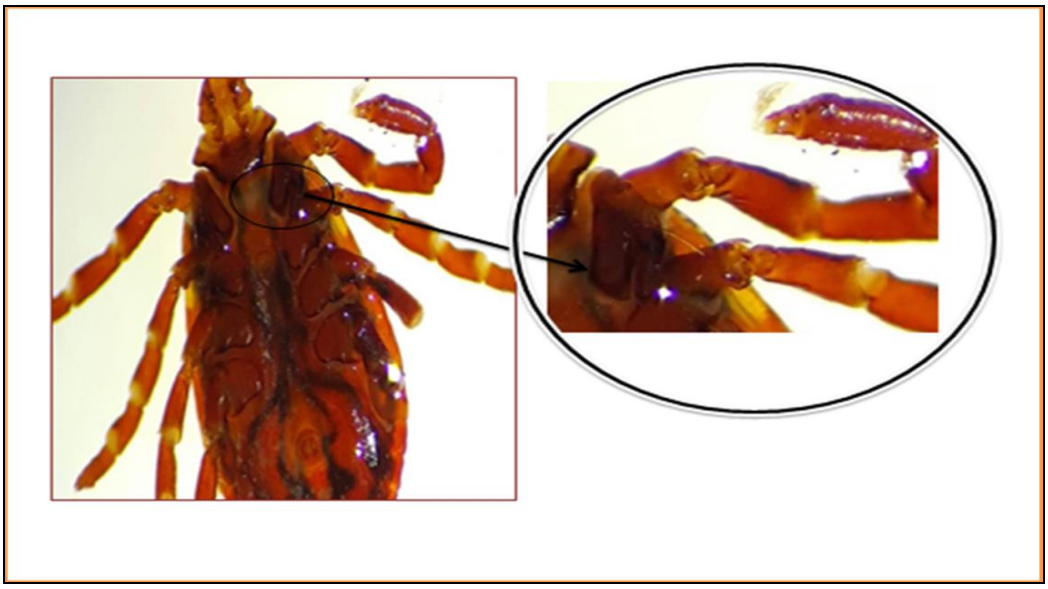


Figure 2.3: Ventral view of male *Rhipicephalus* tick: showing the divided coxa (By Raida Taqatqa).

2.3 DNA extraction

Each tick was removed from the alcohol tube and dried on a tissue paper, placed into a new microfuge tube, and ground mechanically using plastic pestles and then subjected to DNA extraction using DNA extraction kit (genomic DNA from tissue NucleoSpin® Tissue, Germany) following manufacturer's instructions with some modifications: Tick was lysed with 200µl of the tissue lysis buffer and 40 µl of Proteinase K, incubated at 56˚C overnight, then 200µl binding buffer was added and incubated at 70°C for 10 min. For removing the insoluble tissue particles, 100µl isopropanol was added, centrifuged for 1 min at 8000×g. For washing, 500µl wash buffer was added and centrifuged for 1 min at 8000×g repeated three times. Finally, 200µl of pre-warmed elution buffer was added and centrifuged for 1 min at 8000×g. the eluted DNA was kept frozen at -20C until use.

2.4 Amplification of DNA

2.4.1 Primers design and DNA amplification:

Polymerase chain reaction (PCR) was used to amplify partial fragments of the *16S* Ribosomal *rDNA* and *COX-I* genes.

In this study, new primers were designed based on the conserved DNA sequences of three hard tick references exported from the Gene Bank (*R. sanguineus, R. turanicus* and *R.*

bursa). The primer3 website program was used for primer design (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi). Five sets of primers (forward and reverse) were used as shown in Table (2.1). Following optimization, one set of primers (Combined P2) was used in this study.

All sequences were aligned to each other using the website program (http://multalin.toulouse.inra.fr/multalin/) to detect nucleotide variations within these sequences which can be used for further species identification. These variations were used for differentiation of hard tick species using restriction fragment length polymorphism (RFLP) analysis. Virtual DNA digestion using selected discriminated enzyme was done by the followed website program: (http://tools.neb.com/NEBcutter2/).

Table 2.1: The main properties of the primers used in this study.

Gene name	**Primer**	**Primer sequence**	**Primer size bp**	**Amplicon size bp**	**Annealing temperature °C**
16S Ribosomal *rDNA*	(F)	5'-CCCGTTGGCTGAAGTAGG-3	18	520	42
	(R)	5'-CAACGGTGGCTTCGGAGG-3'	18		
COX-1 short	(F)	5'ATAGAATTAGGTCAACCTGGAAC-3'	23	360	57
	(R)	5'TTGAAGAAGCACCAGCAAGA- 3'	20		
COX-1 long	(F)	5'CCGCGATGAATATACTCTACTAAYC-3	25	760	52
	(R)	5'CCAGGATTTGGAATAATTTCTCAAA-3'	25		
COX-1 Combined P1	(F) short	5'ATAGAATTAGGTCAACCTGGAAC-3'	23	655	53
	(R) Long	5'CCAGGATTTGGAATAATTTCTCAAA-3'	25		
COX-1 Combined P*2*	(F) Long	5'CCGCGATGAATATACTCTACTAAYC-3'	25	463	53
	(R) short	5'TTGAAGAAGCACCAGCAAGA- 3'	20		

For DNA amplifications, PCR reactions were performed in 25-µl PCR ready mix (Syntezza, Jerusalem), containing 1.2 µM of each set of primers and 2µl of the extracted DNA. After a denaturation step of 5 min at 95ºC, each of 35 cycles consisted of

denaturation at 95ºC for 20s, annealing at 53ºC for 30s, extension at 72ºC for 2 min with a final extension step of 6 min at 72ºC. Nuclease-free water was used as negative control in each run. PCR products of some (n= 35) of the positive samples were sent for DNA sequencing using the same forward and reverse primers.

2.4.2 Gel electrophoresis:

All PCR products were loaded onto 2 % agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared by dissolving 2g of agarose in 100 ml solution of 1X Tris-acetate EDTA buffer (TAE) (40 mM of Tris acetate and 1mM EDTA). The agarose was dissolved in Erlenmeyer flask using microwave for about 1min till completely dissolved, and then 3.5µl of 10 mg/ml (0.35µg/ml) of Ethidium Bromide was added for DNA staining. The gel was poured in the gel tray in the casting chamber (Bio-Rad Laboratories Inc., USA). Five µl of PCR products were loaded onto the gel. DNA marker ladder of 100bp (Thermo scientific Lithuania) was used in each run. The gel was run at 100V for 45min. The gel images were captured using MiniLumi 1.4 gel documentation system from (DNR Bio-Imaging Systems Ltd, Israel) .

2.4.3 Restriction fragment length polymorphism (RFLP):

Restriction enzyme recognition sites along the mitochondrial *COX-1* gene were mapped using a world wide web based Restriction Mapper program (www.restrictionmapper.org). Using the previously described *COX-1* gene alignment of various *Rhipicephalus* species, *ALU1* restriction enzyme was chosen because it sit is redundant in different *Rhipicephalus* species that were examined in this study. 0.5 µl of*ALU1* restriction enzyme was added to 1.5 µl Assay Buffer for each DNA sample to a total volume of 15 µl to digested DNA samples at 37ºC for 1 hour. The digested samples were loaded onto 3% agarose gel (Agarose LE, Analytical gradient, Promega, Spain). The gel was prepared and documented as mentioned above.

2.4.4 DNA sequencing:

The PCR products of some successfully amplified tick samples (n= 35) were purified and sent for sequencing from both directions using the *COX-I* forward and reverse primers. At HyLab sequencing service (Rehovot, Israel) the PCR reactions and conditions were

performed as described above. In addition, five DNA samples of well identified ticks belonged to the three species (*R. sanguineus*, *R. turanicus* and *R. bursa*) were sent for sequencing to be used as positive controls.

2.5 Genetic data analysis: Alignment, BLAST, and Phylogenetic analysis

2.5.1 DNA sequence analysis, and Phylogenetic analysis:

The obtained sequences were arranged and aligned using BioEdit sequence alignment editor software (Fig. 2.4).

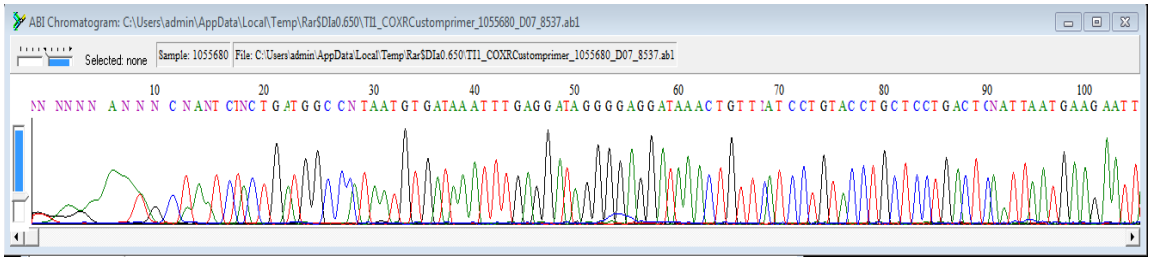


Figure 2.4: BioEdit software for analysis of the obtained DNA sequences. The DNA sequences were analyzed and arranged to prepare them for BLAST analysis step.

BLAST software (http://blast.ncbi. nlm. nih.gov/Blast.cgi) (Fig. 2.5) was used for species identification and for comparison of the obtained DNA sequences in this study to the reference sequences deposited in the database.

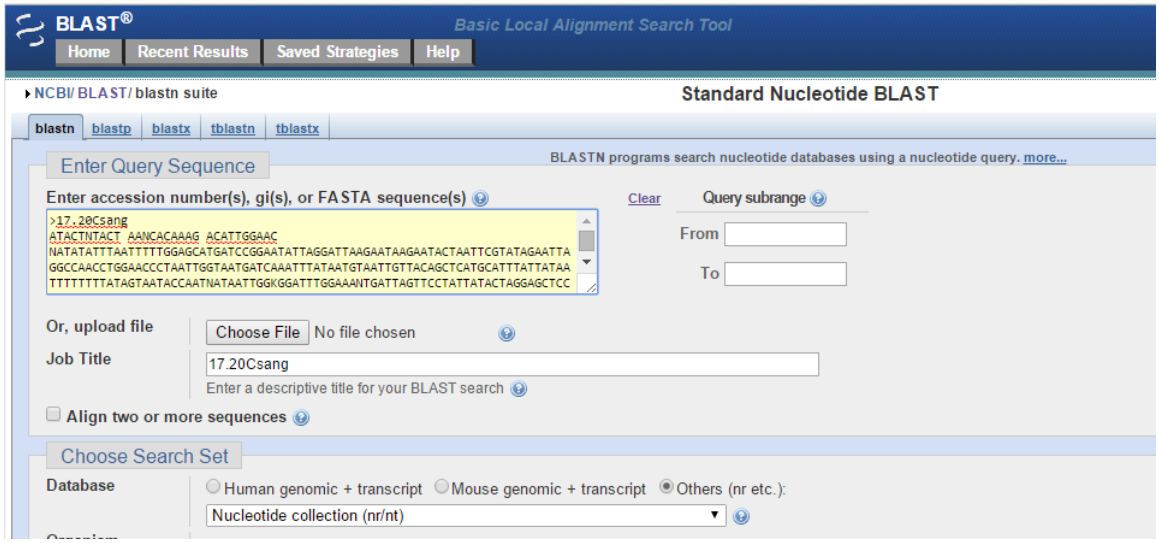


Figure 2.5: Detection of hard ticks species based on sequence homology using BLAST website. (http://blast.ncbi.nlm. nih.gov/ Blast.cgi).

Phylogenetic trees were constructed based on the *COX-1* gene sequences (~450 bp), and IUB (DNA weight matrix) scoring matrix BESTFIT was used for comparison of nucleic acid sequences. The consensus trees were constructed by bootstrap and neighbor-joining method with default repeat number =1000 (http://www.genome.jp/tools/ clustalw/) (Saitou et al., 1987).

2.5.2 Statistical tests:

Statistical analysis was carried out using the SPSS program v13 to find the frequency of the collected ticks, by animal host, genus, developmental stage, and species. Pearson correlation was used to compare the result of the two tests (the newly developed *COX-I*_ PCR_RFLP and microscopic examination). $P< 0.05$ was considered statistically significant.

Chapter 3

Results

3.1 Animal sampling and classification

A total of 351 hard ticks were collected from 105 animals residing in different regions of Palestine: 73 hard ticks were obtained from Jenin, 43 from Jericho, 154 from Nablus, 5 from Qalqilya, 12 from Ramallah and 64 from Tubas (Fig. 3.1). Overall, 163 hard ticks were collected from 41 dogs, 186 hard ticks were sampled from 62 sheep, and two ticks were collected from two goats (Table 3.1). The ticks were identified by microscopy into two main genera: *Rhipicephalus* (n= 342/351; 97.4%), and *Haemaphysalis* (n= 9/351; 2.6%) (Table3.2). This included 165 females, 162 males and 16 nymphs (Table 3.3). Determination of the developmental stage was not possible in eight of the collected ticks as they were damaged during handling. All ticks were further identified up to the species level, the most prevalent species were *R. sanguineus* (n= 279/351; 79.4%) followed by *R. turanicus* (n= 34/351; 9.7%) and *R. bursa* (n= 12/351; 3.4%) while 4.8% of *Rhipicephalus* ticks were identified only to the genus level designated *R.* spp. (n= 17/351). Among *Haemaphysalis* ticks: 0.9% were *H. alderi* (3/351) and 1.6% were *H. parva* (n=6/351) (Table3.4)

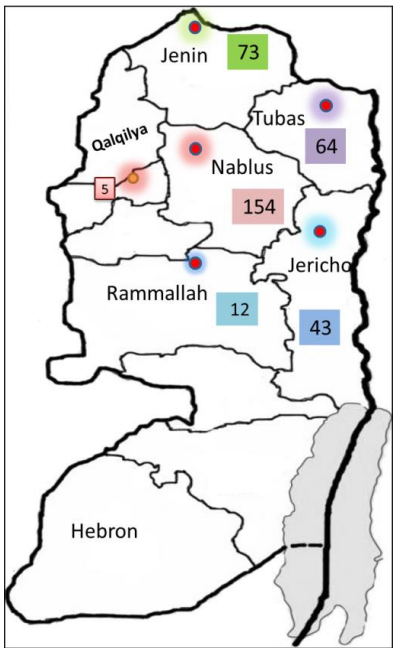


Figure 3.1: The geographic distribution of the hard ticks collected from Palestine, the number of collected ticks per district was indicated.

Table 3.1: Distribution of collected hard ticks by animal host.

Host	**Number of Host**	**Number of collected ticks**
Sheep	62	186
Dog	41	163
Goat	2	2
Total	105	351

Table 3.2: Distribution of collected hard ticks by genus.

Genus	**Number of hard ticks**	**Valid Percent**
Rhipicephalus	342	97.4
Haemaphysalis	9	2.6
Total	351	100

Table 3.3: Distribution of collected hard ticks by developmental stage.

Developmental Stage	**Number of hard ticks**	**Valid Percent**
Female	165	47.0
Male	162	46.2
Nymph	16	4.6
Not determined	8	2.3
Total	351	100

Table 3.4: Distribution of collected hard ticks by species, the identification based on morphological features.

Species	*R. sanguineus*	*R. turanicus*	*R .bursa*	*R.* species	*H. alderi*	*H. parva*	**Total**
Hard ticks	279	34	12	17	3	6	351
Valid Percent	79.2	9.7	3.4	4.8	0.9	1.6	100

3.2 Selection of appropriate gene for identification of *Rhipicephalus* ticks

To determine the proper genetic marker that can be used for species identification of the morphologically related ticks, partial sequence of the *16S* ribosomal *rDNA* gene (520 bp) was amplified using our newly designed primer the forward 5'-CCCGTTGGCTGAAGTAGG-3 and reverse primers 5'-CAACGGTGGCTTCGGAGG-3'. The DNA sample of eight positive controls and three random samples were successfully amplified (Fig.3.2) and sequenced (3/8 of positive control) (see Appendix A: sequences from A1- A10).

Sequencing results showed many nucleotide variations (19 variations) in the sequenced fragment compared to the reference strains imported from the GeneBank of the same species (Fig. 3.3A). However, these variations were not reliable within the same species(Fig. 3.3B) and thus *16S rDNA* was not a suitable genetic marker that can be used to distinguish between different species of *Rhipicephalus* ticks. Therefore, the sequence of *COX-1* gene was studied and further investigated.

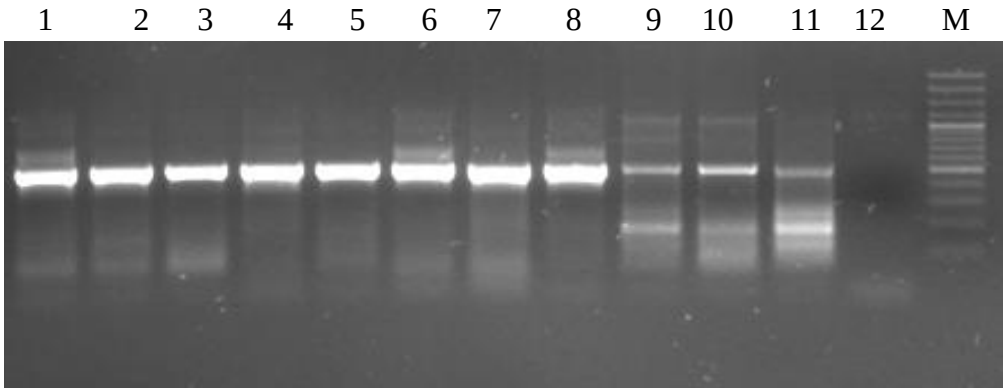


Figure 3.2: PCR analysis of *16S* ribosomal *rDNA*. M: DNA ladder (100 bp), lanes (1, 2, 3 and 4) are positives controls of *R. sanguineus*, lanes (5, 6, 7 and 8) represent positive controls of *R. turanicus*, lanes (9, 10, 11) represent three hard ticks samples, lane 12: negative control, and M: DNA molecular weight marker (100 bp).

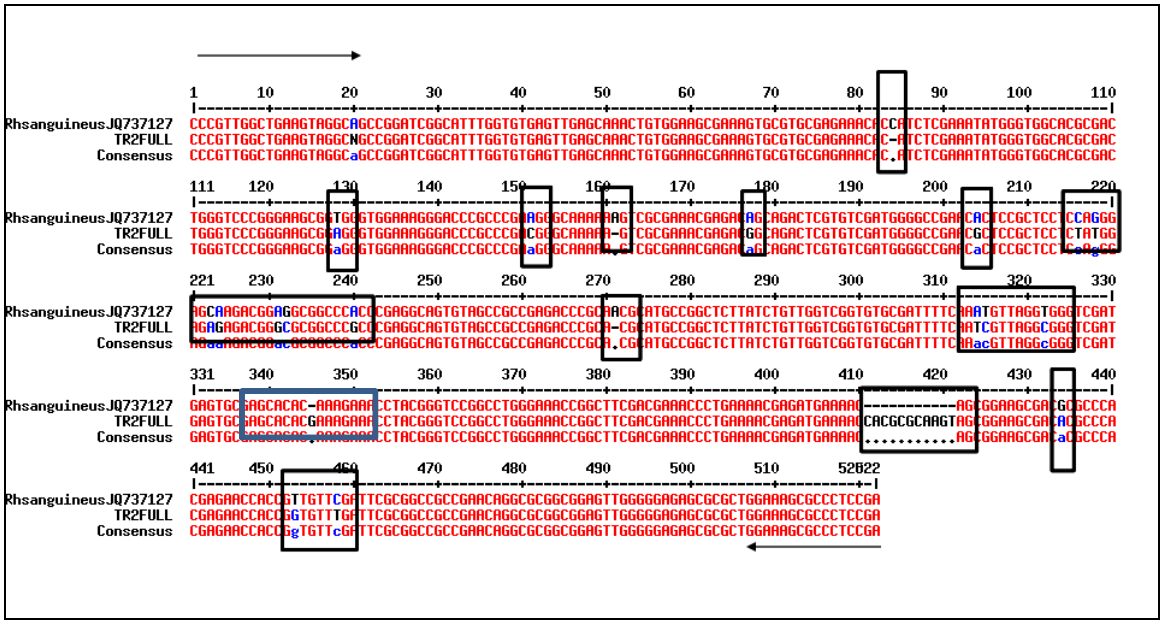


Figure 3.3A: A multiple alignment of reference strains from the GeneBank of *R. sanguineus*, and *R. turanicus* of the *16S* ribosomal *rDNA* gene. There is no 16S ribosomal rDNA sequence of *R. bursa* in the GenebBank. The multiple alignments showing many nucleotide variations (marked in the square). The arrows indicate the location of primers.

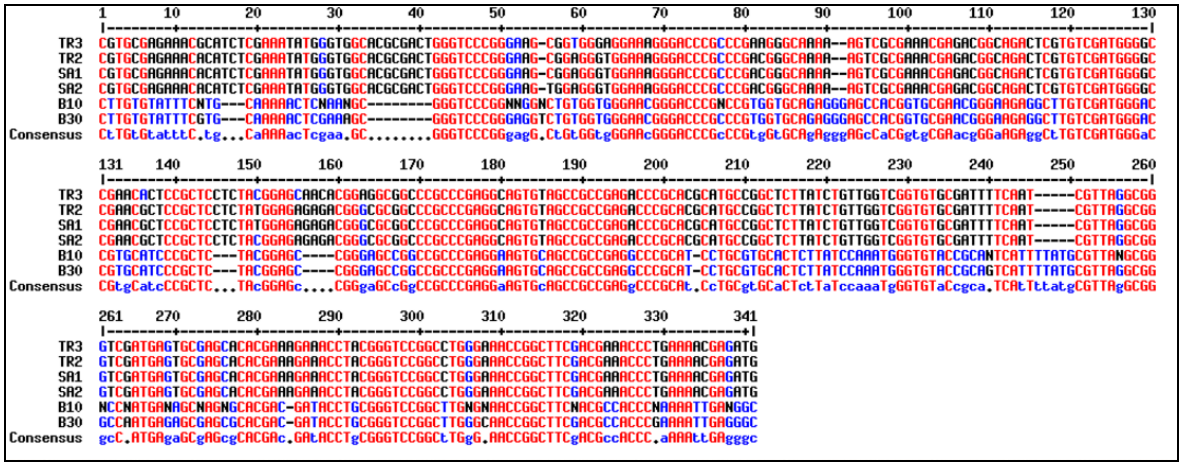


Figure 3.3B: A multiple alignment of sequences from different species (*R. sanguineus, R. turanicus*, and *R. bursa*) of the *16S rDNA* gene: showing many nucleotide variations (71), but are not reliable within the same species. SA1 and SA2 are *R. sanguineus*. TR2 and TR3 are *R. turanicus*. B10 and B30 are *R. bursa*. Red and blue colors showed the differences between the species.

3.3 Primers design targeting *COX-I* gene

During optimization, the newly designed primers (*COX-1*short) (*Table* 2.1) were able to amplify DNA template of *R. sanguineus* and *R. turanicus* samples, but no amplicons were produced from *R. bursa* DNA samples (Fig.3.4B). For the *COX-1* long PCR, nonspecific bands (~ 120 bp) were observed for *R. sanguineus* and *R. turanicus* samples, but no amplicons were produced from *R. bursa* DNA samples (Fig.3.4B). Therefore, two sets of combination primers were used, *COX-1* combined P1 (F (short) +R (long)), and *COX-1* combined P2 (F(long) +R(short)) as shown in Table (2.1). No amplicons were observed using *COX-1* combined P1 for all species (Fig. 3.4C), while *COX-1* combined P2 primer produced amplicons at the size of ~ 450 bp for all examined *Rhipicephalus* species (Fig. 3.4D). Since the amplicon size was the same for all tested samples, suitable restriction enzymes were identified for subsequent RFLP analysis (see section 2.4.3). The sequence of *COX-1* gen and the locations of the two sets of primers the *COX-1*short and *COX-1* long PCR were shown in (Fig.3.4A).

ATTTTACCGCGATGAATATATTCTACTAATCACAAAGACATTGGAACAATATATTTAATTTTTGGAGCAT

GATCCGGAATATTAGGATTAAGAATTAAGAATACTAATTCGTATAGAATTAGGTCAACCTGGAACTCTAAT

TGGTAATGATCAAATTTATAATGTAATTGTTACAGCTCATGCATTTATTATAATTTTTTTTATAGTAATA

CCAATTATAATTGGTGGATTTGGAAACTGATTAGTACCTATTATACTAGGAGCTCCAGATATAGCATTCC

CACGAATAAATAATATAAGATTTTGACTTCTTCCTCCCTCATTATTTATATTAATTAATTCTTCATTAAT

TGAGTCAGGAGCAGGTACAGGATGAACAGTTTATCCTCCCCTATCCTCAAATTTATCACATTATGGGCCA

TCAGTAGATTTAGCTATTTTTTCTCTTCATCTTGCTGGTGCTTCTTCAATTTTAGGTGCAATTAATTTTA

TTACAACTATTGTGAATATACGATCTATTGGAATAACAATAGAACGAATACCATTATTTGTATGATCTGT

TTTAATTACTGCAATTTTATTACTATTATCTTTACCTGTTTTAGCAGGTGCTATTACAATACTATTAACC
GATCGAAATTTTAACACTTCATTTTTTGACCCTTCAGGAGGAGGGGATCCAATTTTATATCAACATTTAT
TTTGATTTTTCGGGCATCCAGAAGTATATATTTTAATCCTTCCAGGATTTGGTATAATTTCTCAAATTAT
TTGTTATAATACAGGTAAAAAAGAACCTTTTGGAAATCTAGGTATAATTTATGCTATAGCAGCAATTGGG
TTATTAGGATTTATTGTGTGAGCTCACCATATATTTACAGTTGGCATAGATGTAGACACTCGAGCTTATT
TTACATCGGCAACAATAATCATTGCCGTTCCTACTGGAATTAAAATTTTTAGTTGACTAGCCACTTTACA
TGGTTCTAACATTAAATTTAATACTTCAATTTTATGAGCTTTAGGATTTGTCTTTTTATTTACAGTAGGA
GGACTTACTGGAATTATATTAGCTAATTCCTCTATTGACATCGTCCTTCATGACACTTATTATGTAGTAG
CTCACTTCCATTACGTATTATCAATAGGAGCAGTATTTGCTATTATAGGAGCTATTATTCATTGATTTCC
TATATTTTTTGGATTAAATTTAAATTCAATATTAACAAAAGTTCAATTTATAATTACATTCATTGGAGTT
AATTTAACTTTTTTTCCACAACATTTTCTAGGCTTAGCTGGAATACCACGTCGTTATTCAGATTACCCAG
ATTTTTTTTCTAAATGAAATTTCGTATCTTCTTTAGGATCTCTTATTTCTTTAACAGGAGTAATCATATT
AATTATTATTATCTGAATTAGAATCGTCGAAAAGAAAATAATTAATTTTCCTTCATTTACCAATTCTTCT
ATTGAATGAATATTAAATTTTCCACCATCAGAACATTCTTTTAACCAAAATAATATTATTCTTAAGTAA

Figure 3.4A: *Rhipicephalus sanguineus* cytochrome oxidase subunit 1 gene (accession number KM494916.1). yellow color represent forward and reverse primers for *COX-1* short, while the green color represent *COX-1* long primers.

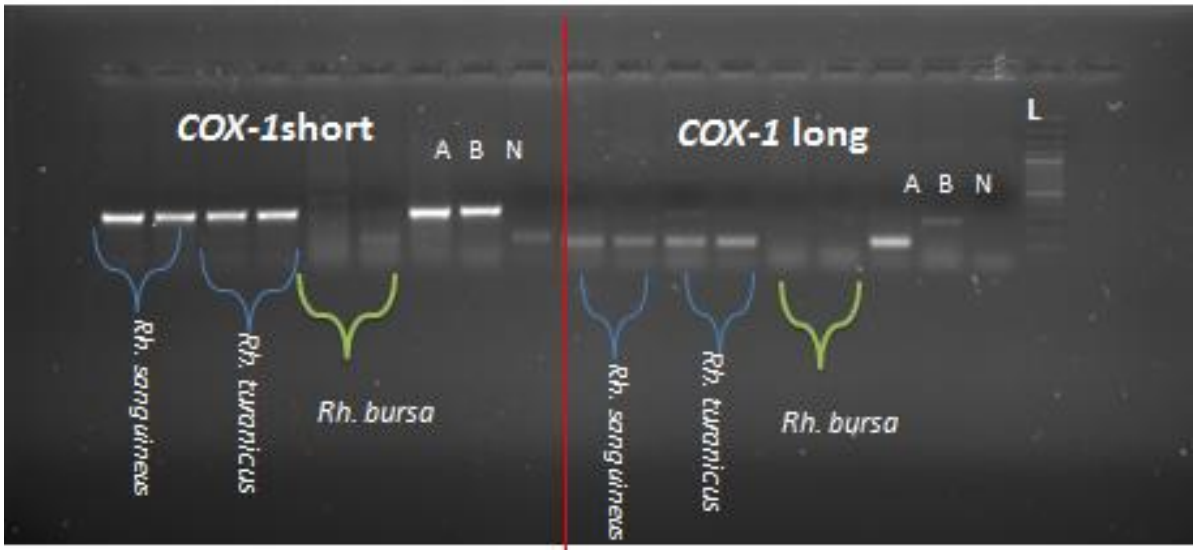


Figure 3.4B: PCR products of (*COX-1*short), and (*COX-1* long) primers of *R. sanguineus* and *R. turanicus.* No products for *R. bursa* tested samples. A: positive control of *R. sanguineus*, B: positive control of *R. turanicus*, N: negative control, L: DNA molecular weight marker (100 bp).

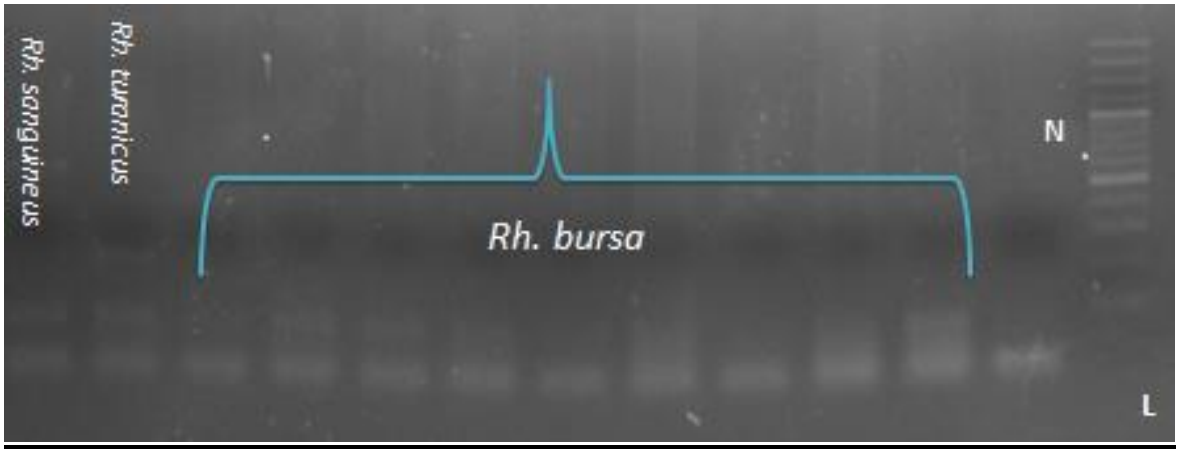


Figure 3.4C: PCR products of (*COX-1* combined P1 (F(short) +R(long)) primers. No products for all species were observed, N: negative control, L: DNA molecular weight marker (100 bp).

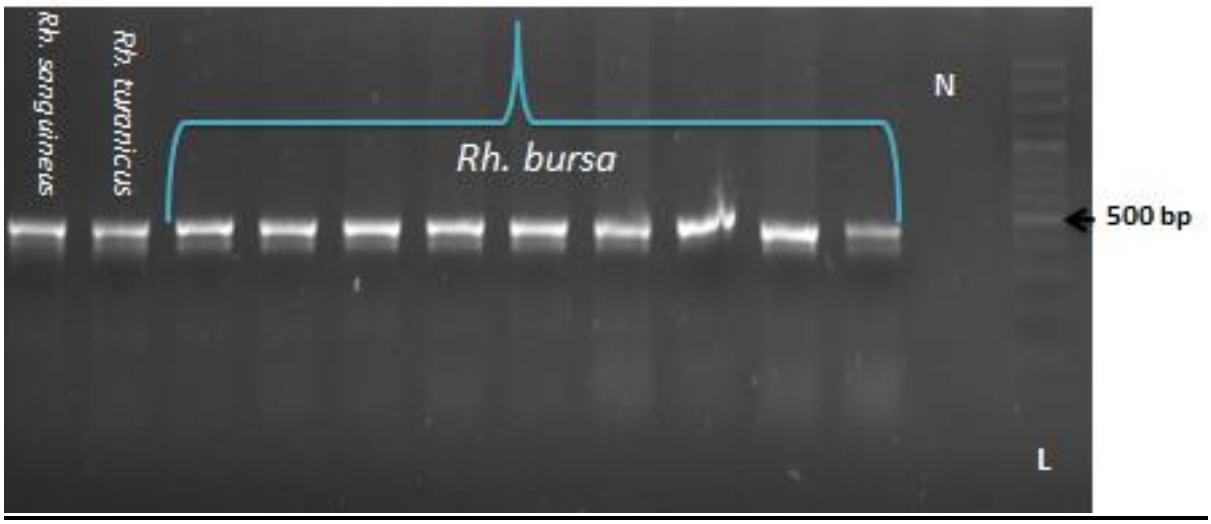
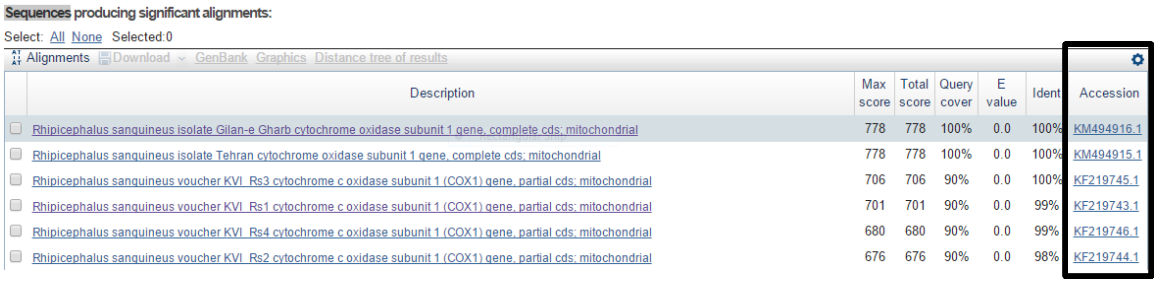


Figure 3.4D: PCR products of (*COX-1* combined P2(F(long) +R(short))primer. All species produced apmlicons at the DNA size~ 450 bp, N: negative control, L: DNA molecular weight marker (100 bp).

3.4 PCR amplification of *COX-1*

To investigate the reliability of using *COX-1* gene as a genetic marker for tick species identification, DNA control samples of the *R. bursa*, *R. sanguineus and R. turanicus* (Kindly provided by Dr. Yuval Gottlieb -The Hebrew University) were amplified and sequenced (see appendix A, sequences A13-A17). BLAST analysis revealed that the control samples had 100% homology with their respective reference sequences of *R. sanguineus*, *R. turanicus* and *R. bursa* deposited in the GeneBank (Fig. 3.5A, B), (Fig. 3.6A, B), (Fig. 3.7). All DNA sequences were aligned to each other using website

software: http://multalin.toulouse- .inra.fr/multalin/. The reference DNA sequences of the three species were downloaded and aligned. In addition three DNA sequences obtained from local ticks representing the species (according to microscopy) were included. Analysis of all sequences revealed some variations between species which can be used to differentiate them by RFLP (Fig. 3.8).



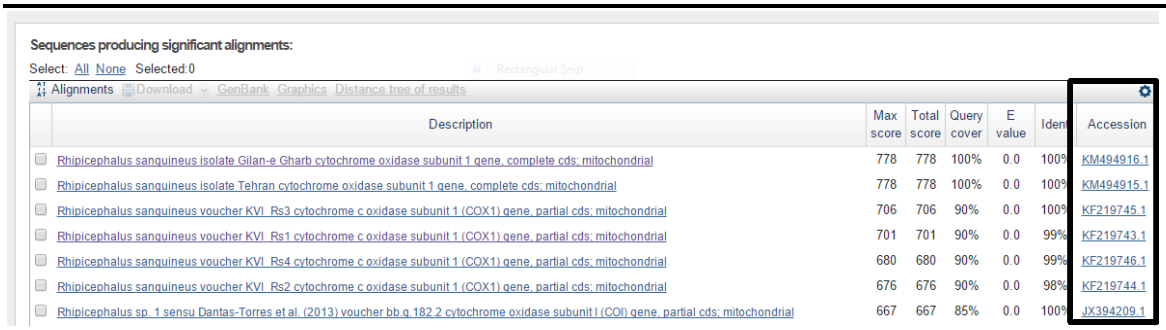
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	778	778	100%	0.0	100%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	778	778	100%	0.0	100%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	90%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	90%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	90%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	90%	0.0	98%	KF219744.1

Figure 3.5A: BLAST of positive control (designated S23S) of *R. sanguineus COX-1* sequence against reference strains sequences (the accession numbers are in box).



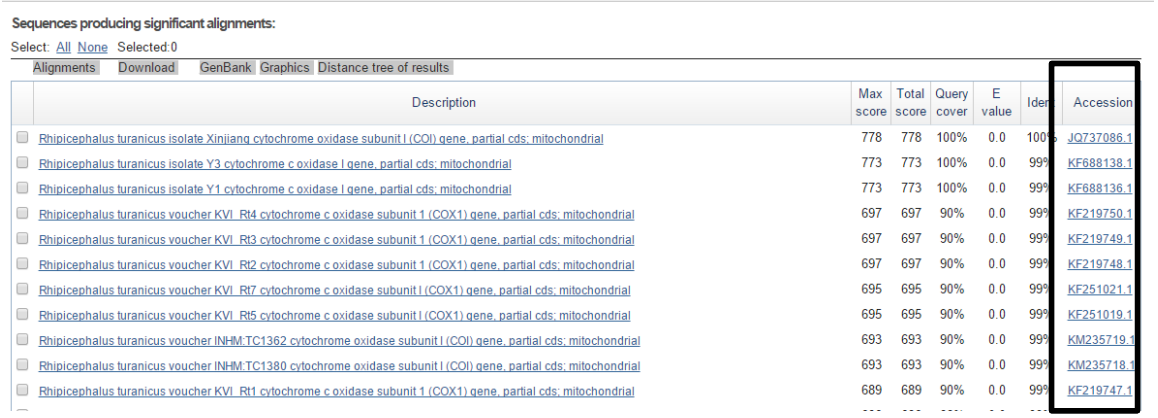
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	778	778	100%	0.0	100%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	778	778	100%	0.0	100%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	90%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	90%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	90%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	90%	0.0	98%	KF219744.1
Rhipicephalus sp. 1 sensu Dantas-Torres et al. (2013) voucher bb.g.182.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	667	667	85%	0.0	100%	JX394209.1

Figure 3.5B: BLAST of positive control (S19S) of *R. sanguineus COX-1* sequence against reference sequences (the accession numbers are in box).



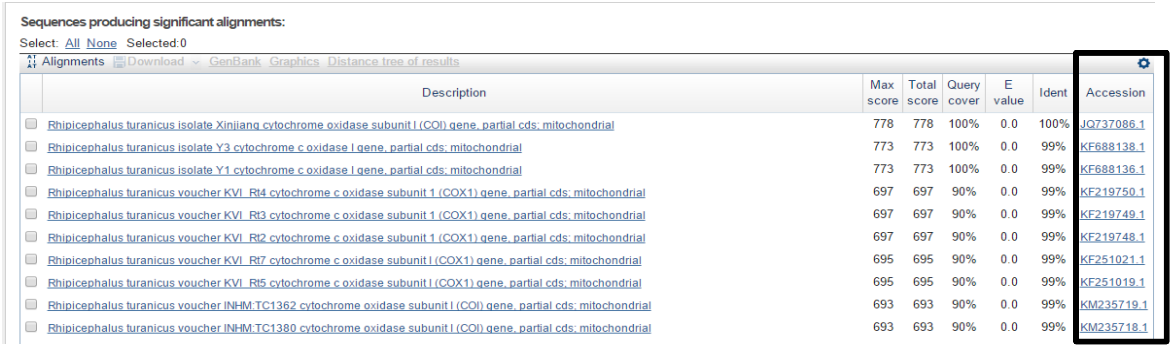
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate Xinjiang cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	778	778	100%	0.0	100%	JQ737086.1
Rhipicephalus turanicus isolate Y3 cytochrome c oxidase I gene, partial cds; mitochondrial	773	773	100%	0.0	99%	KF688138.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	773	773	100%	0.0	99%	KF688136.1
Rhipicephalus turanicus voucher KVI_Rt4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219750.1
Rhipicephalus turanicus voucher KVI_Rt3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219749.1
Rhipicephalus turanicus voucher KVI_Rt2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219748.1
Rhipicephalus turanicus voucher KVI_Rt7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	695	695	90%	0.0	99%	KF251021.1
Rhipicephalus turanicus voucher KVI_Rt5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	695	695	90%	0.0	99%	KF251019.1
Rhipicephalus turanicus voucher INHM:TC1362 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	693	693	90%	0.0	99%	KM235719.1
Rhipicephalus turanicus voucher INHM:TC1380 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	693	693	90%	0.0	99%	KM235718.1
Rhipicephalus turanicus voucher KVI_Rt1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	689	689	90%	0.0	99%	KF219747.1

Figure 3.6A: BLAST of positive control (84T) of *R. turanicus COX-1* sequence against reference sequences (the accession numbers are in box).



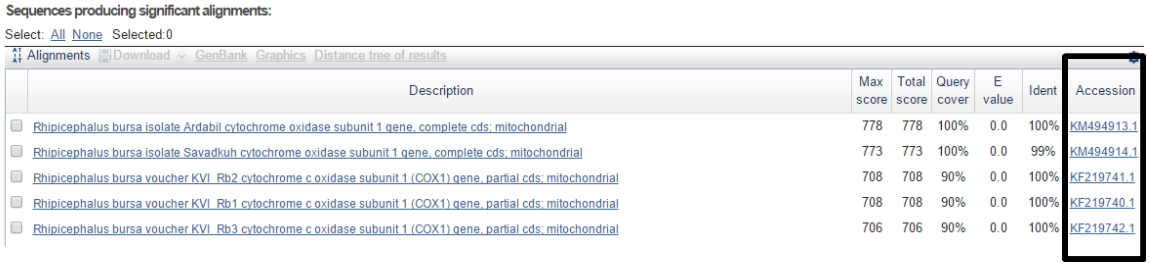
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate Xinjiang cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	778	778	100%	0.0	100%	JQ737086.1
Rhipicephalus turanicus isolate Y3 cytochrome c oxidase I gene, partial cds; mitochondrial	773	773	100%	0.0	99%	KF688138.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	773	773	100%	0.0	99%	KF688136.1
Rhipicephalus turanicus voucher KVI_Rt4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219750.1
Rhipicephalus turanicus voucher KVI_Rt3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219749.1
Rhipicephalus turanicus voucher KVI_Rt2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	697	697	90%	0.0	99%	KF219748.1
Rhipicephalus turanicus voucher KVI_Rt7 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	695	695	90%	0.0	99%	KF251021.1
Rhipicephalus turanicus voucher KVI_Rt5 cytochrome c oxidase subunit I (COX1) gene, partial cds; mitochondrial	695	695	90%	0.0	99%	KF251019.1
Rhipicephalus turanicus voucher INHM:TC1362 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	693	693	90%	0.0	99%	KM235719.1
Rhipicephalus turanicus voucher INHM:TC1380 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	693	693	90%	0.0	99%	KM235718.1

Figure 3.6B: BLAST of positive control (89T) of *R. turanicus COX-1* sequence against reference sequences (the accession numbers are in box).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus bursa isolate Ardabil cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	778	778	100%	0.0	100%	KM494913.1
Rhipicephalus bursa isolate Savadkuh cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	773	773	100%	0.0	99%	KM494914.1
Rhipicephalus bursa voucher KVI_Rb2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	90%	0.0	100%	KF219741.1
Rhipicephalus bursa voucher KVI_Rb1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	90%	0.0	100%	KF219740.1
Rhipicephalus bursa voucher KVI_Rb3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	90%	0.0	100%	KF219742.1

Figure 3.7: BLAST of positive control of *R. bursa* (BC3BRC) *COX-1* sequence against reference strains sequences (the accession numbers are in box).

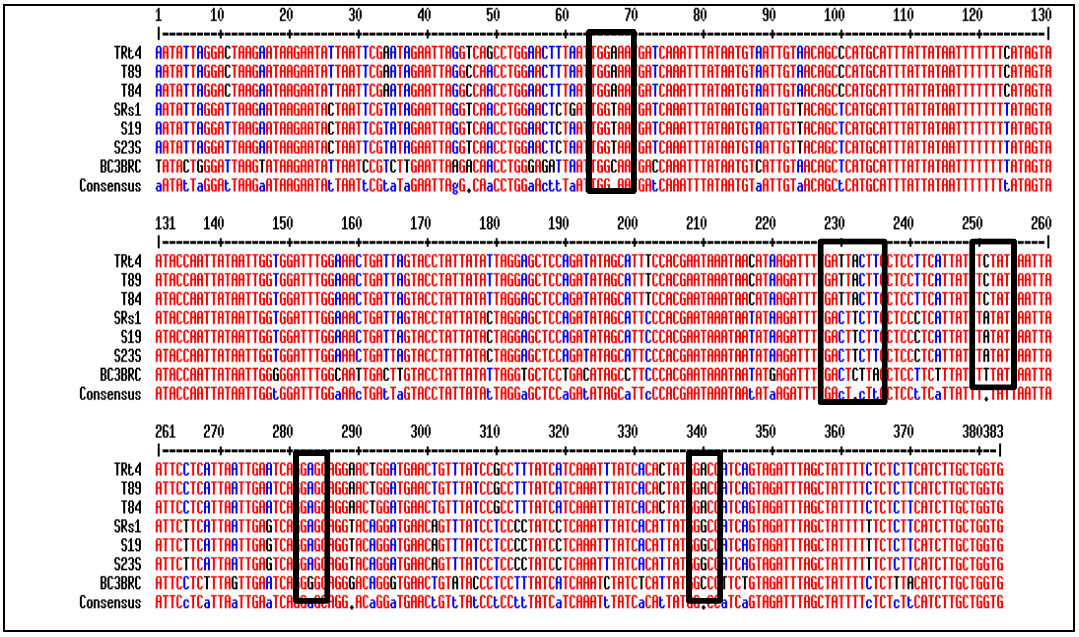


Figure 3.8: A Multiple alignments of positives controls sequences of *COX-1* gene and reference strains from the GeneBank obtained in this study: shows 57 variations between the different *Rhipicephalus* species (marked in black squares). TRt4 is reference strains of *R. turanicus* from the GeneBank, T89, and T84 are positive controls of *R. turanicus*, SRs1: is reference strains of *R. sanguineus* from the GeneBank, S19, and

S23S are positive control of *R. sanguineus*, while BC3BRC is positive control of *R. bursa*. Red color represent similarities between nucleotides.

Afterwards, all DNA extracts of our collected ticks (n=351) were subjected to the *COX-1*- PCR. Figure 3.9 bellow represents an example of the amplificaion results of *COX-1*- PCR; the sample was considered positive when a band of ~450bp was observed on 2% agarose gel, lanes (1-3) represent unidentified hard ticks species while the other lanes represent positive controls (well identified species based on microscopy and DNA sequencing) of *R. bursa, R. sanguineus* and *R. turanicus*, respectively.

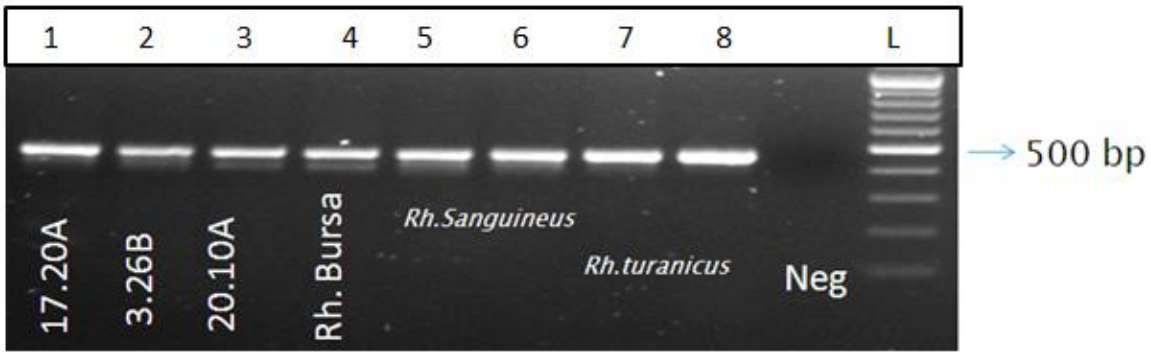


Figure 3.9: PCR result of *COX-1* gene: lanes 1-3 represent three tick samples while the other lanes represent positive controls (well identified species) of *R. bursa, R. sanguineus*, and *R. turanicus*, respectively, Neg: negative control, M: DNA molecular weight marker (100 bp).

3.5 PCR specificity of *COX-1*- PCR

Out of 351 tested samples, 342 samples were successfully amplified by *COX-1*- PCR. Interestingly, all samples of the *Haemaphysalis* species (9/351) 2.6% (six *H. parva* and three *H. adleri*) were PCR negative using the same forward and reverse primers, and the same PCR conditions (Fig. 3.10). Therefore, the described PCR system can be considered as *Rhipicephalus* genus specific.

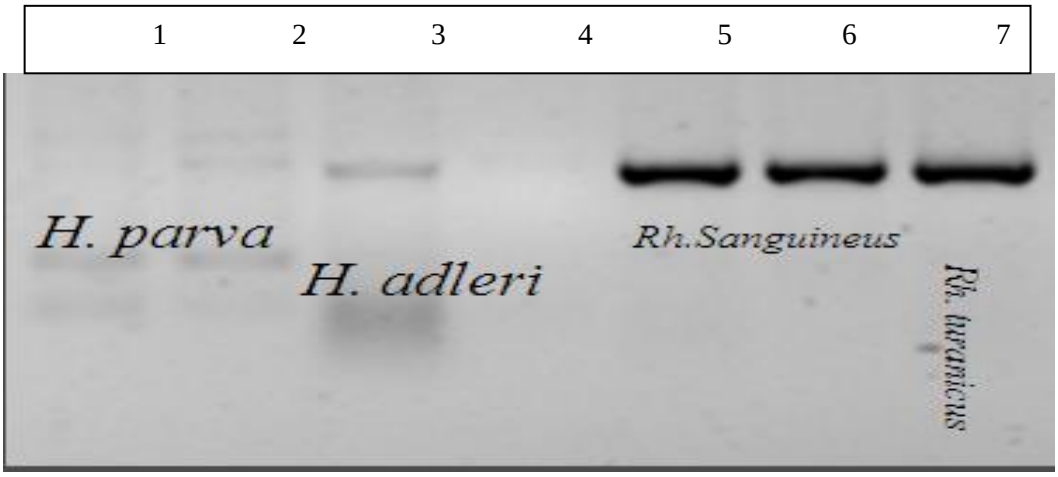


Figure 3.10: PCR specifity of *COX-1* gen*:* Lanes (1and 2) represent *H. parva*, (3and 4) represent *H. adleri,* (5, and 6) are positive controls of *R. sanguineus*, lane (7) represent positive control of *R. turanicus*.

3.6 Virtual cut of sequnces

The PCR products of the control samples were sent for sequencing, the obtained results were used to setup the DNA cuts for correct identification of the tested samples. The restriction site position and fragments length were determined by NEBcutter software (http://nc2.neb.com/NEBcutter2/). The virtual cut of the different tick species, revealed different banding patterns for each species: the expected bands for *R. bursa* were (258, 102, 56 and 27) bp, (223,171, and 27) bp for *R. turanicus*, and (171, 97, 87 and 28) bp bands for *R. sanguineus* (Fig.3.11).

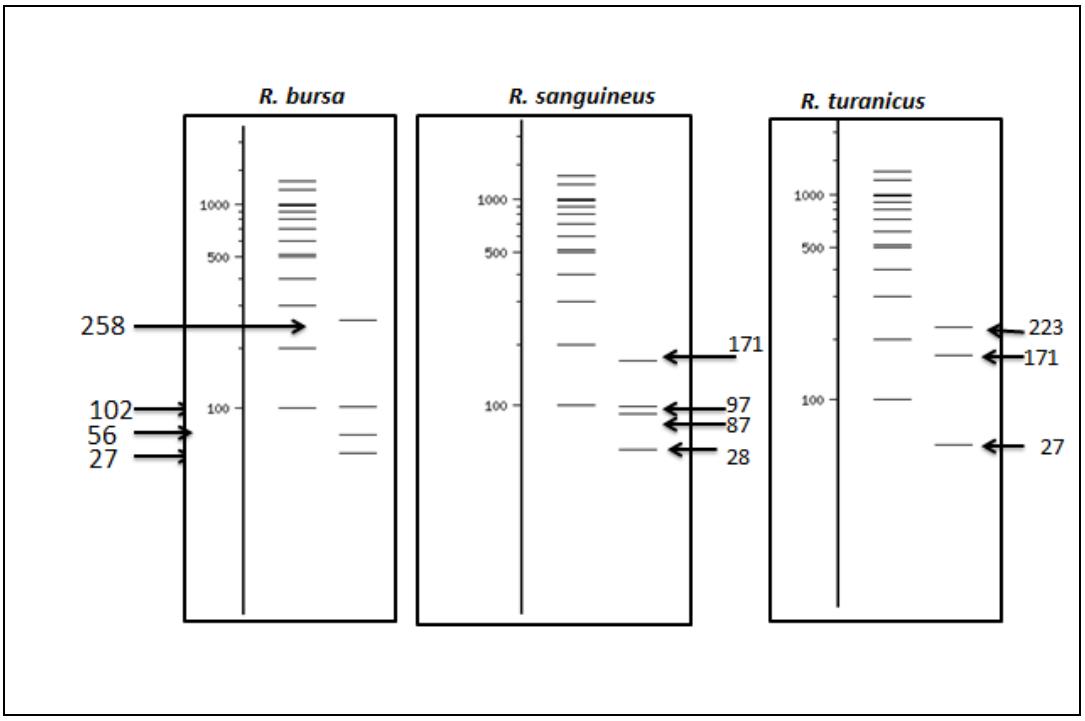


Figure 3.11: virtul cut of *COX-1* gene of *R. bursa*, *R. sanguineus*, and *R. turanicus* sequnces using *ALU1* enzyme.

3.7 Field Ticks identification by PCR-RFLP

Molecular typing of *Rhipicephalus* ticks was performed by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified fragments of the *COX-1* gene using *ALU1* enzyme. The obtained banding patterns were clearly distinguishing the three species as expected from the virtual cut. However, some bands (the small ones 27, and 28 bp) obtained by virtual cut were not visualized on the gel (Fig. 3.11). Following digestion reaction, three bands at the molecular level of (258, 102, 56) bp were observed for *R. bursa* samples, (223, and 171) bp for *R. turanicus*, and (171,97) bp for *R. sanguineus*. The tested samples showed identical RFLP patterns to that of control samples and thus identified accordingly. For example, in figure 3.12, the samples in the first two lanes were identified as *R. sanguineus* as they showed identical patterns (two bands of 171and 98 bp) to the control DNA of *R. sanguineus* (lanes 5 and 6). The sample in the third lane belonged to the *R. bursa*, it showed three bands at the molecular levels of 258, 102 and 56 bp which was identical to the banding pattern of the positive control *R. bursa* (lane 4) while lanes 7 and 8 represent the banding pattern of *R. turanicus*.

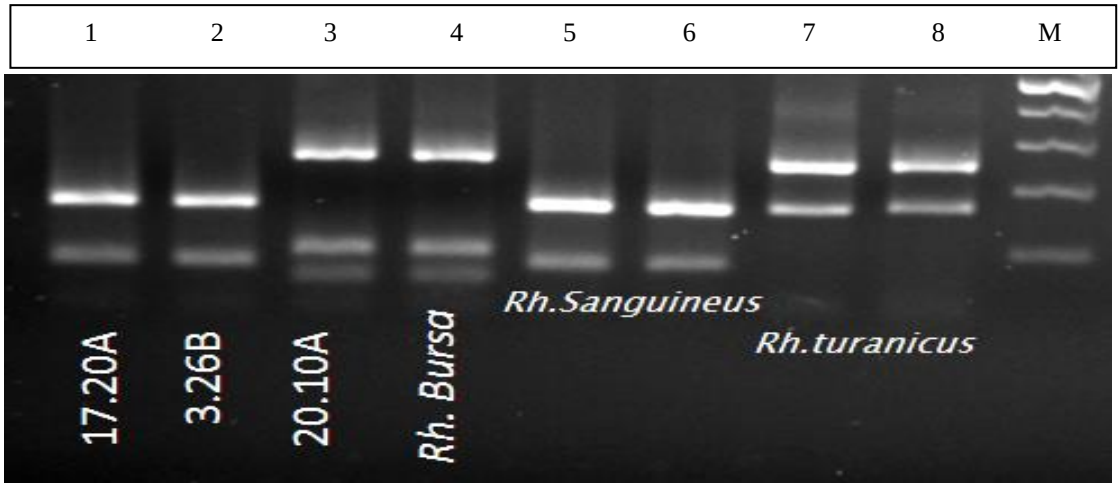
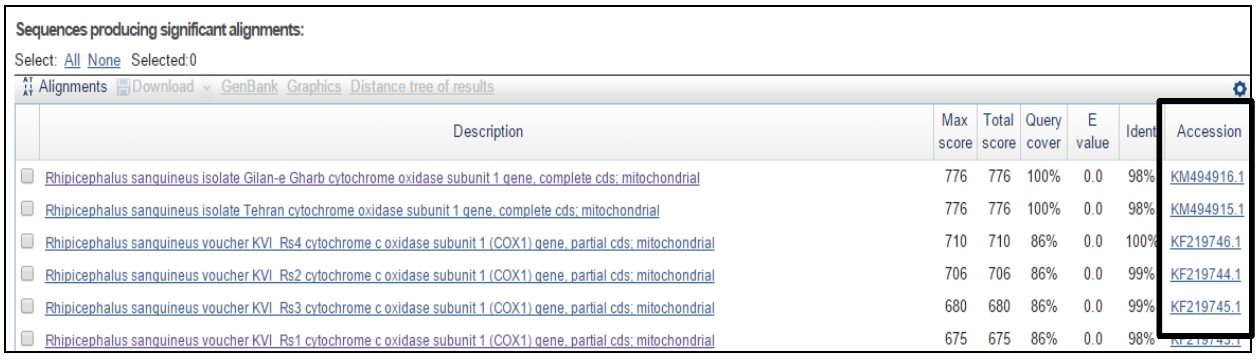


Figure 3.12: Restriction fragment length polymorphism (RFLP) analysis of the *COX-1*- PCR product using *ALU1* enzyme. The digested product was loaded on 3% agarose gel. Lanes 1 and 2: *R. sanguineus* (confirmed by sequencing), lane 3: *R. bursa*, compared to the positives controls of the three species of *R. bursa*, *R. sanguineus* and *R. turanicus*, M: DNA ladder(100 bp).

To confirm the reliability of the PCR- RFLP results, these samples were sent for DNA sequencing. The obtained DNA sequences (see appendix A, sequences: A19, A20, A21) and the subsequent BLAST analysis showed 98-99% homology of these DNA sequences (3.26B and 17.20A) to the reference sequences of *R. sanguineus* in the GeneBank (accession number, KM494916.1). Similarly, the DNA sequence of sample 20.10A showed 99-100% homology to the reference sequence of *R. bursa* (accession number is KM494913.1), as seen in the following figures (Fig. 3.13A,B and C).

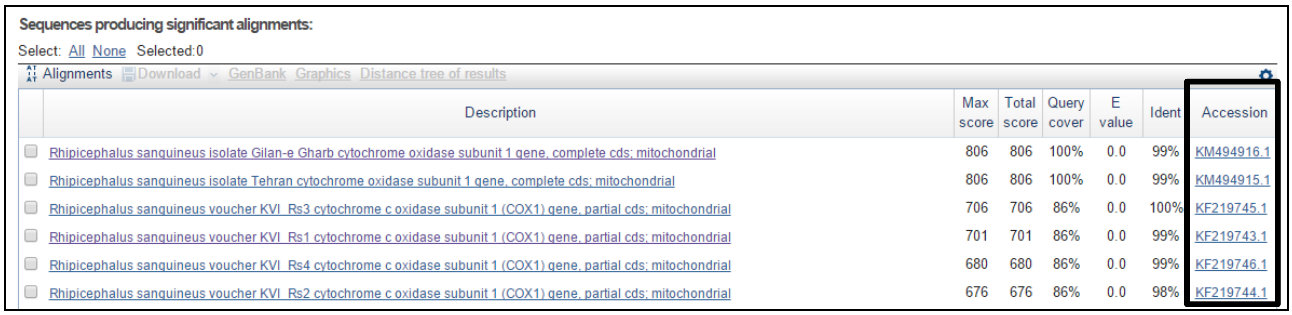

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	776	776	100%	0.0	98%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	776	776	100%	0.0	98%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	710	710	86%	0.0	100%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	99%	KF219744.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	86%	0.0	99%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	675	675	86%	0.0	98%	KF219743.1

Figure 3.13A: BLAST of *R. sanguineus* (designated 3.26B) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).



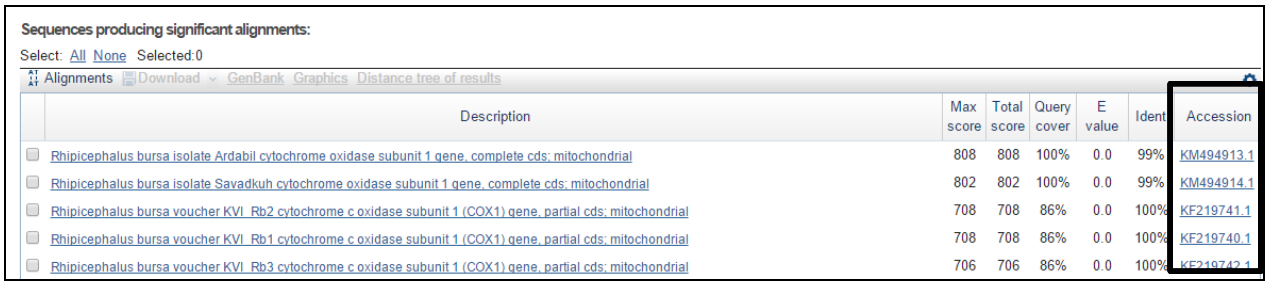
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	806	806	100%	0.0	99%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	806	806	100%	0.0	99%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	86%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	86%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	86%	0.0	98%	KF219744.1

Figure 3.13B BLAST of *R. sanguineus* (designated 17.20A) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus bursa isolate Ardabil cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	808	808	100%	0.0	99%	KM494913.1
Rhipicephalus bursa isolate Savadkuh cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	802	802	100%	0.0	99%	KM494914.1
Rhipicephalus bursa voucher KVI_Rb2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	86%	0.0	100%	KF219741.1
Rhipicephalus bursa voucher KVI_Rb1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	86%	0.0	100%	KF219740.1
Rhipicephalus bursa voucher KVI_Rb3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	100%	KF219742.1

Figure 3.13C: BLAST of *R. bursa* (designated 20.10A) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).

To confirm the identity of the nucleotide sequences, and to compare the sequences of our samples of *Rhipicephalus* ticks to those of the positives controls and to the reference sequences of *R. bursa, R. sanguineus*, and *R. turanicus*, all sequences were aligned to each other using the following website http://multalin.toulouse.inra.fr/multalin/, No genetic variations were obtained among the tested sequences within the same species, the alignment results are shown in (Fig.3.14).

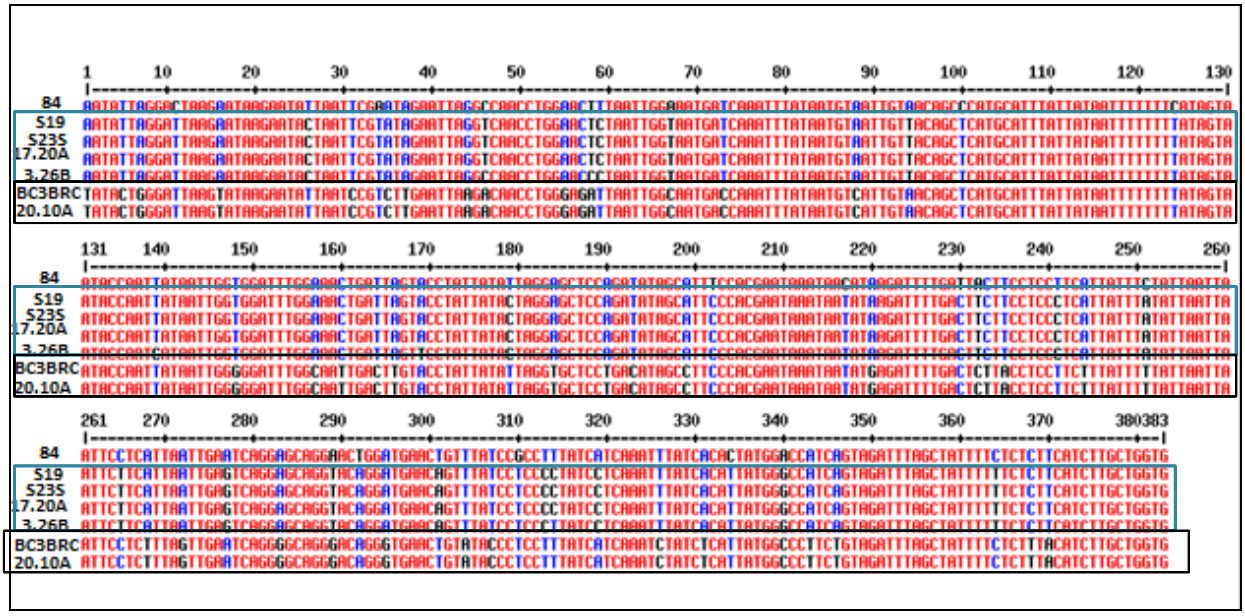


Figure 3.14: A multiple alignment for three tested *Rhipicephalus* ticks species with positive controls of *R. bursa, R. sanguineus* and *R. turanicus* of the *COX -1* gene:

S19 and S23S are positive controls of *R. sanguineus*, T84 is positive control of *R. turanicus*, BC3BRC is positive control of *R. bursa*, and (3.26B, 17.20A, and 20.10A) are tested samples.

A multiple alignment showing that sample (20.10A) is 99% sequence identity with the positive control of *R. bursa* (marked in black squares), while samples (17.20A and 3.26B) are 99% and 98% sequence identity with the positive control of *R. sanguineus* (marked in blue squares), respectively.

Following optimization and validation of our newly developed PCR-RFLP, all field samples (n=342) were subjected to *COX-1*- PCR- RFLP analysis. Among them (n= 277, 80.99%) were *R. sanguineu*s, (n= 11, 3.2%) were *R. turanicus*, and (n= 14, 4.09%) were *R. bursa*. Although the banding patterns were easily distinguishable on the 3% agarose gel, some samples (40/342) showed a single band of approximately 416 bp Figure 3.15 represents an example of tick identification, the tested samples showed identical RFLP patterns to that of control samples and thus identified accordingly. The samples in the first two lanes were identified as *R. sanguineus*, as they showed identical patterns to the control DNA of R. *sanguineus* (lane 10). The samples in lanes (3 and 4) belonged to the *R. bursa*, which were identical to the banding pattern of *R. bursa* (lane 11), while samples in lanes(5 and 6) represent the same banding pattern of *R. turanicus* in (lane 9). Samples in lanes 7 and 8 remained undigested. These results were confirmed by sequencing and blast analysis (Fig. 3.16A, B, C, and D).

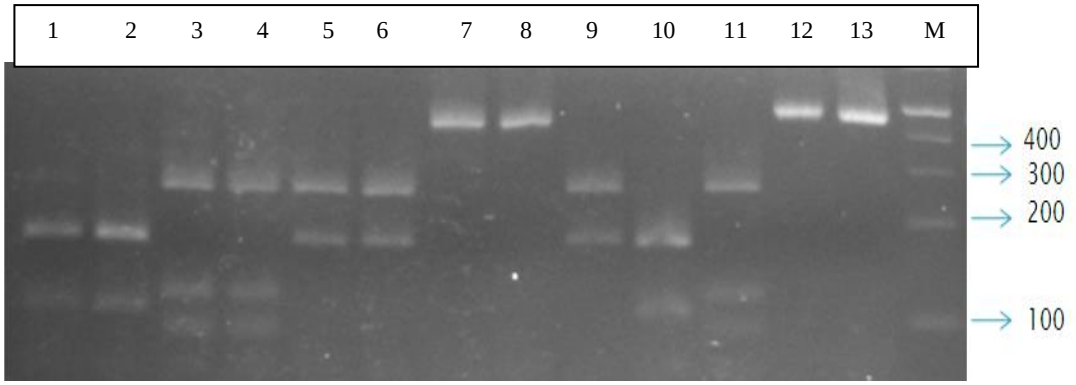


Figure 3.15: Identification of the field samples by *COX-1* PCR-RFLP analysis using *ALU1* enzyme. The digested product was loaded on 3% agarose gel. Lanes 1 and 2 represent samples of *R. sanguineus*, lanes 3 and 4: *R. bursa*, lanes 5 and 6: *R. turanicus*, lanes 7 and 8: uncut by *ALU1* enzyme, (see appendix A, sequences A22, A23, A20, A21, A47, A44, and A45), lanes 9,10 and 11: positive controls of *R. turanicus, R. sanguineus, R. bursa* respectively, lanes 12 and 13 undigested samples, and M: DNA marker (100 bp).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	85%	0.0	99%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	85%	0.0	99%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	73%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	73%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	73%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	73%	0.0	98%	KF219744.1
Rhipicephalus sp. 1 sensu Dantas-Torres et al. (2013) voucher bb.g.182.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	667	667	69%	0.0	100%	JX394209.1

Figure 3.16A: BLAST of tested sample (17.20B, lane 1 on Fig.3.16) of *R. sanguineus COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

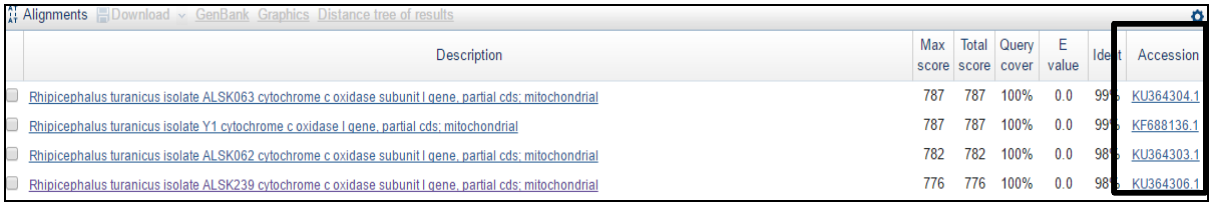
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus bursa isolate Ardabil cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	808	808	100%	0.0	99%	KM494913.1
Rhipicephalus bursa isolate Savadkuh cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	802	802	100%	0.0	99%	KM494914.1
Rhipicephalus bursa voucher KVI_Rb2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	86%	0.0	100%	KF219741.1
Rhipicephalus bursa voucher KVI_Rb1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	708	708	86%	0.0	100%	KF219740.1
Rhipicephalus bursa voucher KVI_Rb3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	100%	KF219742.1

Figure 3.16B: BLAST of *R. bursa* (20.10A, lane 3 on Fig.3.16) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	778	778	96%	0.0	100%	KU364304.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	778	778	96%	0.0	100%	KF688136.1
Rhipicephalus turanicus isolate ALSK062 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	773	773	96%	0.0	99%	KU364303.1
Rhipicephalus turanicus isolate Xinjiang cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	773	773	96%	0.0	99%	JQ737086.1
Rhipicephalus turanicus isolate ALSK239 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	767	767	96%	0.0	99%	KU364306.1

Figure 3.16C: BLAST of *R. turanicus* (21.15C lane 5 on Fig.3.16) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).



Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	787	787	100%	0.0	99%	KU364304.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	787	787	100%	0.0	99%	KF688136.1
Rhipicephalus turanicus isolate ALSK062 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	782	782	100%	0.0	98%	KU364303.1
Rhipicephalus turanicus isolate ALSK239 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	776	776	100%	0.0	98%	KU364306.1

Figure 3.16D: BLAST of uncut tested sample (21.8A lane 7 on Fig.3.16) *COX-1* sequence against reference strains sequences (the accession numbers are marked with black square).

Therefore, the PCR products of some undigested samples (23/40) were purified and sent for DNA sequencing using the same primers described in material and method (section 2.4.1), (see appendix A, sequences from A24 to A46). According to the sequencing and BLAST analysis, these samples were identified as *R. turanicus* (see appendix B, from B3 to B25), the virtual cuts of these sequences showed two bands at the DNA levels of 416, 27bp or 416, 28 bp (Figure 3.17) (see appendix C, C3- C25). These bands (27 or 28 bp) were too small to be visualized on the agarose gel.

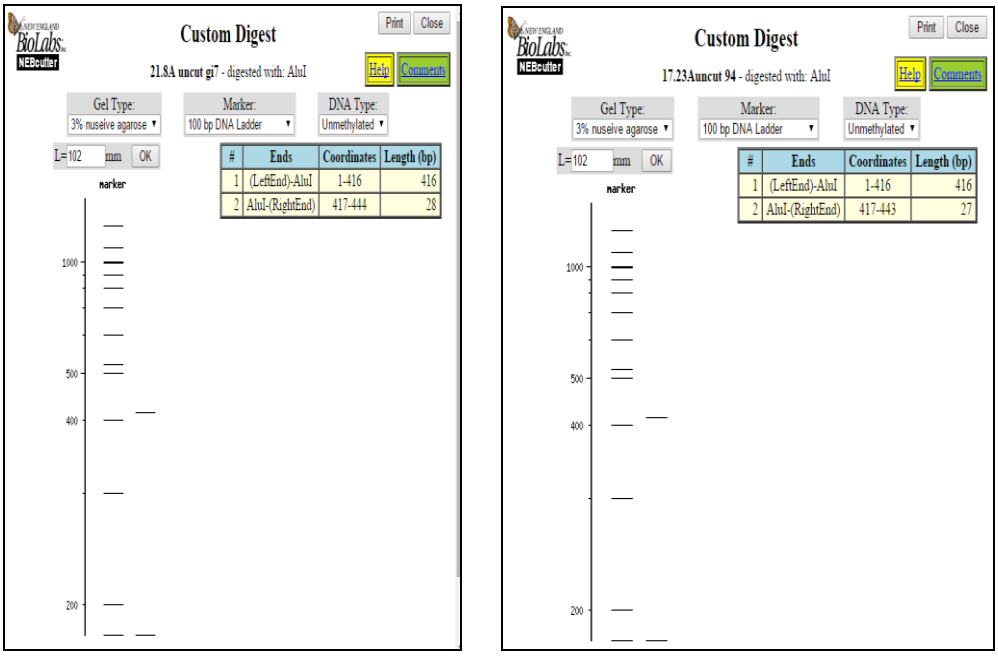


Figure 3.17: Virtul cuts of undigested *COX-1* gene using *ALU1* enzyme**.** Show tow bands at the DNA level size (416 and 28 or 27) bp.

However, all these sequences were aligned to each other and compared with positive controls of *R. turanicus*, the aligment showed several neclutieds variations between the sequncses of the unigestid samples and the positive controles of R. *turanicus* (Fig.3.18).

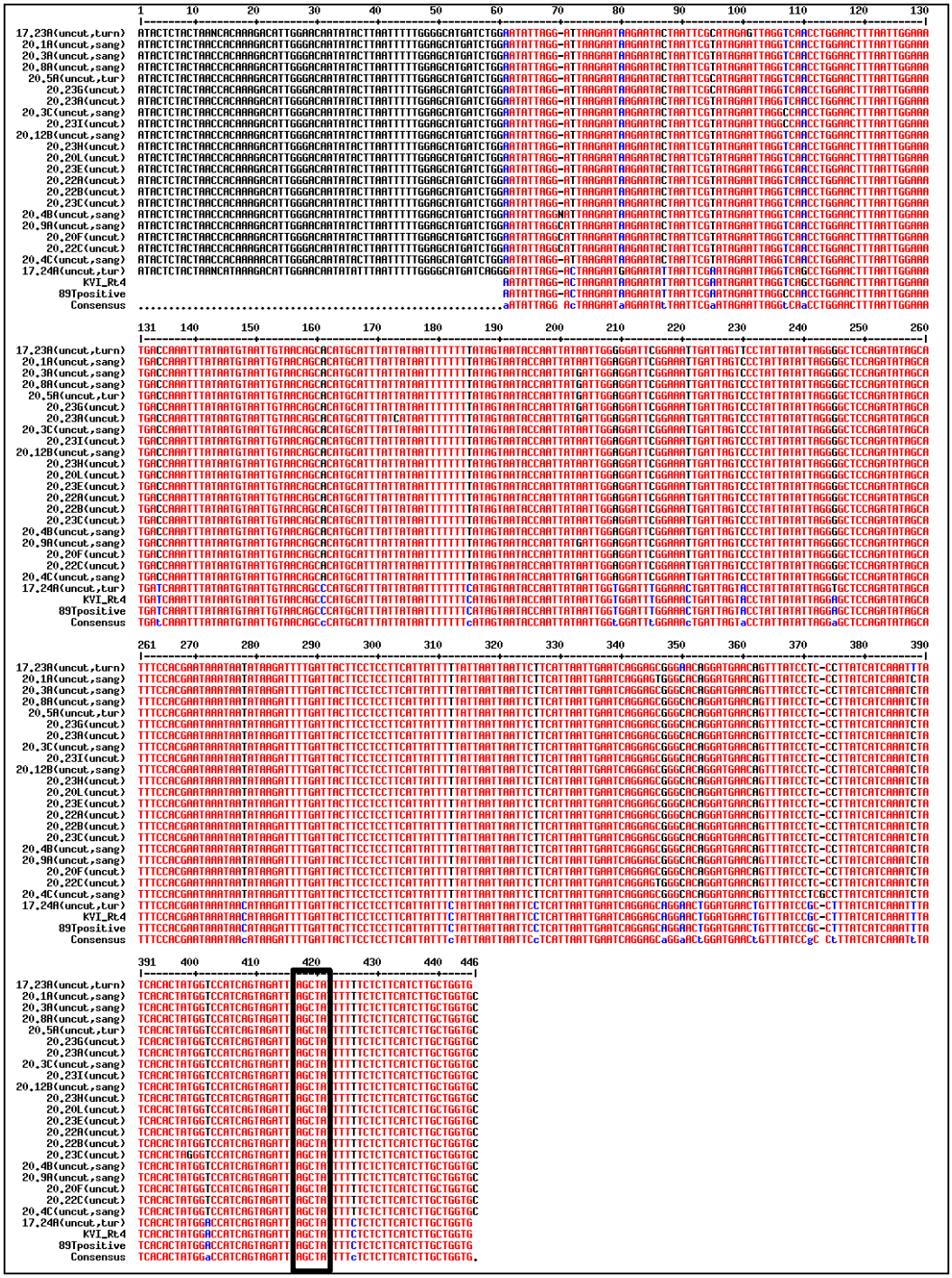


Figure 3.18: A multiple alignment of *DNA COX-1* samples that showed a single band of 416 bp on 3% agarose gel. 89T: positive control of *R. turanicus*, KVI-Rt4: reference strain of ***R****. turanicus* from GeneBank. The multiple alignment shows several variations between the undigested samples and the positive controls and the reference strain of *R. turanicus***.** *(Alu1* site in box). Red color represents similarities between nucleotides. Blue and black colors show nucleotides differences between species.

3.8 Comparison of PCR-RFLP and microscopic examination

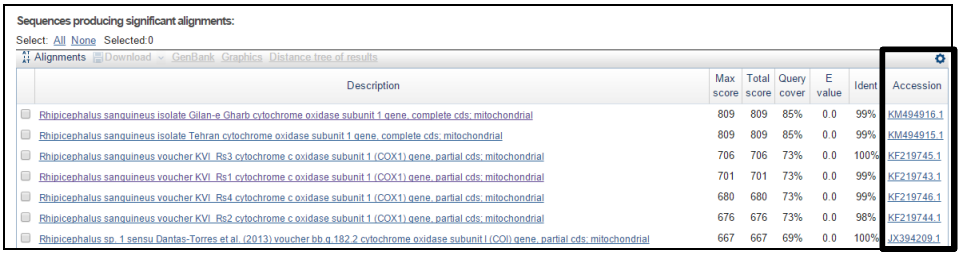
Comparison of the results from the two methods- analysis (*COX-1*- PCR- RFLP and microscopic testing) indicated differences in ticks identification (Table 3.5). By the two methods, 236 of the tested ticks were identified as *R. sanguineus*, tow *R. turanicus*, and 12 *R. bursa*. On the other hand, twenty five ticks which were identified as *R. turanicus* by microscopic examination were identified as *R. sanguineus* by *COX-1*- PCR- RFLP. Nine *R. sanguineus* ticks identified by microscopic examination were identified as *R. turanicus* by *COX-1*- PCR- RFLP. Moreover, 4.67% of *Rhipicephalus* ticks (16/342) were not identified to the species level by microscopic examination. All these samples were identified as *R. sanguineus* by *COX-1*- PCR- RFLP.

To confirm the results, the PCR products of 35 randomly samples were sent for DNA sequencing, the obtained sequences and blast analysis confirmed the results of RFLP. All these samples showed 93-100% homology to the reference DNA sequence of *R. turanicus* (see appendix A, and B).

For example the tick DNA sample designated 17.20B was identified microscopically as *R. turanicus* but it was identified as *R. sanguineus* by RFLP, the sequencing results, Blast analysis and the virtual cuts confirmed the result of *COX-1*-PCR- RFLP as shown (Fig. 3.19A, and B: Comparison of PCR-RFLP and microscopic examination).

COX-1 sequence of 17.20B.

AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTCT 60
AATTGGTAAT GATCAAATTT ATAATGTAAT TGTTACAGCT CATGCATTTA TTATAATTTT 120
TTTTATAGTA ATACCAATTA TAATTGGTGG ATTTGGAAAC TGATTAGTAC CTATTATACT 180
AGGAGCTCCA GATATAGCAT TCCCACGAAT AAATAATATA AGATTTTGAC TTCTTCCTCC 240
CTCATTATTT ATATTAATTA ATTCTTCATT AATTGAGTCA GGAGCAGGTA CAGGATGAAC 300
AGTTTATCCT CCCCTATCCT CAAATTTATC ACATTATGGG CCATCAGTAG ATTTAGCTAT 360
TTTTTCTCTT CATCTTGCTG GTG 383


Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	85%	0.0	99%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	85%	0.0	99%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	73%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	73%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	73%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	73%	0.0	98%	KF219744.1
Rhipicephalus sp. 1 sensu Dantas-Torres et al. (2013) voucher bb.g.182.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	667	667	69%	0.0	100%	JX394209.1

Figure 3.19A: BLAST of *R. sanguineus COX-1* sequence (17.20B) against reference strains sequences (the accession numbers are marked with bold frame).

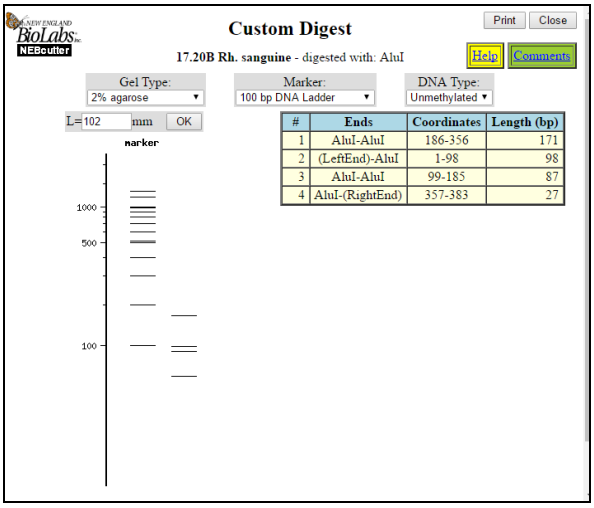


Figure 3.19B: The virtual cut of the sample 17.20B: Showing four bands at the DNA level size (171, 98, 87 and 27) bp. The actual cut of this sample showed two bands at the DNA level of 171 and 98 bp, the band of 27 was not seen on the 3% agarose gel.

The discrepancies between the two tests were revealed by DNA sequencing followed by Blast analysis and virtual cut. The DNA sequences, Blast analysis and the virtual cut of representative samples were described in appendix A, B, and C.

Among 279 *R. sanguineus* ticks identified by microscopy, 32 samples showed one band of ~ 420 bp by *COX-1*- PCR- RFLP. Out of them 19 samples (19/32; 59.37%) were sent for DNA sequencing, the obtained sequences showed a relatively low homology (93%) to the sequence of *R. turanicus* in the GeneBank (accession no. KU364304.1) (see appendix A for sequences: A25, A27, A28, A29, A30, A31, A33, A34, A35, A36, A37, A38, A39, A40, A41, A42, A43, A45, and A46. And appendix B, for blast analysis: B4, B6, B7, B8, B9, B10, B12, B13, B14, B15, B16, B17, B18, B19, B20, B21, B22, B24, and B25).

Furthermore, out of 34 *R. turanicus* ticks identified by microscopy, seven samples remained unidentified by *COX-1*-PCR-RFLP as they showed one band of ~ 420 bp, four of them(4/7; 57.1%) were sent for DNA sequencing, the obtained sequences revealed (93% - 94%) similarity to the sequence of *R. turanicus* deposited in the GeneBank (accession no. KU364304.1) (see appendix A, sequences: A24, A26, A32, and A44, and

appendix B to see the accession no.: B3,B5, B11, and B23).Additionally, two samples were identified as *R. sanguineus* by microscopic examination, but they gave the same banding pattern of *R. bursa* by PCR-RFLP.

Table 3.5: *COX1*-PCR-RFLP results compared to the microscopic examination

Microscopy	**Restriction fragment length polymorphism**				
	R. sanguineus	***R. turanicus***	***R. bursa***	**Un cut**	**Total**
R. sanguineus	**236**	**9**	**2**	**32**	**279**
R. turanicus	**25**	**2**	**0**	**7**	**34**
R. bursa	**0**	**0**	**12**	**0**	**12**
Not identified species	**16**	**0**	**0**	**1**	**17**
Total	**277**	**11**	**14**	**40**	**342**

A highly significant correlation (P= 0.01) was observed between RFLP and microscopy classification.

3.9 Phylogenetic analysis

To infer the genetic relationships between the local *Rhipicephalus* ticks obtained from Palestine and other reference strains published in GenBank/EMBL/DDBJ databases, a phylogenetic tree-based on sequences of *COX-1* gene - was constructed by the neighbor-joining method using the CLUSTAL-X program (http://www.genome.jp/tools/clustalw/).

Phylogenetic analysis revealed four main clusters: *R. bursa* cluster, *R. sanguineus* cluster, and *R. turanicus* cluster genotype 1 (G1), and Genotype 2 (G2). In *R. bursa* cluster sequences were identical to each other and showed 99% sequence identity with *R. bursa* (accession number is KM494913.1; from Iran). In *R. sanguineus* cluster, the nucleotide *COX-1* sequences of *R. sanguineus* identified in this study were identical to each other and showed 98% - 100% homology to the respective *R. sanguineus* reference sequence obtained from Iran (accession no.KM494916.1). *COX-1* sequences of *R. turanicus* (G1)

cluster showed 100% sequence identity to the *COX-1* sequences of *R. turanicus* reference strain (accession no. KU364304.1) obtained from China- Kazakhstan. In cluster IV, the genotype 2 (G2) of *R. turanicus* sequences were identical to each other and showed only 93- 94% homology to the respective *R. turanicus* reference sequence (accession no. KU364304.1) obtained from China. These samples were not identified by RFLP and revealed as one band on agarose gel.

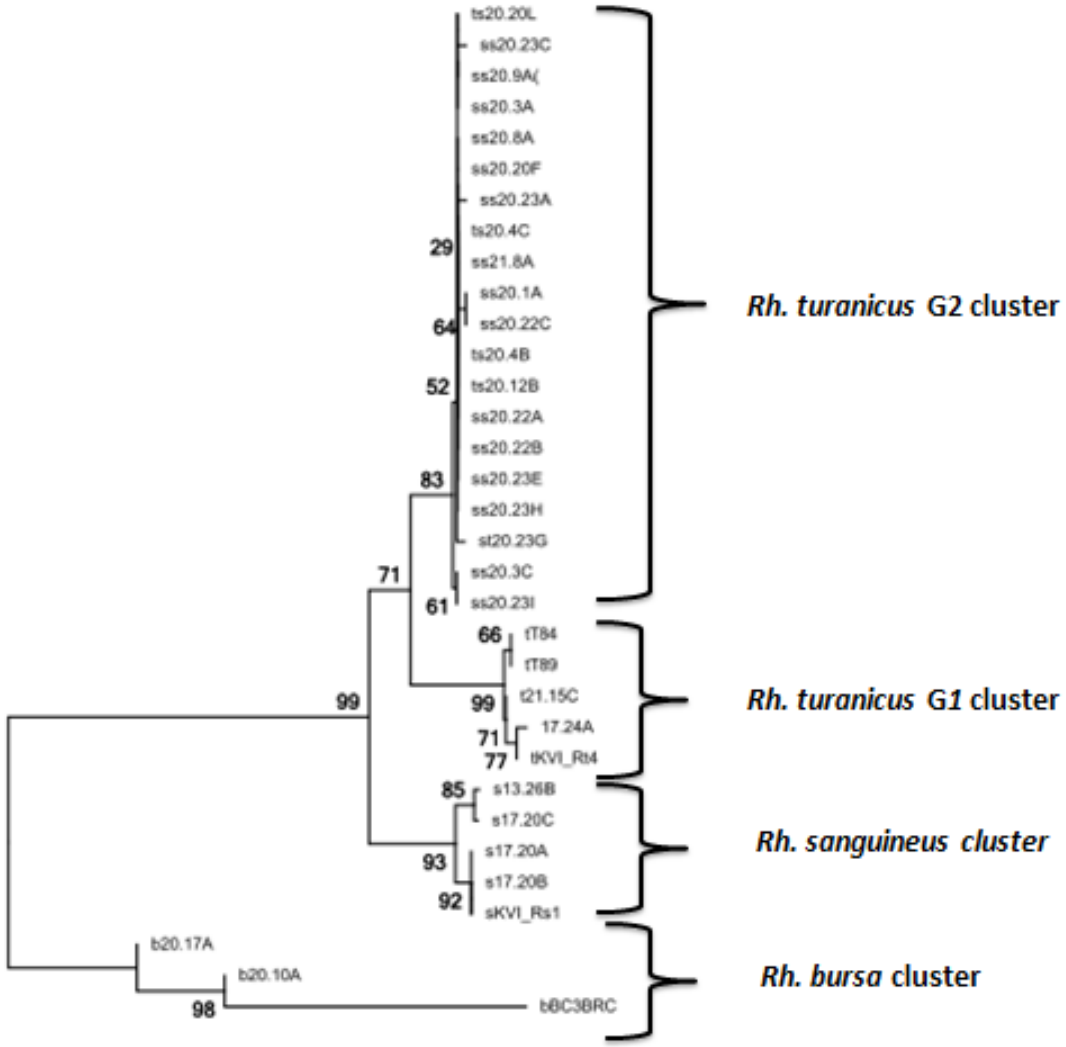


Figure 3.20: Phylogenetic analysis of Palestinian *Rhipicephalus* species based on *COX-1* gene. The tree was constructed by the neighbor-joining method using the CLUSTAL-X program (http://www.genome.jp/tools/ clustalw/) for the alignment of *Rhipicephalus* sequences obtained in this study to those of known *R.* species deposited in the GenBank/EMBL/DDBJ databases. tKV1Rt4: is reference strains of *COX-1* gene of *R. turanicus* from the GeneBank, T89, and T84 are positive controls of *R. turanicus*, SRs1: is reference strains of *R. sanguineus* from the GeneBank, S19, and S23S are positive control of *R. sanguineus*, BC3BRC is positive control of *R. bursa*, while others are different tested samples

Chapter 4

Discussion

Ticks are known to harbor intracellular bacteria. They are among the most efficient vectors of human and animal diseases because they attach firmly when sucking blood, feed slowly, and may remain unnoticed for a considerable time. Ticks can be active in Spring, Summer, and even in cool Winter, thus, several precautions should be taken to avoid tick bites and tick-borne diseases (Estrada-Pena et al., 2004; Inna Ioffe et al., 1997). Failure to control ticks and tick-borne diseases effectively is a major factor limiting livestock production. Ticks identification and differentiation has implications for studying disease transmission, vector-host association as well as geographic habitation range.

Microscopic examination is the most common method that used for tick identification which depends on morphological features of tick developmental stages (Vitale et al., 2006). However, this method is time consuming and the discriminating taxonomic features can be difficult to be used for differentiation, especially if the specimens are damaged, engorged with blood or morphologically incomplete particularly in nymphal and larval ticks. Furthermore, microscopic examination requires the expertise of a dedicated tick taxonomist (Zemtsova et al.,2014). Miss-identification can lead to wrong results and in the case of pathogen transmission, may implicate the incorrect vector species (Parola et al., 2005). Several studies have reported the implication of these ectoparasites in transmition of different pathogens causing several diseases of veterinary and public health importance such as: theileriosis, babesiosis, lyme disease, rocky mountains spotted fever, relapsing fever, tularemia, Colorado tick fever, Crimean-Congo hemorRagic fever, and cytauxzoonosis (Ereqat et al., 2016; Parola et al., 2005). Therfore, accurate differentiation of tick species is necessary.

Recently, several approaches using different genetic markers have been evaluated for the identification and phylogenetic studies of ticks. The extracted DNA from ticks harvested from tick borne disease foci could be used for identification of both the agent and the vector species (Black and Piesman, 1994; Crampton et al., 1996; Black et al., 1997; Klompen et al., 1996; Norris et al., 1997; Dobson and Barker, 1999).

This study included 351 hard ticks collected from dogs, sheep and goats from different cities in Palestine; based on microscopic examination, the most prevalent species was *R. sanguineus* (79.2%) followed by *R. turanicus* (9.7%), R. *bursa* (3.4%), H. *parva* (1.6%), and *H. alderi* (0.9%). Previous morphological studies to identify *Ixodid* ticks to the species level have been reported worldwide. In Iran, 3.16% of the examined ticks were *R. sanguineus* whereas 0.09% and 0.3% were reported from Sudan and Nigeria, respectively (Shemshad et al., 2012; Elghali and Hassan, 2009; Lorusso et al., 2013). Furthermore, the prevalence of *R. turanicus* were (1.2%) in Nigeria and (6.9 %) in Turkey (Lorusso et al., 2013; Keskin et al., 2015). These results were in disagreement with this study. In contrast to our study, *R. turanicus* was reported as the most dominant species (95.5%) in Tunisia (M'ghirbi et al., 2013). The prevalence of *R. turanicus* was (3.4%) in Egypt while *R. sanguineus* was the most prevalent species in occupied Palestine (Asmaa et al., 2014; Wilamowski et al., 1999).

Identification of ticks by molecular techniques using genetic markers have recently been considered to be appropriate approaches for correct identification, especially for population studies or surveys where hundreds of samples are studied. Currently, identification of ticks using genetic markers has not been carried out in Palestine and there is no database of DNA sequences from local tick samples. Therefore, our aim was to genetically identify local ticks collected from different mammalian hosts. This study focused on differentiation of *Rhipicephalus* species, especially on *R. sanguinus*, *R. turanicus* and *R. bursa*, since they are the most common species in Palestine (Ereqat et al., 2016).

The research findings showed that *16SrDNA* was not the suitable gene for differentiation of *Rhipicephalus* tick species. Attempts at creating primers targeting ~520pb of the *16S rDNA* gene were used based on some nucleotides variation observed in the alignment of the reference sequences of *Rhipicephalus* species obtained from Genebank. Unfortunately these variations were not enough to identify the tested ticks down to the species level.

The mitochondrial marker (*COX-1*) gene was used to establish the genetic relationship and generate species-specific restriction maps of the three *Rhipicephalus* species. A major consideration in this investigation was to identify tick species based on a single genetic marker by a relatively simple method compared to other molecular methods and morphological testing. The successful designed set of primers targeting ~463 bp fragment

of *COX-1* gene which specifically amplified *Rhipicephalus* genus DNA leaving both *Haemaphysalis* and *Hyalomma* template DNAs unamplified.

This study focused on the *COX-1* gene as a genetic marker since it is well known that the mitochondrial genome has small size and comparatively fast rate of evolution, compared with the nuclear genome. It was reported that there are a number of nucleotide positions in the *COX-1* sequence with no apparent intra-specific variation but distinct differences among different ticks (Hebert et al., 2003), hence it is considered a good tool to establish relationships of closely related species (Shao and Barker, 2007). Interestingly, *COX-1* sequence variations between related species from the same geographic region were adequate to distinguish between them. The developed *COX1*-PCR- RFLP assay, was able to differentiate between the most common species of *Rhipicephalus* ticks in our area. The restriction digestion profile was easily distinguishable on the 3% agarose gel suggesting that this method is appropriate approach for accurate and rapid identification of *Rhipicephalus* ticks. To the best of my knowledge, this is the first direct molecular method that can identify these hard ticks species.

The assay was optimized and validated against well identified samples and then applied to all collected samples which were identified by microscopy. The agreement between both techniques was significant ($p = 0.01$), indicating that the described assay can be used to identify *Rhipicephalus* ticks in our region. However, 10% (40/342) of tested samples, which were identified, by microscopic testing, as *R. turanicus* (*n=7*), *R. sanguineus*(n= 32), and unidentified species (n=1) uncut by *ALU1* enzyme showing a single band at ~420 bp, thus cannot be identified in this assay. To rule out the possibility of any technical error, these samples were re-amplified, digested and double checked by two investigators. The same results were obtained. To overcome this problem and to identify the undigested samples, the amplified products of these samples were analyzed by DNA sequencing. The BLAST analysis showed 93%- 94% sequence homology with the reference strain sequence of *R. turanicus* (KU364304.1), deposited in the GeneBank. However, the virtual cut of these samples revealed two bands of 416 and 26bp, only one band showed on gel by actual cut since the 26 bp fragment was not observed on agarose gel. These results indicate the presence of different genotypes of *R. turanicus* in our region, this genotype cannot be identified by the described assay. Therefore, DNA sequencing should be applied in this case.

Indeed, there are a growing number of studies in which mitochondrial genes were used as molecular markers for tick identification. A recent study (Erster et al., 2013), succeeded to identify *Rhipicephalus* species (*R. annulatus, R. bursa, R. sanguineus and R. turanicus*) using PCR-RFLP. However, this assay was laborious since it depends on using sequential amplification reactions targeting four mitochondrial markers: *12S rRNA*, *16S rRNA*, *COX-1* and cytochrome b (*CytB*), using two primers for each marker, followed by multiple digestion reactions by different restriction enzymes. In comparison, this study targeted a single marker (*COX-1* gene) followed by one digestion reaction, the obtained banding pattern was clearly differentiate between *R. sanguineus, R. turanicus*, and *R. bursa.* Thus, the presented method was simple, fast and reproducible; hence it can be routinely applied for screening tick species and possible identification.

A novel PCR-RFLP based assay to differentiate between the four most common Metastriate tick genera based on the length of the PCR amplicons and subsequent restriction digestion was conducted. In that study, four tick genera were investigated: *Dermacentor, Amblyomma, Rhipicephalus* and *Haemaphysalis*. Four primers multiplexed was used to amplify the mitochondrial *ITS-1*DNA gene of these ticks, the amplicons were unique for each genus of *A. americanum* and *Dermacentor* species whilst, RFLP analysis using *Tau I* enzyme differentiate *Rhipicephalus* from *Haemaphysalis* and *Ixodes* ticks as they cannot be distinguished directly by their multiplex PCR. The described study was unable to differentiate *Rhipicephalus* species by digestion reaction, unlike this study which could differentiate the *Rhipicephalus* species (Anderson et al., 2004).

Moreover, a study reported from West Africa succeeded to distinguish between the most prevalent *Rhipicephalus* species in their region: *Rhipicephalus* (*Boophilus*), *R.* (*Boophilus) annulatus*, *R. (Boophilus) decoloratus*, *R. (Boophilus) geigyi*, and *R. (Boophilus) microplus* using PCR–RFLP test, based on sequence differences in the second internal transcribed spacer (*ITS2*). The obtained digested profile using *Msp I* restriction enzyme, was able to distinguish between the four *R. (Boophilus)* species. (Lempereur et al., 2010).

Similarly, a PCR-RFLP approach was developed for differentiation of *Haemaphysalis punctata, Haemaphysalis parva, Ixodes ricinus*, and *Dermanyssus gallinae*, based on *16S*

rDNA, using restriction endonuclease *AflI* allowed the differentiation of the five hard tick species in West Africa (Chitimia et al., 2009). In this regard, several molecular approaches have been described using several target genes, in each of these studies, PCR has been employed using specifically designed set of primers, the PCR product is then sequenced , analyzed for nucleotide variations to distinguish one species from another (Qiu et al. 2002). Other studies described species-specific amplification of short products using qPCR differential melting curves and multiplex PCR (Lehmann et al., 2008; Szalanski et al., 2011). These procedures were time consuming, costly and need well equipped laboratories.

In this study and based on phylogenetic analysis four clusters were identified, high genetic homology was observed among three clusters (I, II and III) of the studied samples which showed 99% homology to the reference sequences within the same species. Interestingly, the samples which were unidentified by RFLP assay revealed a different genotype (designated G2) of *R. turanicus* and formed a separate cluster (IV). These sequences showed several nucleotide variations, some of them were in one restriction sites of *ALU 1* enzyme and thus were not recognized.

In conclusion, this is the first study to use molecular approach for identification of *Rhipicephalus* ticks obtained from domestic animals in Palestine. A tool was created that would be useful to investigators in other regions of the Middle East. Study findings provide the foundation for further epidemiological studies on ticks in Palestine. The study succeeded to distinguish between the closely related *Rhipicephalus* species: *R. bursa, R. turanicus*, and *R. sanguineus* regardless of life stage utilized for source DNA. We recommend to use our molecular approach described in this research to discriminate between these *Rhipicephalus* species, quickly, easily and inexpensively, either as a confirmation of microscopic identifications or verification for material processed in the laboratory for vector genetics or pathogen surveillance. Further experimental analysis are still needed to distinguish *R. turanicu*s of genotype G2 and other hard ticks circulating in Palestine.

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Appendix A

Representative sequences of *Rhipicephalus* species

Sequences of the positive controls of *16S rDNA* gene of *R. sanguineus*, *R. turanicus* and *R. bursa* and sequnces of different tested samples

Sequence A1: positive control of *Rhipicephalus sanguineus*(designated 23S)

CNCGTNGGCT GAAGTAGGCA GCCGGATCGG CATTTGGTGT GAGTTGAGCG AACTGTGGAA 60
GCGAAAGTGC GTGCGAGAAA CACATCTCAA AATATGGGTG GCACGCGACT GGGTCCCGGG 120
AAGCGGTGGA GGGAAAGGGA CCCGCCCAAA GGGCAAAAAA GTCGCGAAAC GAGACGGCAG 180
ACTCGTGTCG ATGGGGCCGA ACACTCCGCT CCTCCAGGGA GCAAGACGGA GGCGGCCCAC 240
CCGAGGCAGT GTAGCCGCCG AGACCCGCAA CGCATGCCGG CTCTTATCTG TTGGTCGGTG 300
TGCGATTTTC AAATGTTAGG TGGGTCGATG AGTGCGAGCA CACAAAGAAA CCTACGGGTC 360
CGGCCTGGGA AACCGGCTTC GACGAAACCC TGAAAACGAG ATGAAAAGCA CGCGCGAGTA 420
GCGGAAGCGA CGCGCCCACG AGAACCACCG TTGTTCGATT CGCGGCCGCC GAACAGGCGC 480
GGCGGANTTG GGGGAGAGCG CGCTGGAAAG CGCCCTCCGA 520

Sequence A2: positive control *R. sanguineus* (designated 18S)

TCCCGTNGGC TGAAGTAGGC AGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACCATCTC AAAATATGGG TGGCACGCGA CTGGGTCCCG 120
GGAAGCGGTG GGTGGAAAGG GACCCGCCCG AAGGGCAAAA AAGTCGCGAA ACGAGACAGC 180
AGACTCGTGT CGATGGGGCC GAACACTCCG CTCCTCCAGG GAGCAAGACG GAGGCGGCCC 240
ACCCGAGGCA GTGTAGCCGC CGAGACCCGC AACGCATGCC GGCTCTTATC TGTTGGTCGG 300
TGTGCGATTT TCAAATGTTA GGTGGGTCGA TGAGTGCGAG CACACAAAGA AACCTACGGG 360
TCCGGCCTGG GAAACCGGCT TCGACGAAAC CCTGAAAACG AGATGAAAAG CACGCGCGAG 420
TAGCGGAAGC GACGCGCCCA CGAGAACCAC CGTTGTTCGA TTCGCGGCCG CCGAACAGGC 480
GCGGCGGAGT TGGGGGAGAG CGCGCTGGAA AGCGCCCTCC GA 522

Sequence A3: positive control of *R. turanicus* (designated 89T)

TCCCGTNGGC TGAAGTAGGC AGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACCATCTC AAAATATGGG TGGCACGCGA CTGGGTCCCG 120
GGAAGCGGTG GGTGGAAAGG GACCCGCCCG AAGGGCAAAA AAGTCGCGAA ACGAGACAGC 180
AGACTCGTGT CGATGGGGCC GAACACTCCG CTCCTCCAGG GAGCAAGACG GAGGCGGCCC 240
ACCCGAGGCA GTGTAGCCGC CGAGACCCGC AACGCATGCC GGCTCTTATC TGTTGGTCGG 300
TGTGCGATTT TCAAATGTTA GGTGGGTCGA TGAGTGCGAG CACACAAAGA AACCTACGGG 360
TCCGGCCTGG GAAACCGGCT TCGACGAAAC CCTGAAAACG AGATGAAAAG CACGCGCGAG 420
TAGCGGAAGC GACGCGCCCA CGAGAACCAC CGTTGTTCGA TTCGCGGCCG CCGAACAGGC 480
GCGGCGGAGT TGGGGGAGAG CGCGCTGGAA AGCGCCCTCC GAAGCCNCCG TTG 533

Sequence A4: sequence of tested sample designated 20.11A (*R. bursa*)

TCCCGTTGGC TGAAGTAGGC AGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACATCTCG AAATATGGGT GGCACGCGAC TGGGTCCCGG 120
GAAGCGGAGG GTGGAAAGGG ACCCGCCCGA CGGGCAAAAA GTCGCGAAAC GAGACGGCAG 180
ACTCGTGTCG ATGGGGCCGA ACGCTCCGCT CCTCTATGGA GAGAGACGGG CGCGGCCCGC 240
CCGAGGCAGT GTAGCCGCCG AGACCCGCAC GCATGCCGGC TCTTATCTGT TGGTCGGTGT 300
GCGATTTTCA ATCGTTAGGC GGGTCGATGA GTGCGAGCAC ACGAAAGAAA CCTACGGGTC 360
CGGCCTGGGA AACCGGCTTC GACGAAACCC TGAAAACGAG ATNAAAAGCA CGCGCAAGTA 420
GCGGAAGCGA CACGCCCACG AGAACCACCG GTGTTNNATT CGCGNCCGCC GAACAGGCGC 480
NGN 483

Sequence A5: sequence of tested sample designated 20.10A (*R. bursa*)

GGCAANNTGC GGANGCGAAN NCTTGTGTAT TTCNTGCAAA AACTCNAANG CGGGTCCCGG 60
NNGGNCTGTG GTGGGAACGG GACCCGNCCG TGGTGCAGAG GGAGCCACGG TGCGAACGGG 120
AAGAGGCTTG TCGATGGGAC CGTGCATCCC GCTCTACGGA GCCGGGAGCC GGCCGCCCGA 180
GGAAGTGCAG CCGCCGAGGC CCGCATCCTG CGTGCACTCT TATCCAAATG GGTGTACCGC 240
ANTCATTTTA TGCGTTANGC GGNCCNATGA NAGCNAGNGC ACGACGATAC CTGCGGGTCC 300
GGCTTGNGNA ACCGGCTTCN ACGCCACCCN AAAATTGANG GCAAGCAAGC 350

Sequence A6: sequence of tested sample designated 20.20C (*R. bursa*)

GTTGGNTNAA NNANGCAGCN GNNTCGGCAN TTTGGTGTGC GGTGGCAAAC TGCGGATGNG 60
AAAG-CTTGT GTATTTCGTG CAAAAACTCG AAAGCGGGTC CCGGGAGGTC TGTGGTGGGA 120
ACGGGACCCG CCCGTGGTGC AGAGGGAGCC ACGGTGCGAA CGGGAAGAGG CTTGTCGATG 180
GGACCGTGCA TCCCGCTCTA CGGAGCCGGG AGCCGGCCGC CCGAGGAAGT GCAGCCGCCG 240
AGGCCCGCAT CCTGCGTGCA CTCTTATCCA AATGGGTGTA CCGCAGTCAT TTTATGCGTT 300
AGGCGGGCCA ATGAGAGCGA GCGCACGACG ATACCTGCGG GTCCGGCTTG GGCAACCGGC 360
TTCGACGCCA CCCGAAAATT GAGGGCAAGC AAGCACGAAA GCACTCGCAA GTAGCGGAAG 420
CGAAACGCCG TCCGAAACAC ACCGGTGNNN GGTTCGCGGC AGCNGAACAG GCGCN 475

Sequence A7: sequence of tested sample designated 20.12C (*R. turanicus*)

CCCGTNNGCT GAAGTAGGCA GCCGGATCGG CATTTGGTGT GAAGTGAGCA AACTGTGGAA 60
GCGAAAGTGC GTGCGAGAAA CGCATCTCGA AATATGGGTG GCACGCGACT GGGTCCCGGG 120
AAGCGGTGGG AGGAAAGGGA CCCGCCCGAA GGGCAAAAAG TCGCGAAACG AGACGGCAGA 180
CTCGTGTCGA TGGGGCCGAA CACTCCGCTC CTCTACGGAG CAACACGGAG GCGGCCCGCC 240
CGAGGCAGTG TAGCCGCCGA GACCCGCACG CATGCCGGCT CTTATCTGTT GGTCGGTGTG 300
CGATTTTCAA TCGTTAGGCG GGTCGATGAG TGCGAGCACA CGAAAGAAAC CTACGGGTCC 360
GGCCTGGGAA ACCGGCTTCG ACGAAACCCT GAAAACGAGA TGAAAAGCAC GCGCAAGTAG 420
CGGAAGCGAC GCGCCCACGA GAACCACCGG TGTTCGATTC GCGGCCGCCG AACAGGCGCG 480
GCGGAGTTGG GGGAGAGCGC GCTGGAAAGC GCCCTCCGAA 520

Sequence A8: sequence of tested sample designated 20.12B (*R. turanicus*)

TCCCGTTGGC TGAAGTAGGC NGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACATCTCG AAATATGGGT GGCACGCGAC TGGGTCCCGG 120
GAAGCGGAGG GTGGAAAGGG ACCCGCCCGA CGGGCAAAAA GTCGCGAAAC GAGACGGCAG 180
ACTCGTGTCG ATGGGGCCGA ACGCTCCGCT CCTCTATGGA GAGAGACGGG CGCGGCCCGC 240
CCGAGGCAGT GTAGCCGCCG AGACCCGCAC GCATGCCGGC TCTTATCTGT TGGTCGGTGT 300
GCGATTTTCA ATCGTTAGGC GGGTCGATGA GTGCGAGCAC ACGAAAGAAA CCTACGGGTC 360
CGGCCTGGGA AACCGGCTTC GACGAAACCC TGAAAACGAG ATGAAAAGCA CGCGCAAGTA 420
GCGGAAGCGA CACGCCCACG AGAACCACCG GTGTTTGATT CGCGGCCGCC GAACAGGCGC 480
GGCGGAGTTG GGGGAGAGCG CGCTGGAAAG CGCCCTCCGA 520

Sequence A9: sequence of tested sample designated 20.15A (*R. sanguineus*)

TCCCGTTGGC TGAAGTAGGC NGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACATCTCG AAATATGGGT GGCACGCGAC TGGGTCCCGG 120
GAAGTGGAGG GTGGAAAGGG ACCCGCCCGA CGGGCAAAAA GTCGCGAAAC GAGACGGCAG 180
ACTCGTGTCG ATGGGGCCGA ACGCTCCGCT CCTCTACGGA GAGAGACGGG CGCGGCCCGC 240
CCGAGGCAGT GTAGCCGCCG AGACCCGCAC GCATGCCGGC TCTTATCTGT TGGTCGGTGT 300
GCGATTTTCA ATCGTTAGGC GGGTCGATGA GTGCGAGCAC ACGAAAGAAA CCTACGGGTC 360
CGGCCTGGGA AACCGGCTTC GACGAAACCC TGAAAACGAG ATGAGAAGCA CGCGCAAGTA 420
GCGGAAGCGA CACGCCCACG AGAACCACCG GTGTTTGATT CGCGGCCGCC GAACAGGCGC 480
GGCGGAGTTG GGGGAGAGCG CGCTGGAAAG CGCCCTCCGA 520

Sequence A10: sequence of tested sample designated 20.15B (*R. sanguineus*)

TCCCGTNGGC TGAAGTAGGC AGCCGGATCG GCATTTGGTG TGAGTTGAGC AAACTGTGGA 60
AGCGAAAGTG CGTGCGAGAA ACACATCTCG AAATATGGGT GGCACGCGAC TGGGTCCCGG 120
GAAGCGGAGG GTGGAAAGGG ACCCGCCCGA CGGGCAAAAA GTCGCGAAAC GAGACGGCAG 180

ACTCGTGTCG ATGGGGCCGA ACGCTCCGCT CCTCTATGGA GAGAGACGGG CGCGGCCCGC 240
CCGAGGCAGT GTAGCCGCCG AGACCCGCAC GCATGCCGGC TCTTATCTGT TGGTCGGTGT 300
GCGATTTTCA ATCGTTAGGC GGGTCGATGA GTGCGAGCAC ACGAAAGAAA CCTACGGGTC 360
CGGCCTGGGA AACCGGCTTC GACGAAACCC TGAAAACGAG ATGAAAAGCA CGCGCAAGTA 420
GCGGAAGCGA CACGCCCACG AGAACCACCG GTGTTTGATT CGCGGCCGCC GAACAGGCGC 480
GGCGGAGTTG GGGGAGAGCG CGCTGGAAAG CGCCCTCCGA 520

Sequences of the reference strains from the GeneBank, and the sequences of the positive controls of *COX-1* gene of *R. sanguineus*, *R. turanicus* and *R. bursa*

Sequence A11: KVI_Rt4 (reference strain of *R. turanicus* from the GeneBank)

AATATTAGGA CTAAGAATAA GAATATTAAT TCGAATAGAA TTAGGTCAGC CTGGAACTTT 60
AATTGGAAAT GATCAAATTT ATAATGTAAT TGTAACAGCC CATGCATTTA TTATAATTTT 120
TTTCATAGTA ATACCAATTA TAATTGGTGG ATTTGGAAAC TGATTAGTAC CTATTATATT 180
AGGAGCTCCA GATATAGCAT TTCCACGAAT AAATAACATA AGATTTTGAT TACTTCCTCC 240
TTCATTATTT CTATTAATTA ATTCCTCATT AATTGAATCA GGAGCAGGAA CTGGATGAAC 300
TGTTTATCCG CCTTTATCAT CAAATTTATC ACACTATGGA CCATCAGTAG ATTTAGCTAT 360
TTTCTCTCTT CATCTTGCTG GTG
383

Sequence A12: SRs1 (reference strain of *R. sanguineus* from the GeneBank)

ATATTAGGAT TAAGAATAAG AATACTAATT CGTATAGAAT TAGGTCAACC TGGAACTCTG 60
ATTGGTAATG ATCAAATTTA TAATGTAATT GTTACAGCTC ATGCATTTAT TATAATTTTT 120
TTTATAGTAA TACCAATTAT AATTGGTGGA TTTGGAAACT GATTAGTACC TATTATACTA 180
GGAGCTCCAG ATATAGCATT CCCACGAATA AATAATATAA GATTTTGACT TCTTCCTCCC 240
TCATTATTTA TATTAATTAA TTCTTCATTA ATTGAGTCAG GAGCAGGTAC AGGATGAACA 300
GTTTATCCTC CCCTATCCTC AAATTTATCA CATTATGGGC CATCAGTAGA TTTAGCTATT 360
TTTTCTCTTC ATCTTGCTGG TGC 383

Sequence A13: S19S positive control of *R. sanguineus*

AA TATTAGGATT AAGAATAAGA 60
ATACTAATTC GTATAGAATT AGGTCAACCT GGAACTCTAA TTGGTAATGA TCAAATTTAT 120
AATGTAATTG TTACAGCTCA TGCATTTATT ATAATTTTTT TTATAGTAAT ACCAATTATA 180
ATTGGTGGAT TTGGAAACTG ATTAGTACCT ATTATACTAG GAGCTCCAGA TATAGCATTC 240
CCACGAATAA ATAATATAAG ATTTTGACTT CTTCCTCCCT CATTATTTAT ATTAATTAAT 300
TCTTCATTAA TTGAGTCAGG AGCAGGTACA GGATGAACAG TTTATCCTCC CCTATCCTCA 360
AATTTATCAC ATTATGGGCC ATCAGTAGAT TTAGCTATTT TTTCTCTTCA TCTTGCTGGT 420
G 421

Sequence A14: positive control of *R. sanguineus* designated (S23S)

AA TATTAGGATT AAGAATAAGA 60
ATACTAATTC GTATAGAATT AGGTCAACCT GGAACTCTAA TTGGTAATGA TCAAATTTAT 120
AATGTAATTG TTACAGCTCA TGCATTTATT ATAATTTTTT TTATAGTAAT ACCAATTATA 180
ATTGGTGGAT TTGGAAACTG ATTAGTACCT ATTATACTAG GAGCTCCAGA TATAGCATTC 240
CCACGAATAA ATAATATAAG ATTTTGACTT CTTCCTCCCT CATTATTTAT ATTAATTAAT 300
TCTTCATTAA TTGAGTCAGG AGCAGGTACA GGATGAACAG TTTATCCTCC CCTATCCTCA 360
AATTTATCAC ATTATGGGCC ATCAGTAGAT TTAGCTATTT TTTCTCTTCA TCTTGCTGGT 420
G 421

Sequence A15: positive control of *R. turanicus* designated (T84)

AA TATTAGGACT AAGAATAAGA 60
ATATTAATTC GAATAGAATT AGGCCAACCT GGAACTTTAA TTGGAAATGA TCAAATTTAT 120
AATGTAATTG TAACAGCCCA TGCATTTATT ATAATTTTTT TCATAGTAAT ACCAATTATA 180
ATTGGTGGAT TTGGAAACTG ATTAGTACCT ATTATATTAG GAGCTCCAGA TATAGCATTT 240
CCACGAATAA ATAACATAAG ATTTTGATTA CTTCCTCCTT CATTATTTCT ATTAATTAAT 300
TCCTCATTAA TTGAATCAGG AGCAGGAACT GGATGAACTG TTTATCCGCC TTTATCATCA 360
AATTTATCAC ACTATGGACC ATCAGTAGAT TTAGCTATTT TCTCTCTTCA TCTTGCTGGT 420

G 421

Sequence A16: positive control of *R. turanicus* designated (T89)

AA TATTAGGACT AAGAATAAGA 60
ATATTAATTC GAATAGAATT AGGCCAACCT GGAACTTTAA TTGGAAATGA TCAAATTTAT 120
AATGTAATTG TAACAGCCCA TGCATTTATT ATAATTTTTT TCATAGTAAT ACCAATTATA 180
ATTGGTGGAT TTGGAAACTG ATTAGTACCT ATTATATTAG GAGCTCCAGA TATAGCATTT 240
CCACGAATAA ATAACATAAG ATTTTGATTA CTTCCTCCTT CATTATTTCT ATTAATTAAT 300
TCCTCATTAA TTGAATCAGG AGCAGGAACT GGATGAACTG TTTATCCGCC TTTATCATCA 360
AATTTATCAC ACTATGGACC ATCAGTAGAT TTAGCTATTT TCTCTCTTCA TCTTGCTGGT 420
G 421

Sequence A17: positive control for *R. bursa* designated(BC3BRC)

ATACTCTACT AACCATAAAG ACATTGGAAC AATATATTTA ATTTTTGGCG CATGAGCTGG 60
TATACTGGG- ATTAAGTATA AGAATATTAA TCCGTCTTGA ATTAAGACAA CCTGGGAGAT 120
TAATTGGCAA TGACCAAATT TATAATGTCA TTGTAACAGC TCATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGGG GATTTGGCAA TTGACTTGTA CCTATTATAT 240
TAGGTGCTCC TGACATAGCC TTCCCACGAA TAAATAATAT GAGATTTTGA CTCTTACCTC 300
CTTCTTTATT TTTATTAATT AATTCCTCTT TAGTTGAATC AGGGACAGGG ACAGGGTGAA 360
CTGTATACCC TC-CTTTATC ATCAAATTTA TCTCATTATG GCCCTTCTGT AGATTTAGCT 420
ATTTTCTCTT TACATCTTGC TGGTG 445

Sequence A18: sequence of tested sample designated 3.26B (*R. sanguineus*)

AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGCCAAC CTGGAACCCT 120
AATTGGTAAT GATCAAATTT ATAATGTAAT TGTTACAGCT CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATCA TAATTGGTGG ATTTGGAAAC TGATTAGTTC CTATTATACT 240
AGGAGCTCCA GATATAGCAT TCCCACGAAT AAATAATATA AGATTTTGAC TTCTTCCTCC 300
CTCATTATTT ATATTAATTA ATTCTTCATT AATTGAGTCA GGAGCAGGTA CAGGATGAAC 360
AGTTTATCCT CCCTTATCCT CAAATTTATC ACATTATGGG CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTG 444

Sequence A19: sequence of tested sample designated 17.20A (*R. sanguineus*)

AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTCT 120
AATTGGTAAT GATCAAATTT ATAATGTAAT TGTTACAGCT CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGTGG ATTTGGAAAC TGATTAGTAC CTATTATACT 240
AGGAGCTCCA GATATAGCAT TCCCACGAAT AAATAATATA AGATTTTGAC TTCTTCCTCC 300
CTCATTATTT ATATTAATTA ATTCTTCATT AATTGAGTCA GGAGCAGGTA CAGGATGAAC 360
AGTTTATCCT CCCCTATCCT CAAATTTATC ACATTATGGG CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTG 443

Sequence A20: sequence of tested sample designated 20.10A (*R. bursa*)

ATACTCTACT AACCATAAAG ACATTGGAAC AATATATTTA ATTTTTGGCG CATGAGCTGG 60
TATACTGGGA TTAAGTATAA GAATATTAAT CCGTCTTGAA TTAAGACAAC CTGGGAGATT 120
AATTGGCAAT GACCAAATTT ATAATGTCAT TGTAACAGCT CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGGGG ATTTGGCAAT TGACTTGTAC CTATTATATT 240
AGGTGCTCCT GACATAGCCT TCCCACGAAT AAATAATATG AGATTTTGAC TCTTACCTCC 300
TTCTTTATTT TTATTAATTA ATTCCTCTTT AGTTGAATCA GGGGCAGGGA CAGGGTGAAC 360
TGTATACCCT CCTTTATCAT CAAATCTATC TCATTATGGC CCTTCTGTAG ATTTAGCTAT 420
TTTCTCTTTA CATCTTGCTG GTG 443

Sequence A21: sequence of tested sample designated 20.17A (*R. bursa*)

ATACTCTACT AACCATAAAG ACATTGGAAC AATATATTTA ATTTTTGGCG CATGAGCTGG 60
TATACTGGG- ATTAAGTATA AGAATATTAA TCCGTCTTGA ATTAAGACAA CCTGGGAGAT 120
TAATTGGCAA TGACCAAATT TATAATGTCA TTGTAACAGC TCATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGGG GATTTGGCAA TTGACTTGTA CCTATTATAT 240

TAGGTGCTCC TGACATAGCC TTCCCACGAA TAAATAATAT GAGATTTTGA CTCTTACCTC 300
CTTCTTTATT TTTATTAATT AATTCCTCTT TAGTTGAATC AGGGGCAGGG ACAGGGTGAA 360
CTGTATACCC TC-CTTTATC ATCAAATTTA TCTCATTATG GCCCTTCTGT AGATTTAGCT 420
ATTTTCTCTT TACATCTTGC TGGTG 445

Sequence A22: sequence of tested sample designated 17.20B (*R. sanguineus*)

AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTCT 120
AATTGGTAAT GATCAAATTT ATAATGTAAT TGTTACAGCT CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGTGG ATTTGGAAAC TGATTAGTAC CTATTATACT 240
AGGAGCTCCA GATATAGCAT TCCCACGAAT AAATAATATA AGATTTTGAC TTCTTCCTCC 300
CTCATTATTT ATATTAATTA ATTCTTCATT AATTGAGTCA GGAGCAGGTA CAGGATGAAC 360
AGTTTATCCT CCCCTATCCT CAAATTTATC ACATTATGGG CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTG 443

Sequence A23: sequence of tested sample designated 17.20C (*R. sanguineus*)

AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGCCAAC CTGGAACCCT 120
AATTGGTAAT GATCAAATTT ATAATGTAAT TGTTACAGCT CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATNA TAATTGGKGG ATTTGGAAAN TGATTAGTTC CTATTATACT 240
AGGAGCTCCA GATATAGCAT TCCCACGAAT AAATAATATA AGATTTTGAC TTCTTCCTCC 300
CTCATTATTT ATATTAATTA ATTCTTCATT AATTGAGTCA GGAGCAGGTA CAGGATGAAC 360
AGTTTATCCT CCCTTATCCT CAAATTTATC ACATTATGGA CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTG 443

Sequence A24: sequence of tested sample designated 17.23A (uncut)

ATACTCTACT AANCACAAAG ACATTGGAAC AATATACTTA ATTTTTGGGG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGCATAGAG TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGGGG ATTCGGAAAT TGATTAGTTC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGAA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATTTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTG 443

Sequence A25: sequence of tested sample designated 21.8A (uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A26: sequence of tested sample designated 17.24A(uncut)

ATACTCTACT AANCATAAAG ACATTGGAAC AATATATTTA ATTTTTGGGG CATGATCAGG 60
GATATTAGGA CTAAGAATGA GAATATTAAT TCGAATAGAA TTAGGTCAGC CTGGAACTTT 120
AATTGGAAAT GATCAAATTT ATAATGTAAT TGTAACAGCC CATGCATTTA TTATAATTTT 180
TTTCATAGTA ATACCAATTA TAATTGGTGG ATTTGGAAAC TGATTAGTAC CTATTATATT 240
AGGTGCTCCA GATATAGCAT TTCCACGAAT AAATAACATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT CTATTAATTA ATTCCTCATT AATTGAATCA GGAGCAGGAA CTGGATGAAC 360
TGTTTATCCG CCTTTATCAT CAAATTTATC ACACTATGGA CCATCAGTAG ATTTAGCTAT 420
TTTCTCTCTT CATCTTGCTG GTG 443

Sequence A27: sequence of tested sample designated 20.1A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60

AATATTAGG- ATTAAGAATA AGAATACTAA TTCGTATAGA ATTAGGTCAA CCTGGAACTT 120
TAATTGGAAA TGACCAAATT TATAATGTAA TTGTAACAGC ACATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGAG GATTCGGAAA TTGATTAGTC CCTATTATAT 240
TAGGGGCTCC AGATATAGCA TTTCCACGAA TAAATAATAT AAGATTTTGA TTACTTCCTC 300
CTTCATTATT TTTATTAATT AATTCTTCAT TAATTGAATC AGGAGTGGGC ACAGGATGAA 360
CAGTTTATCC TCCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A28: sequence of tested sample designated 20.3A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A29: sequence of tested sample designated 20.3C(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGCCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A30: sequence of tested sample designated 20.4B(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGN ATTAAGAATA AGAATACTAA TTCGTATAGA ATTAGGTCAA CCTGGAACTT 120
TAATTGGAAA TGACCAAATT TATAATGTAA TTGTAACAGC ACATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGAG GATTCGGAAA TTGATTAGTC CCTATTATAT 240
TAGGGGCTCC AGATATAGCA TTTCCACGAA TAAATAATAT AAGATTTTGA TTACTTCCTC 300
CTTCATTATT TTTATTAATT AATTCTTCAT TAATTGAATC AGGAGCGGGC ACAGGATGAA 360
CAGTTTATCC TCCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A31: sequence of tested sample designated 20.4C(uncut)

ATACTCTACT AACCACAAAA ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CGCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A32: sequence of tested sample designated 20.5A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGCATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300

TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A33: sequence of tested sample designated 20.8A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A34: sequence of tested sample designated 20.9A(uncut)

ATACTCTACT AACCACAAAG ACATTGGAAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGC ATTAAGAATA AGAATACTAA TTCGTATAGA ATTAGGTCAA CCTGGAACTT 120
TAATTGGAAA TGACCAAATT TATAATGTAA TTGTAACAGC ACATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATGATTGGAG GATTCGGAAA TTGATTAGTC CCTATTATAT 240
TAGGGGCTCC AGATATAGCA TTTCCACGAA TAAATAATAT AAGATTTTGA TTACTTCCTC 300
CTTCATTATT TTTATTAATT AATTCTTCAT TAATTGAATC AGGAGCGGGC ACAGGATGAA 360
CAGTTTATCC TCCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A35: sequence of tested sample designated 20.12B(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A36: sequence of tested sample designated 20.20F(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGC ATTAAGAATA AGAATACTAA TTCGTATAGA ATTAGGTCAA CCTGGAACTT 120
TAATTGGAAA TGACCAAATT TATAATGTAA TTGTAACAGC ACATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGAG GATTCGGAAA TTGATTAGTC CCTATTATAT 240
TAGGGGCTCC AGATATAGCA TTTCCACGAA TAAATAATAT AAGATTTTGA TTACTTCCTC 300
CTTCATTATT TTTATTAATT AATTCTTCAT TAATTGAATC AGGAGCGGGC ACAGGATGAA 360
CAGTTTATCC TCCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A37: sequence of tested sample designated 20.20L(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A38: sequence of tested sample designated 20.22A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A39: sequence of tested sample designated 20.22B(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A40 : sequence of tested sample designated 20.22C(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGC ATTAAGAATA AGAATACTAA TTCGTATAGA ATTAGGTCAA CCTGGAACTT 120
TAATTGGAAA TGACCAAATT TATAATGTAA TTGTAACAGC ACATGCATTT ATTATAATTT 180
TTTTTATAGT AATACCAATT ATAATTGGAG GATTCGGAAA TTGATTAGTC CCTATTATAT 240
TAGGGGCTCC AGATATAGCA TTTCCACGAA TAAATAATAT AAGATTTTGA TTACTTCCTC 300
CTTCATTATT TTTATTAATT AATTCTTCAT TAATTGAATC AGGAGTGGGC ACAGGATGAA 360
CAGTTTATCC TCCCTTATCA TCAAATCTAT CACACTATGG TCCATCAGTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A41: sequence of tested sample designated 20.23A(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TCATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A42: sequence of tested sample designated 20.23C(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTAGGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A43: sequence of tested sample designated 20.23E(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120

AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A44: sequence of tested sample designated 20.23G(uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGCATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TGATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A45: sequence of tested sample designated 20.23H (uncut)

ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGTCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCAGTAG ATTTAGCTAT 420
TTTTTCTCTT CATCTTGCTG GTGC 444

Sequence A46: sequence of tested sample designated 20.23I(uncut)

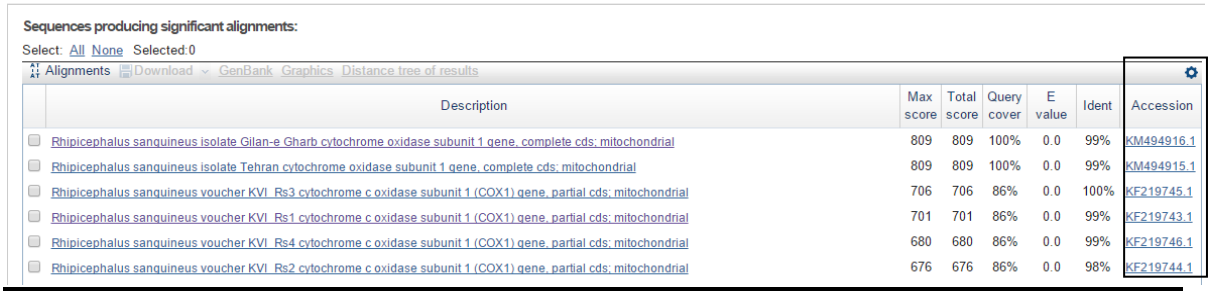
ATACTCTACT AACCACAAAG ACATTGGGAC AATATACTTA ATTTTTGGAG CATGATCTGG 60
AATATTAGGA TTAAGAATAA GAATACTAAT TCGTATAGAA TTAGGCCAAC CTGGAACTTT 120
AATTGGAAAT GACCAAATTT ATAATGTAAT TGTAACAGCA CATGCATTTA TTATAATTTT 180
TTTTATAGTA ATACCAATTA TAATTGGAGG ATTCGGAAAT TGATTAGTCC CTATTATATT 240
AGGGGCTCCA GATATAGCAT TTCCACGAAT AAATAATATA AGATTTTGAT TACTTCCTCC 300
TTCATTATTT TTATTAATTA ATTCTTCATT AATTGAATCA GGAGCGGGCA CAGGATGAAC 360
AGTTTATCCT CCCTTATCAT CAAATCTATC ACACTATGGT CCATCA-GTA GATTTAGCTA 420
TTTTTTCTCT TCATCTTGCT GGTGC 445

Sequence A47: sequence of tested sample designated 21.15C (*R. turanicus*)

ATTGGAACAA TATATTTAAT TTTTGGGGCA TGATCAGGAA TATTAGGACT AAGAATAAGA 60
ATATTAATTC GAATAGAATT AGGTCAACCT GGAACTTTAA TTGGAAATGA TCAAATTTAT 120
AATGTAATTG TAACAGCCCA TGCATTTATT ATAATTTTTT TCATAGTAAT ACCAATTATA 180
ATTGGTGGAT TTGGAAACTG ATTAGTACCT ATTATATTAG GAGCTCCAGA TATAGCATTT 240
CCACGAATAA ATAACATAAG ATTTTGATTA CTTCCTCCTT CATTATTTCT ATTAATTAAT 300
TCCTCATTAA TTGAATCAGG AGCAGGAACT GGATGAACTG TTTATCCGCC TTTATCATCA 360
AATTTATCAC ACTATGGACC ATCAGTAGAT TTAGCTATTT TCTCTCTTCA TCTTGCTGGT 420
G 421

Appendix B

BLAST analysis of *COX-1* sequences of different *Rhipicephalus* species obtained in this study compared with reference DNA sequences of *Rhipicephalus* species deposited in the GeneBank



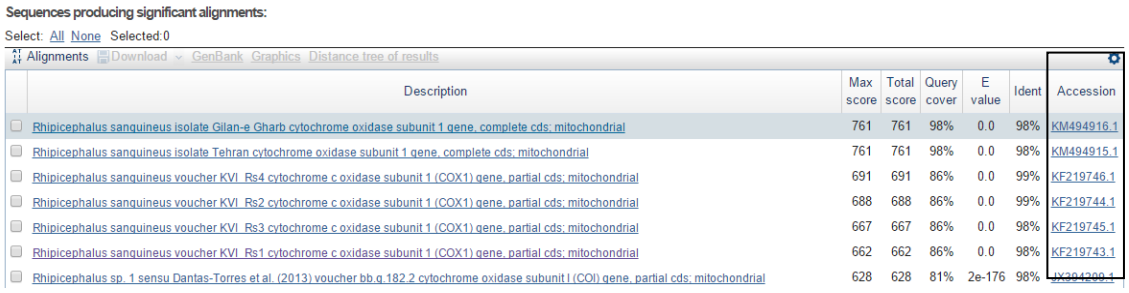
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	100%	0.0	99%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	809	809	100%	0.0	99%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	100%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	86%	0.0	99%	KF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	86%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	86%	0.0	98%	KF219744.1

B1: BLAST of (17.20B *R. sanguineus* by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	761	761	98%	0.0	98%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	761	761	98%	0.0	98%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	691	691	86%	0.0	99%	KF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	688	688	86%	0.0	99%	KF219744.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	667	667	86%	0.0	98%	KF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	662	662	86%	0.0	98%	KF219743.1
Rhipicephalus sp. 1 sensu Dantas-Torres et al. (2013) voucher bb.g.182.2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	628	628	81%	2e-176	98%	JX394289.1

B2: BLAST of (17.20C *R. sanguineus* by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).

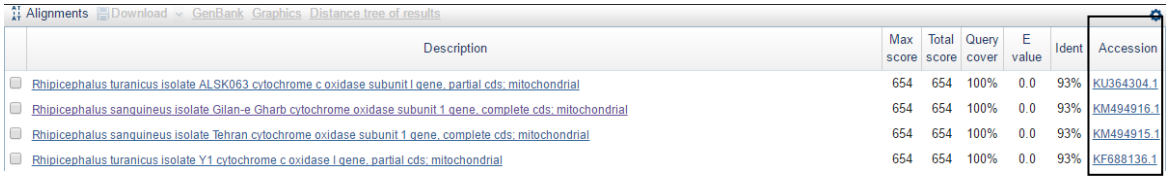
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

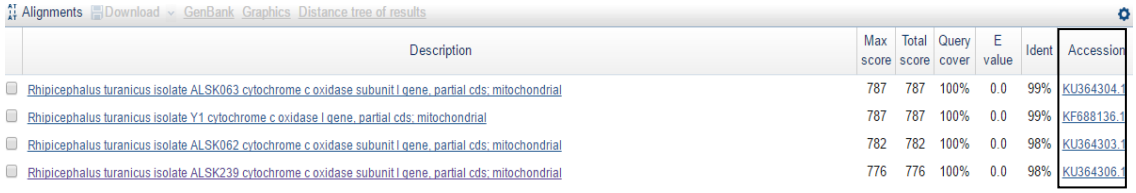
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	671	671	100%	0.0	94%	KF688136.1
Rhipicephalus turanicus isolate Y3 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688138.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Xinjiang cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	645	645	96%	0.0	94%	JQ737086.1

B3: BLAST of (17.23A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



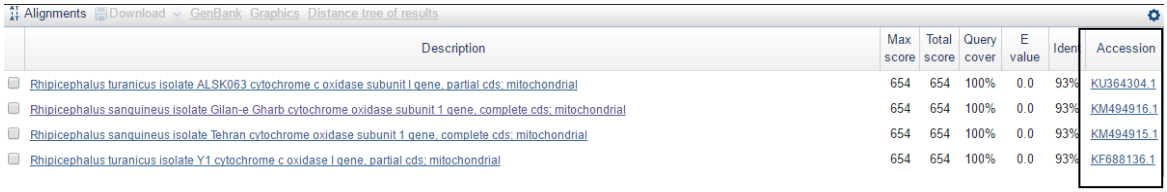
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

B4: BLAST of (21.8A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



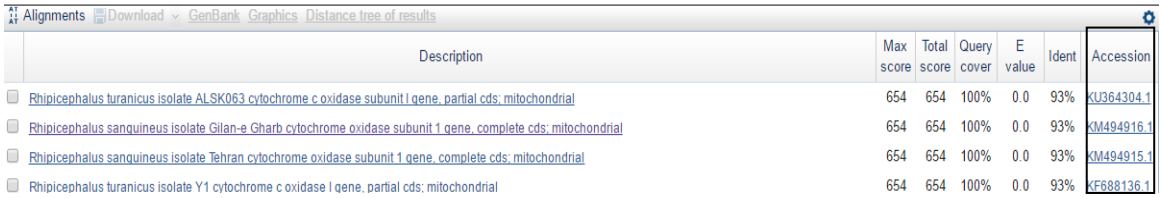
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	787	787	100%	0.0	99%	KU364304.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	787	787	100%	0.0	99%	KF688136.1
Rhipicephalus turanicus isolate ALSK062 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	782	782	100%	0.0	98%	KU364303.1
Rhipicephalus turanicus isolate ALSK239 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	776	776	100%	0.0	98%	KU364306.1

B5: BLAST of (17.24A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



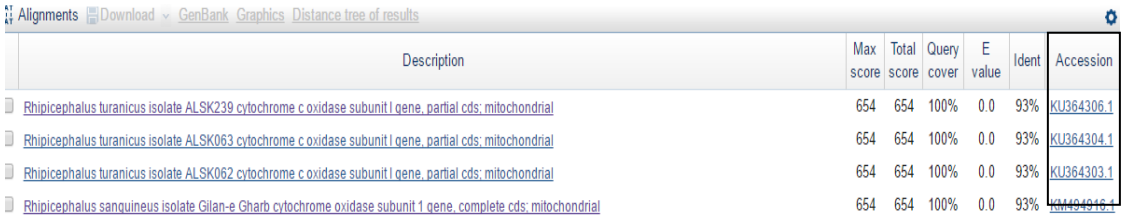
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

B6: BLAST of (20.1A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



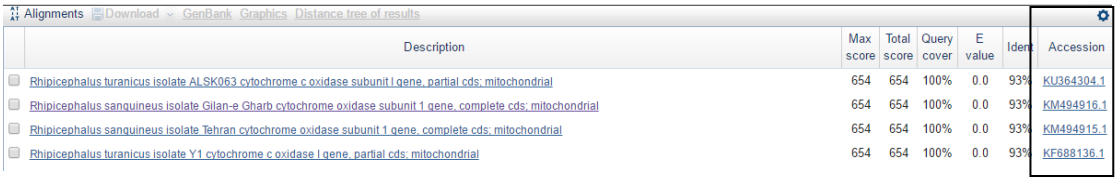
Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

B7: BLAST of (20.3A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK239 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364306.1
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus turanicus isolate ALSK062 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364303.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1

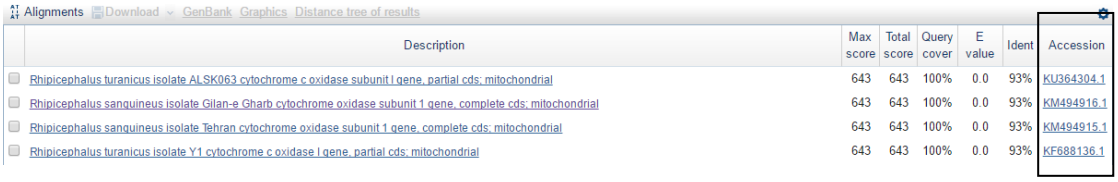
B8: BLAST of (20.3C uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

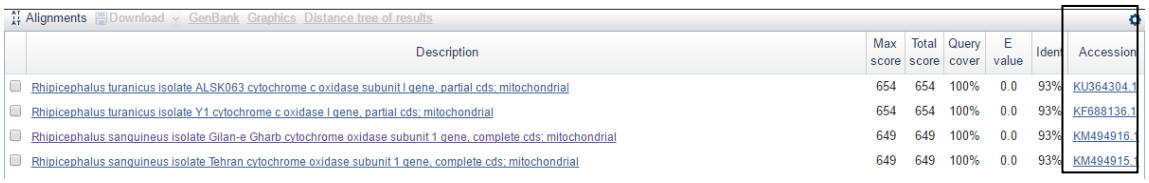
B9: BLAST of (20.4B uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	643	643	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	643	643	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	643	643	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	643	643	100%	0.0	93%	KF688136.1

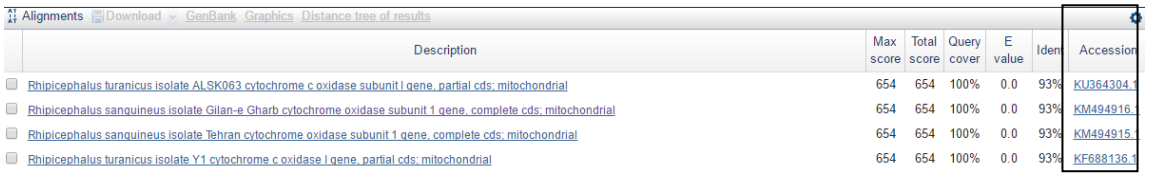
B10: BLAST of (20.4C uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494915.1

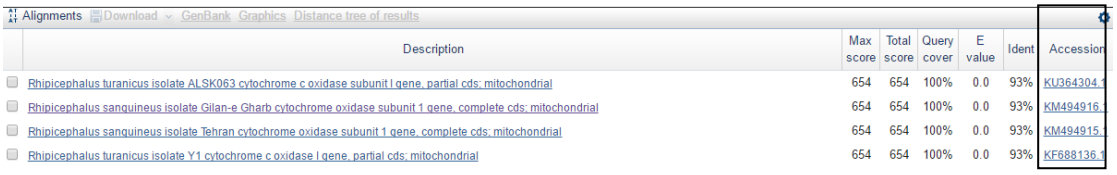
B11: BLAST of (20.5A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

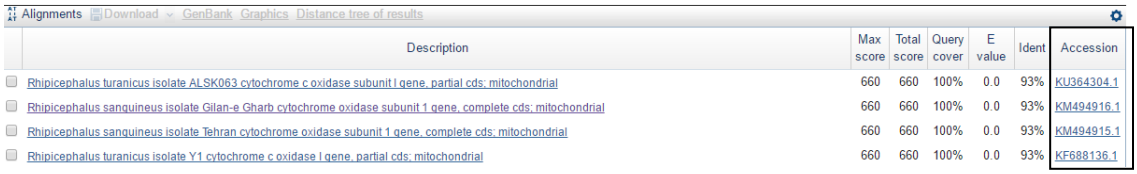
B12: BLAST of (20.8A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

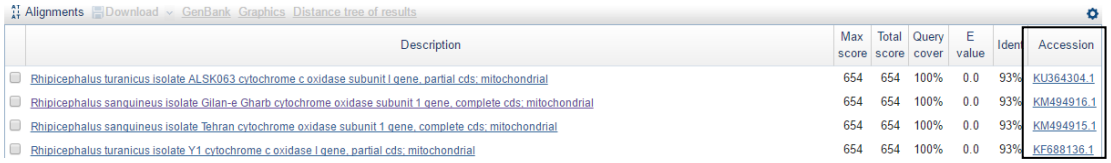
B13: BLAST of (20.9A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

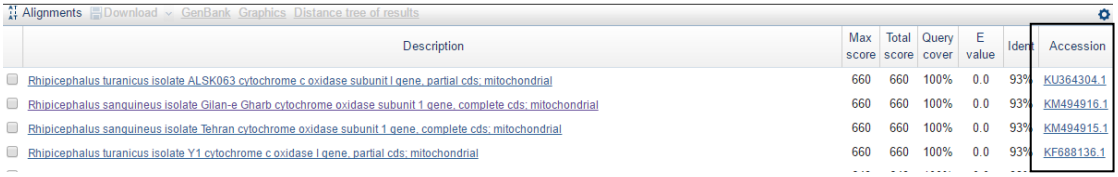
B14: BLAST of (20.12B 20.3A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

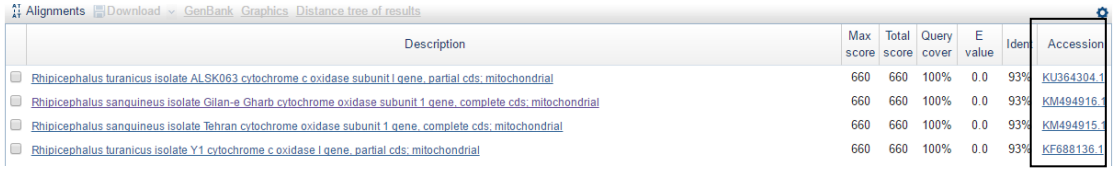
B15: BLAST of (20.20F uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

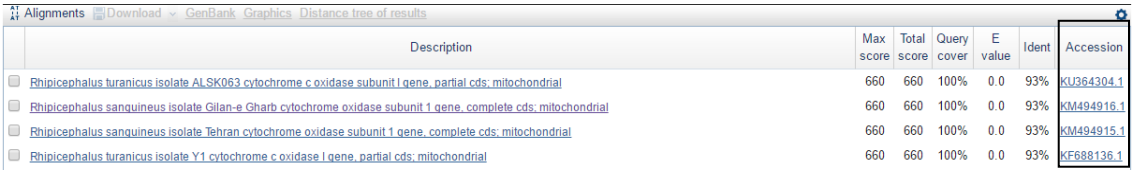
B16: BLAST of (20.20L uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

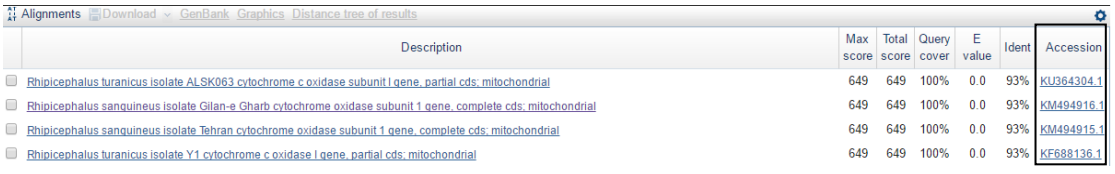
B17: BLAST of (20.22A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

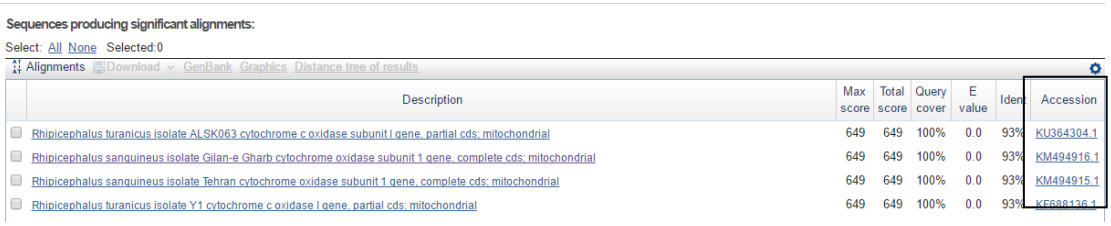
B18: BLAST of (20.22B uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	649	649	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	649	649	100%	0.0	93%	KF688136.1

B19: BLAST of (20.22C uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



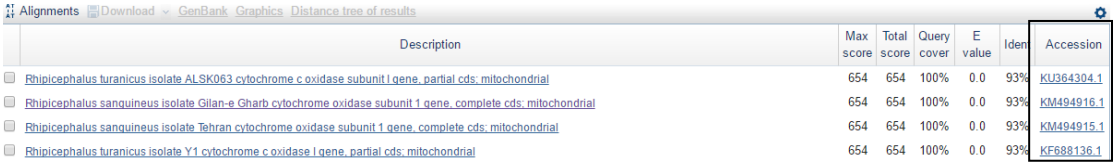
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	649	649	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	649	649	100%	0.0	93%	KF688136.1

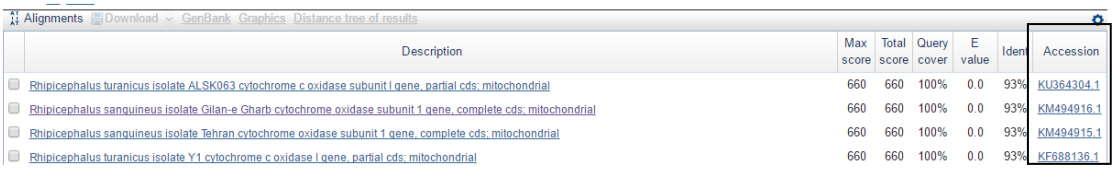
B20: BLAST of (20.3A uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1

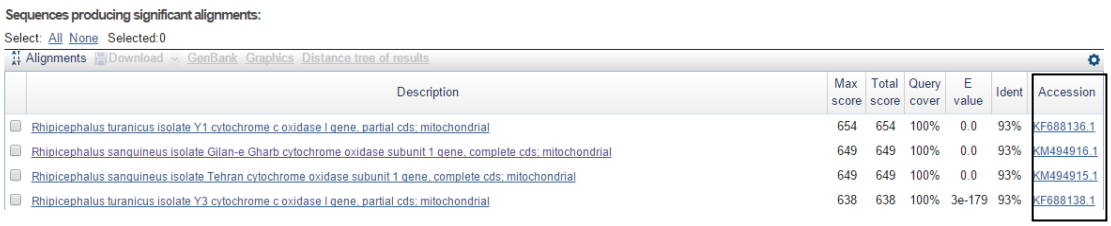
B21: BLAST of (20.23C uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

B22: BLAST of (20.23E uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



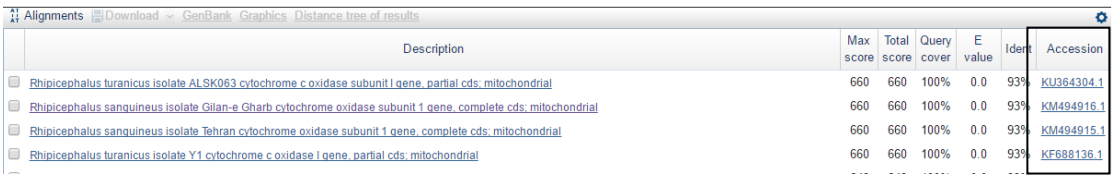
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KF688136.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	649	649	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y3 cytochrome c oxidase I gene, partial cds; mitochondrial	638	638	100%	3e-179	93%	KF688138.1

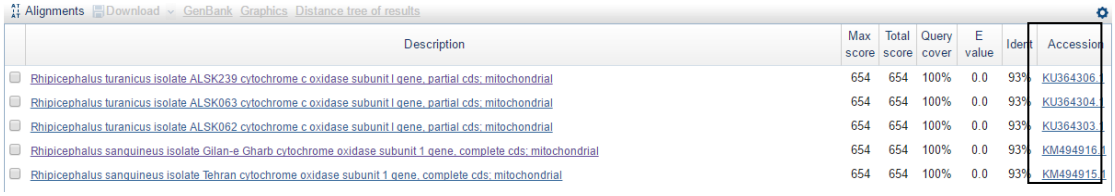
B23: BLAST of (20.23G uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KU364304.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	660	660	100%	0.0	93%	KM494915.1
Rhipicephalus turanicus isolate Y1 cytochrome c oxidase I gene, partial cds; mitochondrial	660	660	100%	0.0	93%	KF688136.1

B24: BLAST of (20.23H uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).



Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus turanicus isolate ALSK239 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364306.1
Rhipicephalus turanicus isolate ALSK063 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364304.1
Rhipicephalus turanicus isolate ALSK062 cytochrome c oxidase subunit I gene, partial cds; mitochondrial	654	654	100%	0.0	93%	KU364303.1
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	654	654	100%	0.0	93%	KM494915.1

B25: BLAST of (20.23I uncut *Rhipicephalus* species by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).

Sequences producing significant alignments:

Select: All None Selected:0

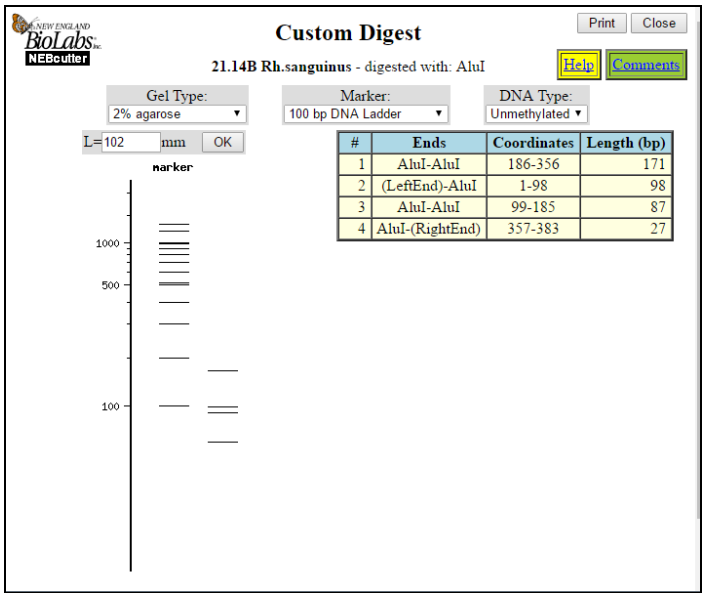
Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Rhipicephalus sanguineus isolate Gilan-e Gharb cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	806	806	100%	0.0	99%	KM494916.1
Rhipicephalus sanguineus isolate Tehran cytochrome oxidase subunit 1 gene, complete cds; mitochondrial	806	806	100%	0.0	99%	KM494915.1
Rhipicephalus sanguineus voucher KVI_Rs3 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	706	706	86%	0.0	100%	EF219745.1
Rhipicephalus sanguineus voucher KVI_Rs1 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	701	701	86%	0.0	99%	EF219743.1
Rhipicephalus sanguineus voucher KVI_Rs4 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	680	680	86%	0.0	99%	EF219746.1
Rhipicephalus sanguineus voucher KVI_Rs2 cytochrome c oxidase subunit 1 (COX1) gene, partial cds; mitochondrial	676	676	86%	0.0	98%	EF219744.1

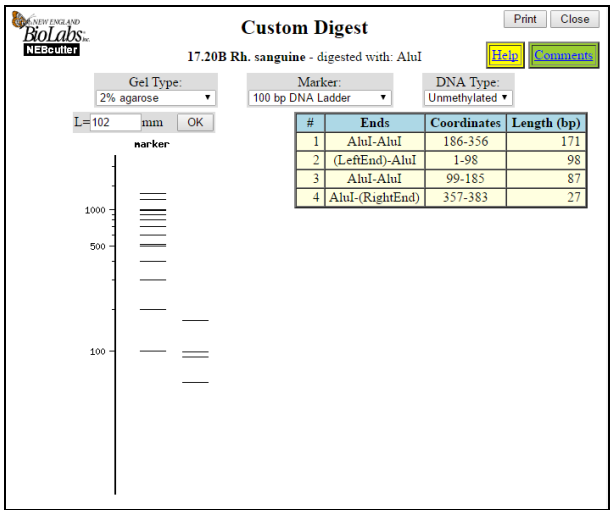
B26: BLAST of (17.20A *R. sanguineus* by RFLP) *COX-1*sequence against reference strains sequences. (The accession numbers are marked with black square).

Appendix C

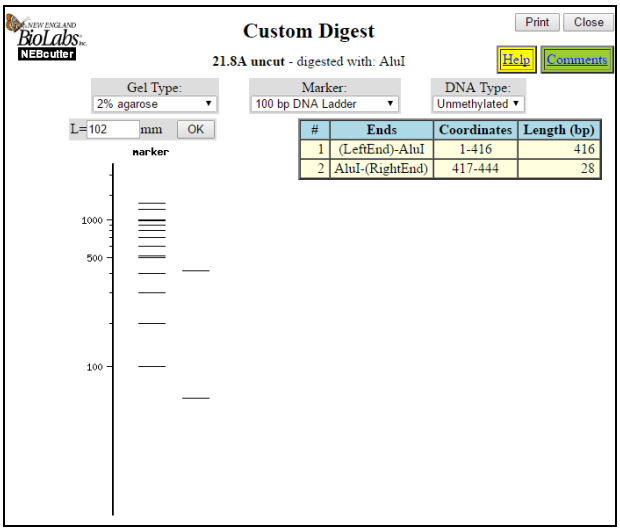
Virtual cuts of *COX-1* gene sequences of different *Rhipicephalus* species obtained in this study



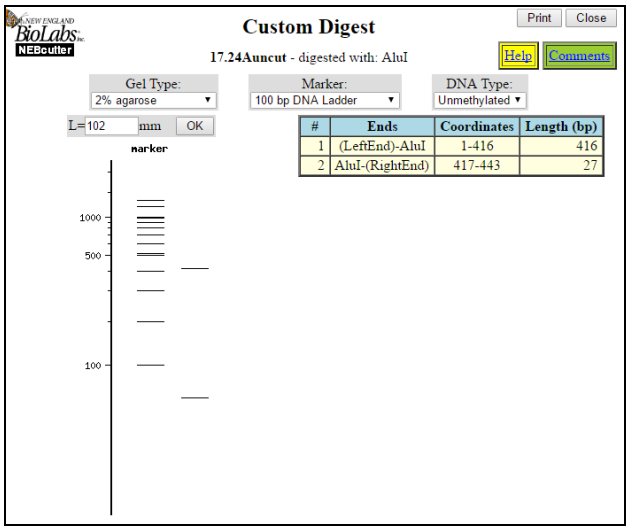
C1: 21.14B *R. sanguineus*



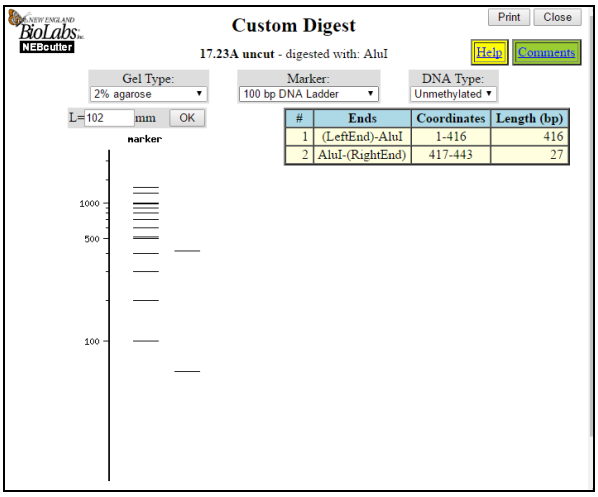
C2: 17.20B *R. sanguineus*



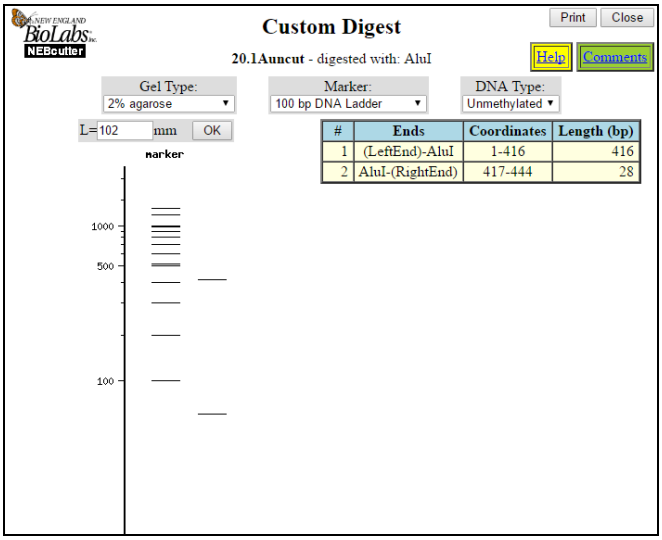
C3: 21.8A(uncut)



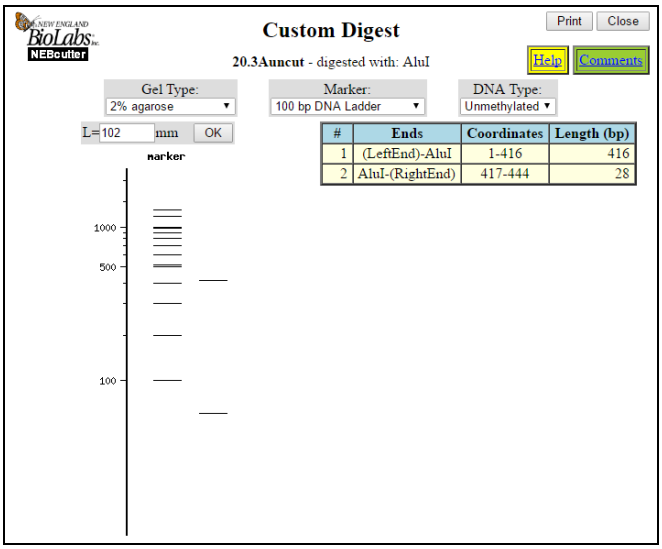
C4: 17.24A (uncut)



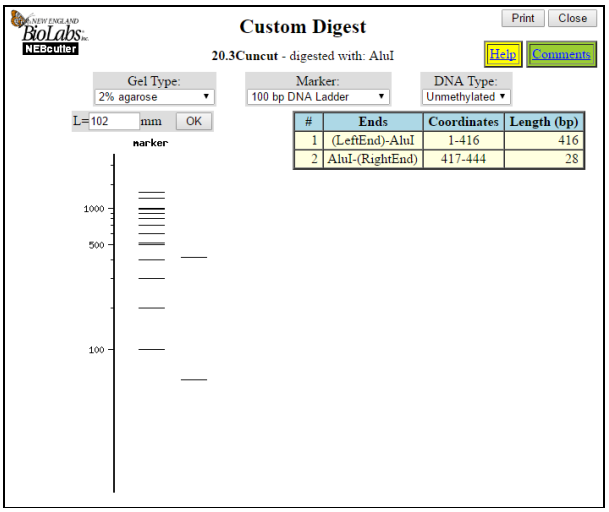
C5: 17.23A uncut



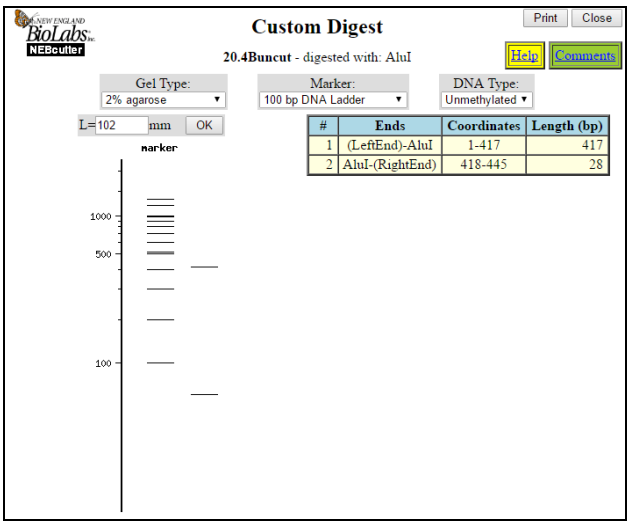
C6: 20.1A(uncut)



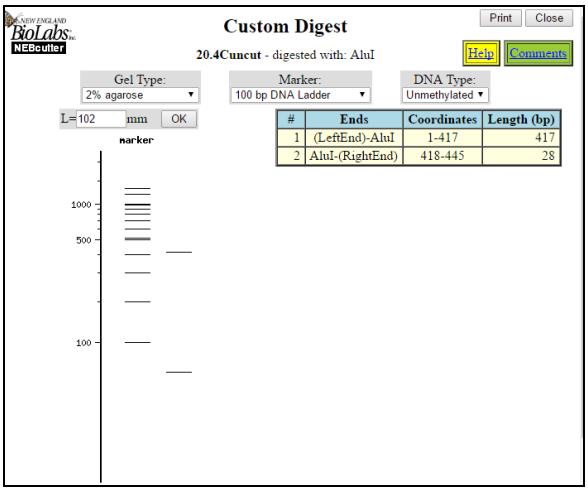
C7: 20.3A



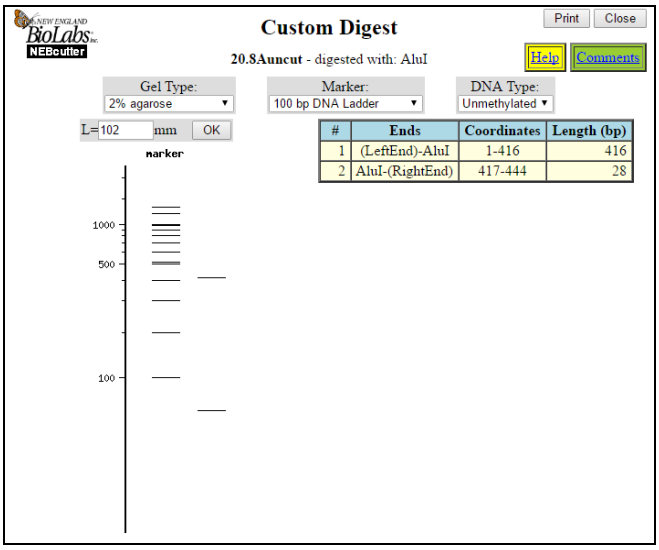
C8: 20.3C(uncut)



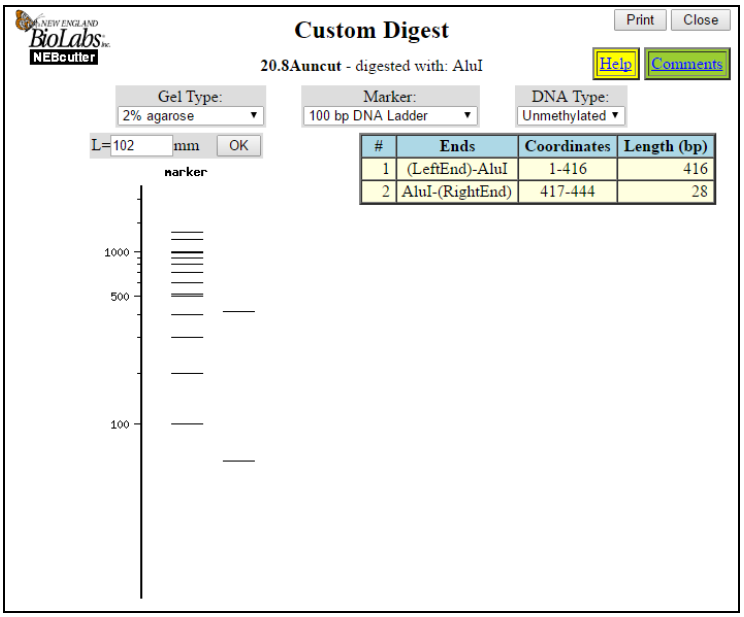
C9: 20.4B(uncut)



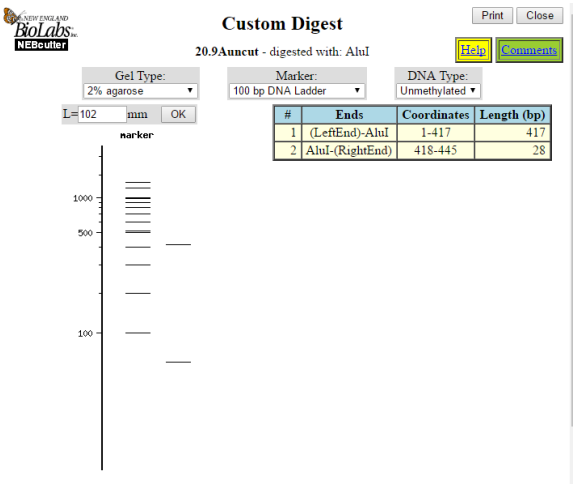
C10: 20.4C(uncut)



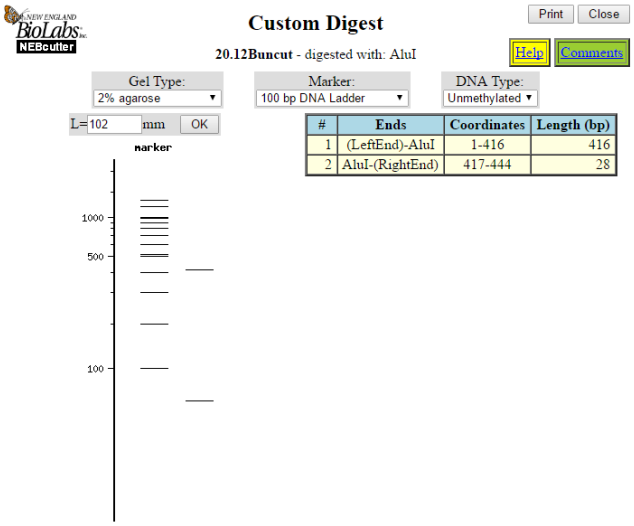
C11: 20.5A(uncut)



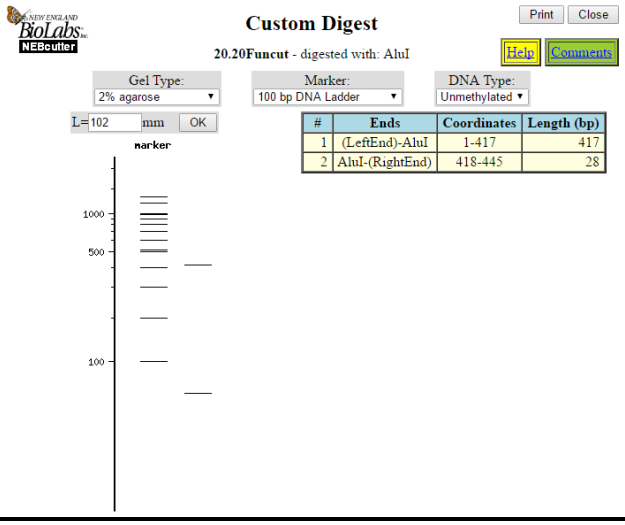
C12: 20.8A(uncut)



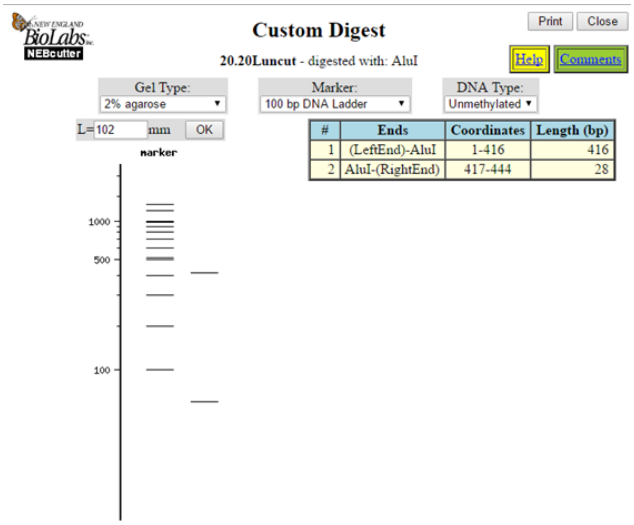
C13: 20.9A(uncut)



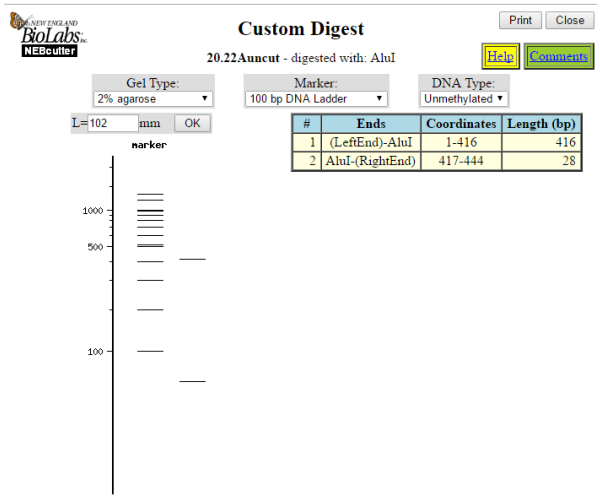
C14: 20.12B (uncut)



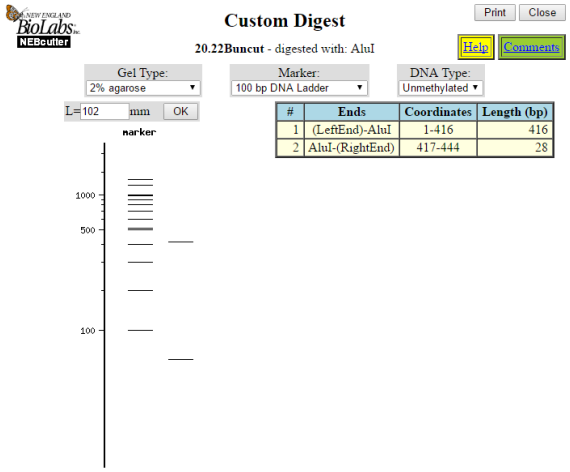
C15: 20.20F(uncut)



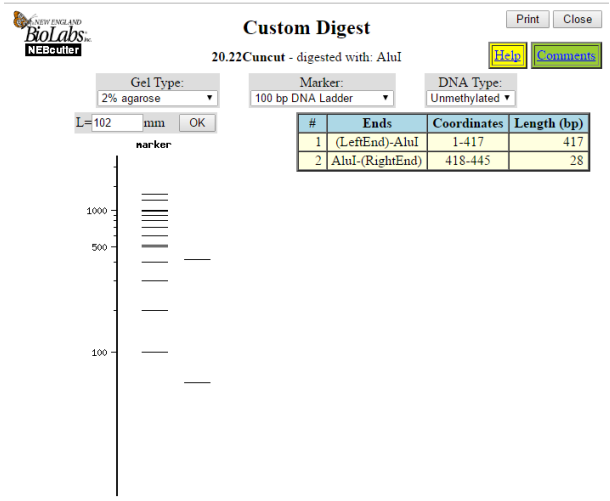
C16: 20.20L (uncut)



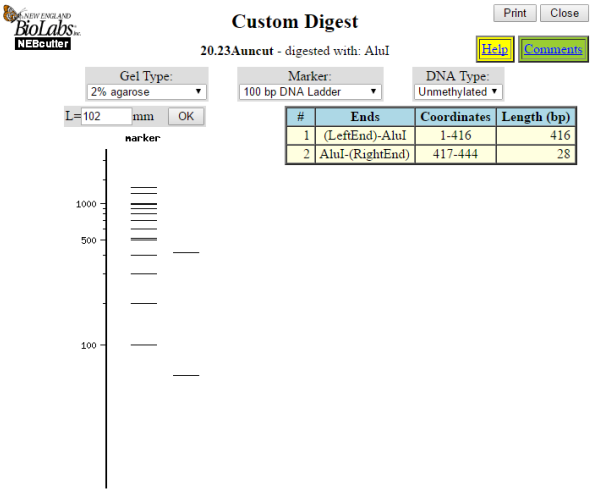
C17: 20.22A (uncut)



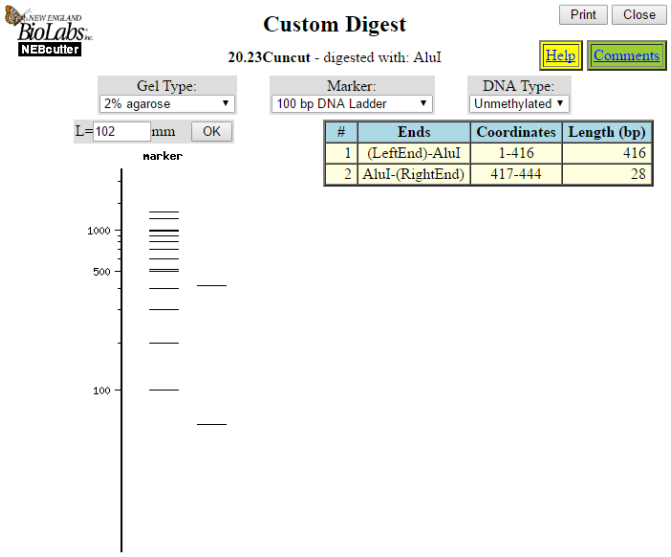
C18: 20.22B (uncut)



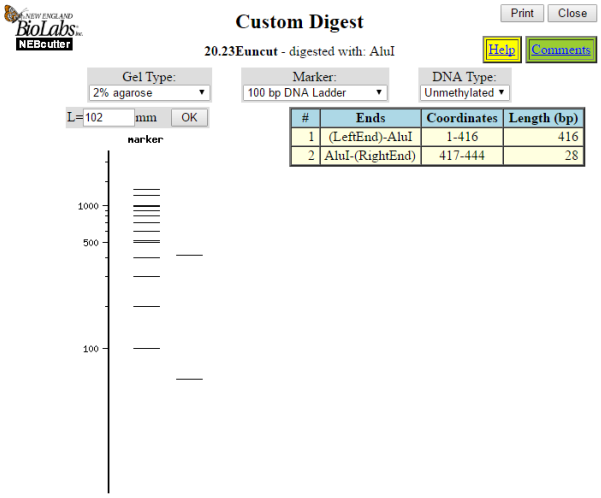
C19: 20.22C(uncut)



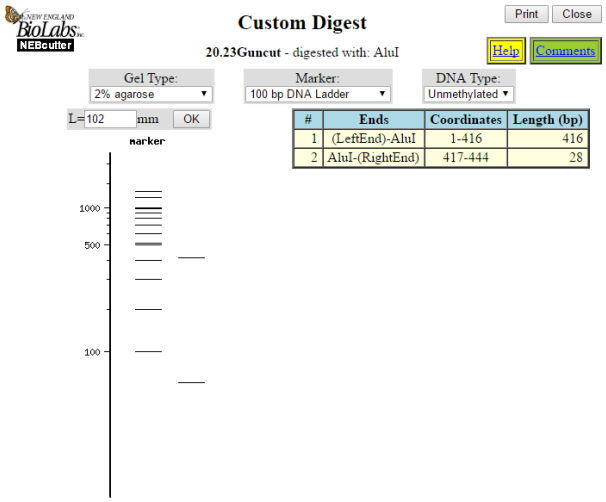
C20: 20.23A(uncut)



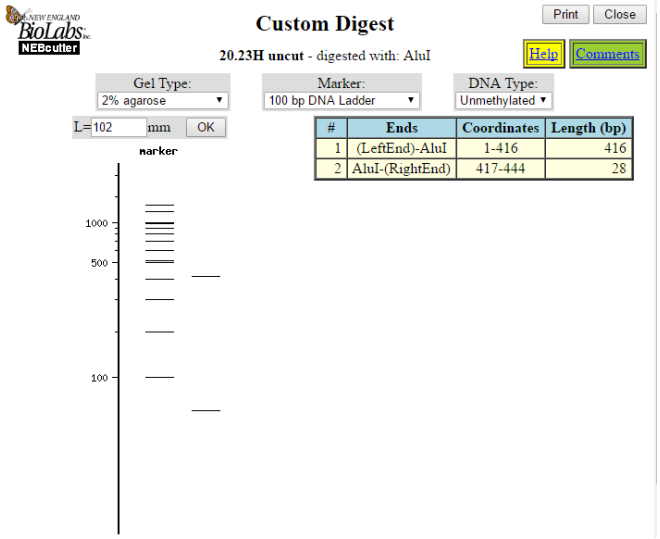
C21: 20.23C(uncut)



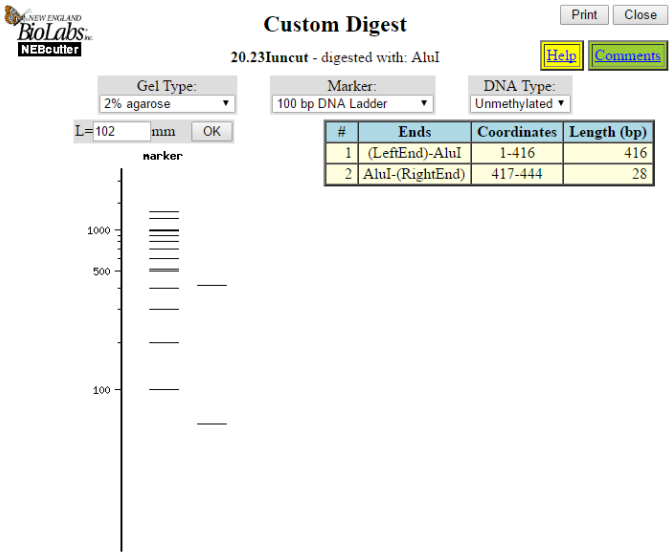
C22: 20.23E(uncut)



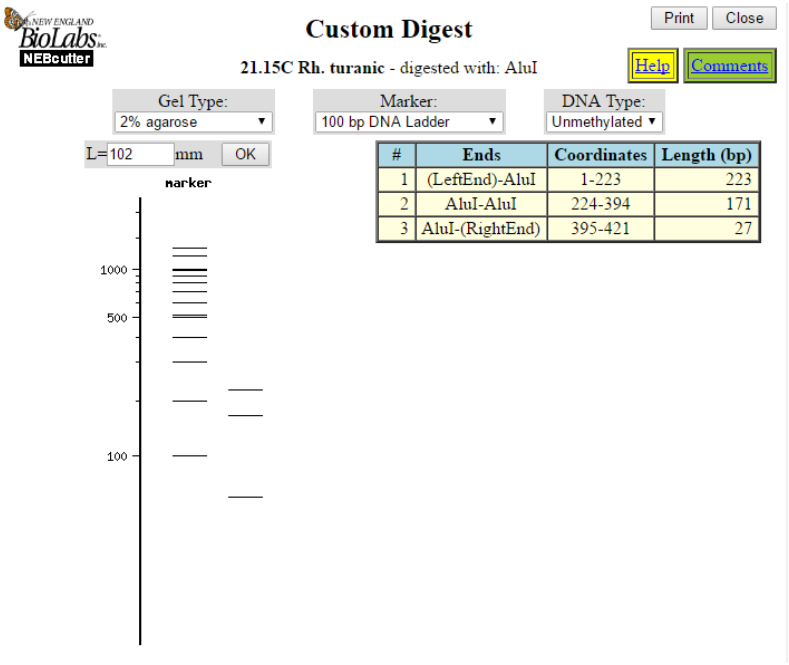
C23: 20.23G(uncut)



C24: 20.23H (uncut)



C25: 20.23I(uncut)



C26:21.15C

التعرف على الفصائل والشجرة الوراثية للقراد الصلب (*Rhipicephalus*) بواسطة التحليل الجيني الجزيئي من عدة محافظات فلسطينية مختلفة.

إعداد: رائدة سالم احميدان طقاطقة

إشراف: د. سهير عريقات و د. عبد المجيد ناصر الدين

الملخص:

القراد (Ticks) اسم يُطلق على أحد الكائنات المتطفلة التي تقوم بامتصاص دماء كل من الفقاريات ، بما في ذلك البرمائيات والزواحف والطيور والثدييات.

يقسم القراد الى عائليتين وهما: (Soft ticks أو Argasid)، و (*Ixodid* أو Hard ticks).عائلة (Ixodidae) تقسم الى مجموعتين بناء على الصفات الشكلية لها : (Metastriata) و(Prostriata). و يعتبر القراد(Ticks) ثاني أهم ناقل لمسببات الامراض، و في عائلة (Ixodidae) تعتبر الأجناس التالية مهمة طبيا (*Ixodes*) و (*Amblyomma*) و (*Dermacentor*) و (*Rhipicephalus*) .

جنس (*Rhipicephalus*) والذي هو من مجموعة ال (Metastriata)، يضم 84 فصيلا ،منها (*R. sanguineus*) و (*R. turanicus*) و(*R. bursa*) وهي الأكثر شيوعا في فلسطين، وتعتبر هذه الفصائل الثلاثة من النواقل المهمة طبيا، لذا فإنه من الضروري تصنيفها بدقة. يعتبر التصنيف الشكلي بين فصائل القراد صعب وخاصة في حالة تلف(damaged) بعض الاجزاء، أو احتقان (امتصاص) القراد بالدماء(بعد التغذية)، أو في المراحل غير الناضجة.

هذه الدراسة تهدف الى التعرف على أكثر الفصائل شيوعا في فلسطين باستخدام الطرق الجزيئية التقليدية و لتطوير طريقة جزيئية للتمييز بين هذه الأنواع التي تتطفل على الأغنام والكلاب ولدراسة التباين الوراثي في كل نوع مقارنة بأنواع محلية وعالمية.

بتصنيف القراد(Hard tick)، فإنه يمكن تقييم بل والسيطرة على المخاطر المحتملة التي قد تصيب الحيوانات والانسان.

تم جمع 351 عينة من القراد(Ixodidae) من الأغنام و الماعز والكلاب، في الفترة ما بين آذار و تشرين أول من عام 2014. تم تصنيف هذه العينات الى جنسين رئيسين بناء على الصفات الشكلية لها وهي: 97.4% (*Rhipicephalus*) و 2.6% (*Haemaphysalis*)، ومن ثم تم تصنيفها الى مستوى الفصيل وكانت النتائج كالتالي:

79.2% (*R.sanguineus*)، و9.7%(*R. turanicus*) و 3.4%(*R. bursa*) ، و 0.9% (*H. alderi*) ، و 1.6%(*H. parva*). ثم تم تصنيف هذه العينات بتحليل جين *COX-1*باستخدام تفاعل سلسلة البوليميرية (PCR) و (RFLP) باستخدام انزيم القطع المتخصص (*AluI*).

أظهرت النتائج توافق وارتباط عال بين التصنيف باستخدام (RFLP) و التصنيف باستخدام المجهر($p<0.01$).

عند تحليل جين (*COX-1*) لإنشاء شجرة ارتباطات سلالات *فصائل (Rhipicephalus*)، اظهر التحليل أربع مجموعات جينية رئيسة: تحت مجموعة (*R. bursa*)، ومجموعة (*R. sanguineus*)، و مجموعتي (G1,G2) (*R. turanicus*)، وهذا يؤكد أهمية استخدام جين *COX-1* للتفريق بين هذه الانواع.

بحدود معرفتي هذه الدراسة هي الأولى في العالم التي نجحت بتصنيف هذه الفصائل الثلاثة تصنيفا مباشرا بتحليل جين (*COX-1*) باستخدام (PCR- RFLP).