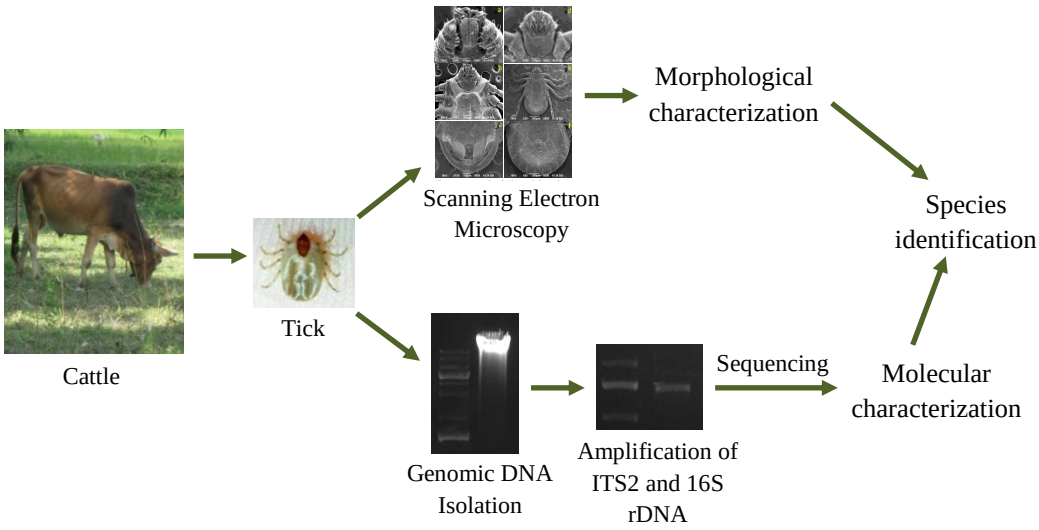
Chapter 2
Collection and identification of cattle-ticks

Graphical abstract



2.1 Introduction

The conventional method of identifying tick specimen relies mostly on morphological characters and ecological distributions. Species determination using conventional methods (morphology-ecology) are limited for morphologically similar taxa, damaged specimens, and where immature stages are not described or are engorged [225]. This method is cumbersome and difficult since ticks vary marginally in size and in morphological characters [226]. This can be overcome by using ultrastructural observation of external features and molecular markers such as ITS and 16S rDNA which are highly conserved and easy to amplify using polymerase chain reaction (PCR). In this chapter, identification of tick species collected from Napaam village has been described. This is the first report on identification of the ticks

prevalent in this region using morphological and well-defined DNA markers. The ticks were identified as *Rhipicephalus* (*B*) *microplus* and *Haemaphysalis bispinosa* based on morphological characters. The phylogenetic status of the two ticks was also established using ITS2 and 16S ribosomal DNA. Moreover, a diagnostic tool based on PCR-RFLP of ITS2 sequence has been described to distinguish between the two tick species.

2.2 Materials and methods

2.2.1 Materials

DyNAzyme II DNA polymerase, restriction enzymes and T4 DNA ligase were from Thermo Scientific® (Pittsburgh, USA). DNeasy blood and tissue kit and QIAquick gel extraction kit were from Qiagen (Hilden, Germany). All other chemicals and reagents used were of analytical grade and were procured from Sigma (MO, USA), Merck (Mumbai, India) and Himedia (Mumbai, India).

2.2.2 Source of ticks

Tick at various developmental stages such as nymph, adult and fully-engorged were collected from Napaam village, Assam India (Latitude 26.70420^0 N and Longitude 92.83340^0 E), from cattle and goats sharing common pastures. The tick specimens were washed by sonication for 2-5 min in 70% ethanol. Washed ticks were placed in single vials, labeled and preserved in 70% ethanol until morphological identification and genomic DNA extraction. For salivary gland isolation, ticks were immediately dissected in the laboratory after collection.

2.2.3 Scanning Electron Microscopy (SEM)

Scanning electron microscopy was used to visualize the external features of the adult ticks. For SEM analysis, tick specimens were cleaned by sonication in 70% ethanol and washing twice in deionized water followed by fixation with 2.5% glutaraldehyde solution for 5 h. These were dehydrated with a gradient of 60-100% ethanol and critical-point dried [227]. The tick specimens were then fixed to metal stub attached with a conductive carbon tape, and sputter coated with gold in a JFC

1800 fine ion coater (JEOL Ltd., Tokyo, Japan). The specimens were observed and photographed under a JEOL 6510 scanning electron microscope (JEOL Ltd.) at the Sophisticated and Analytical Instrumentation Centre (SAIC) of Tezpur University, Tezpur. The external features were analyzed and recorded for identification.

2.2.4 Morphological identification of ticks

The ticks were identified to species level on the basis of their morphological characters as described by Miranpuri and Gill [228] and Geevarghes and Misra [203]. Several identification keys such as shape and size of gnathostome, basis capituli, palpi, position and shape of sex organs, anal groove and spiracles, presence or absence of eyes, festoons, ornamentation etc. were used for the correct identification of tick species.

2.2.5 DNA extraction from ticks

Total genomic DNA was isolated from the whole adult ticks using DNeasy Blood and Tissue kit (Qiagen). Briefly, individual ticks were dissected into pieces and suspended in 180 µl lysis buffer ATL and homogenized using tissue homogenizer (IKA, Bangalore, India) for 2-3 min. The homogenate was centrifuged and DNA was extracted using the kit, following manufacturer's instructions. Briefly, 20 µl Proteinase K was added to the homogenate and incubated at 56ºC for 2-4 h. 200 µl buffer AL and 200 µl 100% ethanol was added and mixed thoroughly. The mixture was applied to spin column supplied with the kit and centrifuged at 8000 rpm for 1 min, followed by washing with buffer AW1 and AW2. The DNA was eluted in 50-100 µl of elution buffer. The isolated DNA was run on 0.8% agarose gel to check its integrity and the concentration was determined spectrophotometrically (260/280 ratio) in a NanoDrop 2000 spectrophotometer (DE, USA).

2.2.6 DNA amplification by Polymerase Chain Reaction **(PCR)**

The genes were amplified using gene-specific primers. ITS2 was amplified using primers flanking 5.8S and 28S rDNA sequences as described previously [229], while for amplification of 16S ribosomal DNA (rDNA), primers designed from

consensus sequence were used (Table 2.1). A total of 0.2 µM of the primer sets and 1-2 µg of template DNA were used in a 25 µl PCR reaction mixture. Polymerase Chain Reaction (PCR) was performed as follows: One cycle of 94ºC for 5 min; 30 cycles of 94ºC for 30 s, 55ºC for 1 min, 72ºC for 2 min; and final extension of 72ºC for 10 min. The amplified DNA was electrophoresed on 0.8% agarose gel and visualized under UV light.

Table 2.1 Primers for amplification of ITS2 and 16S rDNA

Gene	Primer name	Primer sequence (5` to 3`)
ITS2	F1-ITS2	CGAGACTTGGTGTGAATTGCA
	R1-ITS2	TCCCATACACCACATTTCCCG
16S rDNA	F-RMI16S	AATTGCTGTAGTATTTTGAC
	R-RMI16S	TCTGAACTCAGATCAAGTAG

2.2.7 Gel extraction of PCR products

PCR products were run on agarose gel and then extracted using QIAquick gel extraction kit. The DNA fragment was excised from the gel with a fresh scalpel. The gel slice was weighed in a clean eppendorf tube and 3 volumes of buffer QG was added to it. This was incubated at 50°C for about 10 min till the gel was completely dissolved. 1 gel volume of isopropanol was added to the tube. The sample was then applied to a spin column and centrifuged at 13,000 rpm for 30-60 s. The flow-through was discarded and the column washed with 750 µl buffer PE. The DNA was eluted with 30-50 µl of buffer EB in a clean tube by centrifugation. The eluted DNA was analyzed on 0.8% agarose gel.

2.2.8 Sequencing of DNA

The genes were sequenced using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, MA, USA). 15 µl reactions were prepared using 3 pmol sequencing primer, containing approximately 3-10 ng of PCR product. Amplification was over 30 cycles at 95ºC for 10 s, 50ºC for 10 s, and 60ºC for 4 min. The PCR product was cleaned up by BigDye Terminator clean-up method. Briefly, 25 mM EDTA was added to the reaction, to which 2 µl 3 M sodium acetate (pH 4.6) and 50 µl

of ethanol was added and incubated for 15 min at room temperature. The mixture was centrifuged at 12,000 g for 20 min and the pellet washed with 70% ethanol. The pellet was dissolved in 15 µl Hi-Di formamide, transferred to sample tubes covered with septa, denatured and snap chilled. This was electrophoresed in an automated sequencer (Genetic Analyzer 3010, Applied Biosystems) and sequence obtained from the sequence analyzer (Applied Biosystems). The sequence obtained from the sequence analyzer was analysed using GENERUNNER.

2.2.9 Sequence analysis

The DNA sequences were submitted to GenBank using BankIt submission tool of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers were obtained (Table 2). The ITS2 and 16S rDNA sequences were aligned with sequences of different tick species obtained from the NCBI database using ClustalW program with default parameter settings [230]. Neighbor-Joining trees were constructed with bootstraps of 1,000 replicates based upon the alignment of ITS2 and 16S rDNA using MEGA program (version 5) [231]. The evolutionary distances were computed using the Kimura 2-parameter method [232] and are in the units of the number of base substitutions per site. Pairwise distance was calculated using MEGA5. All ambiguous positions were removed for each sequence pair.

2.2.10 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) of ITS2

For PCR-RFLP analysis the restriction sites of the sequences were mapped using the online tools of Rebase® (http://rebase.neb.com/rebase/rebase.html) and RestrictionMapper (http://www.restrictionmapper.org/) and suitable restriction enzyme was selected. PCR products were digested by *Hin*dIII enzyme (20 U) and run on 1% agarose gel stained with Nancy 520 (Sigma, MO, USA) along with Generuler 1 kb plus DNA ladder and documented.

2.3 Results

2.3.1 Morphological analysis of cattle-ticks

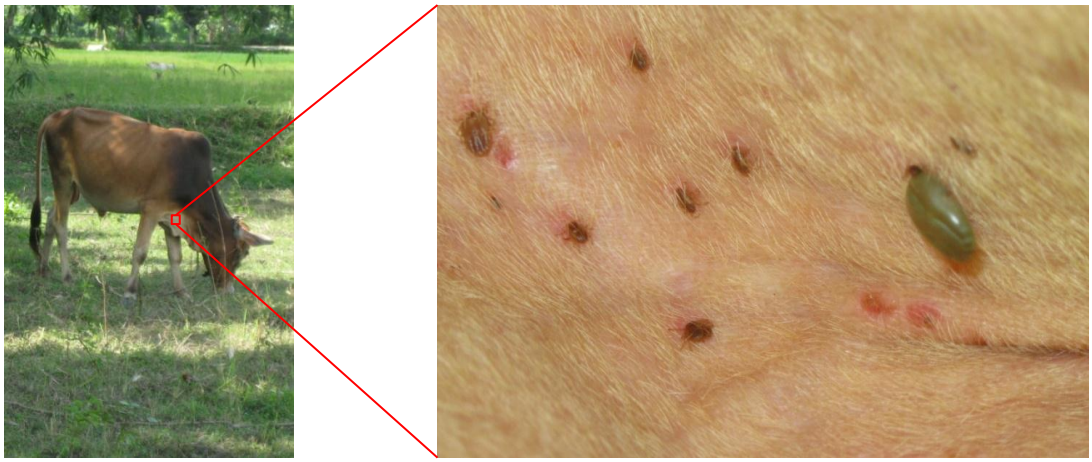


Figure 2.1 Representative of cattle for collection of ticks. Right panel: Ticks at different stages of feeding on host skin.

About 250 ticks (nymph, adult and full-engorged) were collected from throat and dewlap region of cattle (Fig. 2.1) and about 60 ticks (nymph, adult and full-engorged) from goats using tweezers. Based on morphological characters like shape of the capitulum, hypostomal dentition, bristle-bearing protuberance on palpal segment, spurs, cornua etc. these ticks were categorized. The male and female ticks were distinguished by the shape and position of genital operculum, presence of caudal appendages and adanal plates (Rhipicephaline ticks) and shape and size of festoons and spiracles (Haemaphysalinae ticks). The body weight of female Rhipicepahline ticks increased to about 130 times after feeding on host blood, while that of Haemaphysalinae ticks to about 110 times; the sizes of both the ticks increased to about 4-4.5 times after feeding (Fig. 2.2).

a

b

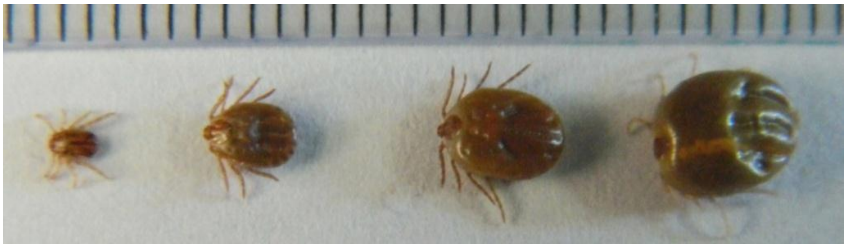


Figure 2.2 Female ticks (a: Rhipicephaline and b: Haemaphysalinae) at different feeding stages (Scale: 1mm).

Based on these characters the cattle ticks were morphologically identified as *Rhipiciphalus* (*Boophilus*) *microplus* and *Haemaphysalis bispinosa*, and the ticks collected from goats were found to be *H. bispinosa*. Further based on the scanning electron micrographs the adult ticks had 4/4 hypostomal dentition in both *R. microplus* and *H. bispinosa* (Fig. 2.3a, 2.3d). *R.* (*B*) *microplus* had a hexagonal basis capitulum, unlike that of *H. bispinosa* which had a rectangular basis capitulum. The ticks were identified to species level using following diagnostic morphological characters.

Rhipicephalus (B) microplus

The species of genus *Rhipicephalus* was confirmed to be *R.* (*B*) *microplus* based on characteristics like hypostomal dentition in 4 + 4 columns and absence of bristle bearing protuberance on the internal margin of the palpal segment 1, both in male and females (Fig. 2.3a). Coxa 1 spurs were distinct and genital aperture posterior lip was U-shaped in female *R.* (*B*) *microplus* (Fig. 2.3b). Male *R.* (*B*) *microplus* was identified based on the presence of caudal appendage and lack of distinct spur like extension of the adanal plate (Fig. 2.3c).

Differential diagnosis

The *R.* (*B*) *microplus* was distinguished from *R.* (*B*) *decoloratus* and *R.* (*B*). *annulatus* based on distinctive features of the ticks, as both are reported to occur in India. *R.* (*B*) *microplus* has a 4 + 4 column of teeth on its hypostome while *R.* (*B*) *decoloratus* has only a 3 + 3 column. *R. microplus* had no protuberance bearing setae on the inner margin of palp segment 1 but this protuberance is present in *R.* (*B*)

decoloratus. To differentiate *R.* (*B*) *microplus* from *R.* (*B*) *annulatus*, the caudal appendage in a male is the most conspicuous feature which was observed in *R.* (*B*) *microplus*, but absent in *R.* (*B*) *annulatus.*

Haemaphysalis bispinosa

This species was characterized by the absence of well-developed cornua. The lateral borders of the palpae were not widely salient and the tick showed prominent postero-dorsal and postero-ventral (Fig. 2.3d) spurs at palpal segment 3 in both, male and female tick. The postero-ventral spur of palpal segment 3 broadly triangular and blunt in both the sexes, overlapping anterior 1/3rd of palpal segment 2 in females (Fig. 2.3d) and anterior 1/2 of palpal segment 2 in males. The hypostome showed 4/4 rows of teeth (Fig. 2.3d) in both the sexes. Genital operculum in females was widely triangular in shape (Fig. 2.3e). The festoons in males were twice as long as broad. The spiracle in male was sub-oval, longer than broad with its dorsal and ventral sides parallel while in female spiracle was subcircular and as broad as long (Fig. 2.3f). Each coxa had a spure; coxa 1 possessed large spure (Fig. 2.3d and 2.3e) in both the sexes.

Differential diagnosis

Haemaphysalis bispinosa was differentiated from *H. anomala* and *H. intermedia*, as they share similar host range and geographical range based on distinctive feature between the tick species. *H. intermedia* is differentiated from both *H. bispinosa* and *H. anomala* by extra-long ventral spur on the palpal segment 2 which reached at the base of palpal segment 3 in case of male and slightly short of it in case of females while the spurs are shorter in the other two species, never crossing ½ of the 2nd palpal segment. Posterior salience on palpal segment 2 can be used to differentiate all three species. Palpae of *H. intermedia* lack posterior salience while it is widely salient in *H. anomala* and moderately salient in *H. bispinosa*. *H. anomala* and *H. bispinosa* males can be differentiated on the basis of spur of the coxa IV. It was short and single in *H. bispinosa*, while it is double and long in *H. anomala*. Punctations on scutum were few in *H. bispinosa* as compared to numerous punctuations on *H. anomala*, both in male and females.

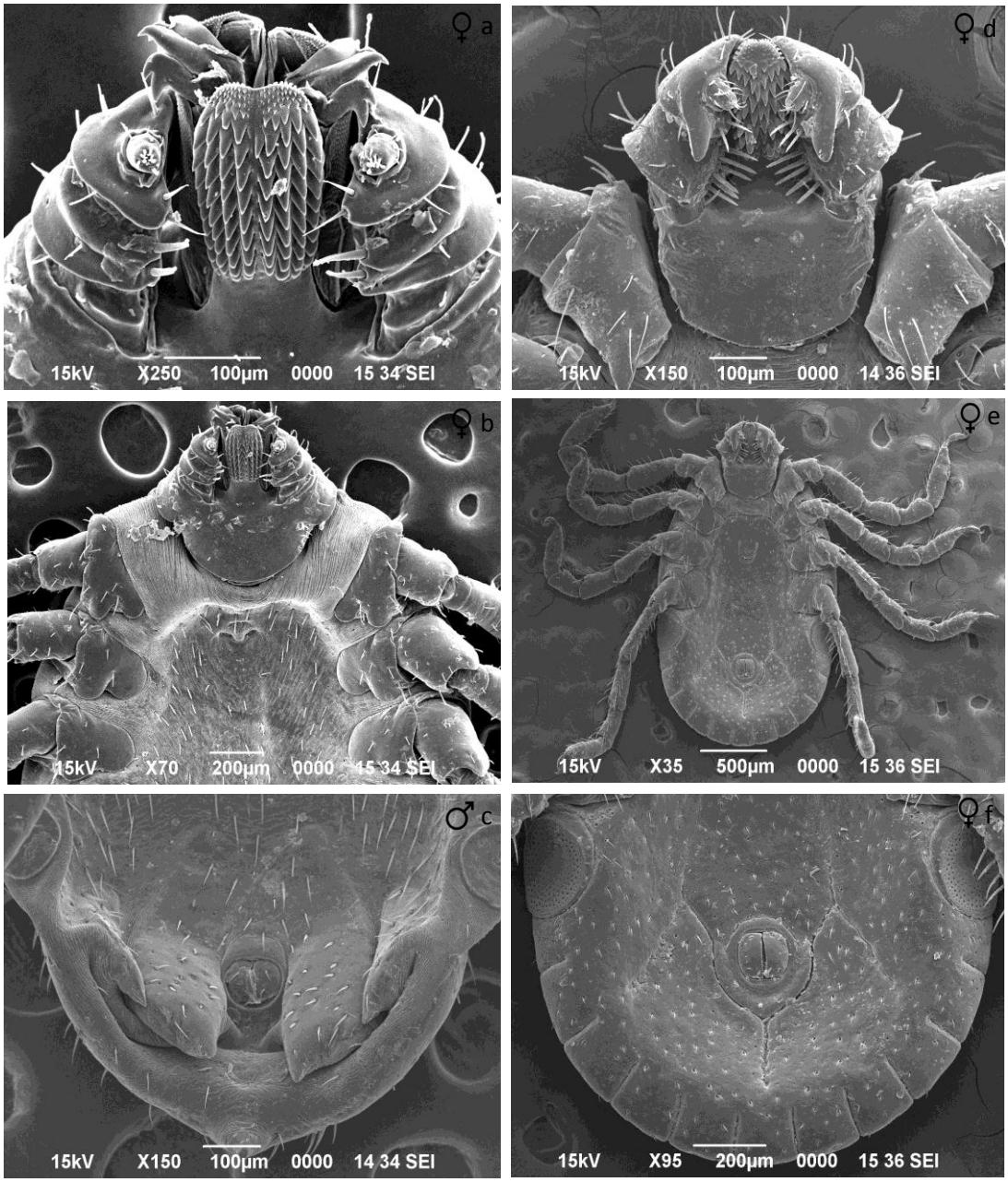


Figure 2.3 Electron micrographs of adult female *Rhipicephalus (B). microplus* (a, and b), adult male *R. (B). microplus* (c), and adult female *Haemaphysalis bispinosa* (d, e, and f).

Table 2.2 Some morphological characters of *R.* (*B*) *microplus* and *H. bispinosa.*

Morphological keys	**Tick species**	
	R. microplus	***H. bispinosa***
Basis capituli	Hexagonal	Rectangular
Scutum	Inornate	Inornate
Hypostome	Very short	Short and broad

Eyes	Present	Absent
Hypostomal dentition	4+4	4+4
Palpi	Wider than long, no protuberence on palpal segment 1	Wider than long
Spurs	Distinct coxa 1 spurs	Prominent postero-dorsal and postero-ventral spurs at palpal segment 3
Anal groove	Below anus	Below anus
Festoons	None	Present, twice as long as broad in male
Ornation	None	None
Genitalia	U-shaped	Triangular in shape
Adanal plates	Present in males	Absent
Spiracles	Bluntly or elongate comma-shaped	Sub-oval in male, subcircular in female

2.3.2 DNA extraction from ticks

Genomic DNA was isolated from whole tick using DNeasy blood and tissue kit. The 260/280 ratios of isolated DNA samples from tick specimens were found to be 1-1.15, which was due to RNA contamination, as the samples were not treated with RNase after cell lysis. A prominent band for the isolated gDNA was observed at ~ 20 kb when run on 0.8% agarose gel (Fig. 2.4).

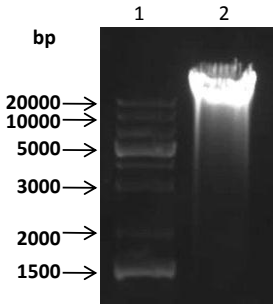


Figure 2.4 0.8% Agarose gel of tick genomic DNA. Lane 1: 1 kb plus DNA ladder; lane 2: genomic DNA.

2.3.3 Amplification and sequencing of ITS2 and 16S rDNA

The ITS2 gene amplified from these tick specimens were found to be approximately 1500 bp and 1700 bp for *R.* (*B*) *microplus* and *H. bispinosa*,

respectively (Fig. 2.5). While, amplified products of about 450 bp were obtained for mitochondrial 16S rDNA for each of these ticks (Fig 2.6). The ITS2 and 16S rDNA genes of 3 individuals of both *R. microplus* and *H. bispinosa* were sequenced using BigDye terminator cycle sequencing (Fig. 2.7) and submitted to GenBank of the National Center for Biotechnology Information (NCBI) (Table 2.3).

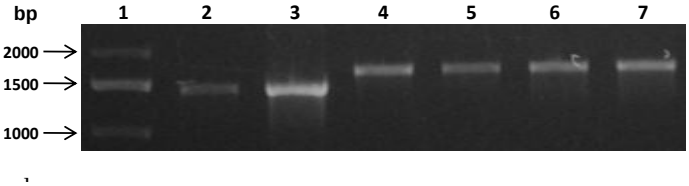


Figure 2.5 0.8% agarose gel of ITS2 PCR products. Lane: 1 kb plus DNA ladder; lane 2-3: ITS2 amplified from *R.* (*B*) *microplus*; lane 4-5: ITS2 amplified from *H. bispinosa.*

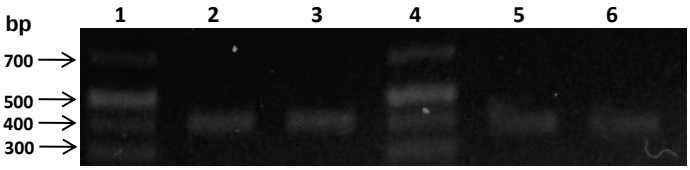


Figure 2.6 0.8% agarose gel of 16S rDNA PCR products. Lane 1 and 4**:** 1 kb plus DNA ladder; lane 2-3: *R.* (*B*) *microplus* 16S rDNA PCR product; lane 5-6: *H. bispinosa* 16S rDNA PCR product.

Table 2.3 Sequences submitted to NCBI database with GenBank™ accession numbers.

Species	Specimen voucher no.	Host	GenBank accession no. ITS2	16S
Rhipicephalus (*B*) *microplus*	TUTEZ-121	Cattle	JX974346	JX974347
Rhipicephalus (*B*) *microplus*	TUTEZ-R1301	Cattle	KC853417	KC853421
Rhipicephalus (*B*) *microplus*	TUTEZ-R13V4	Cattle	KC879264	KC953868
Haemaphysalis bispinosa	TUTEZ-G135	Goat	KC853416	KC853420
Haemaphysalis bispinosa	TUTEZ-R1324	Cattle	KC853414	KC853419
Haemaphysalis bispinosa	TUTEZ-R1320	Cattle	KC853415	KC853418

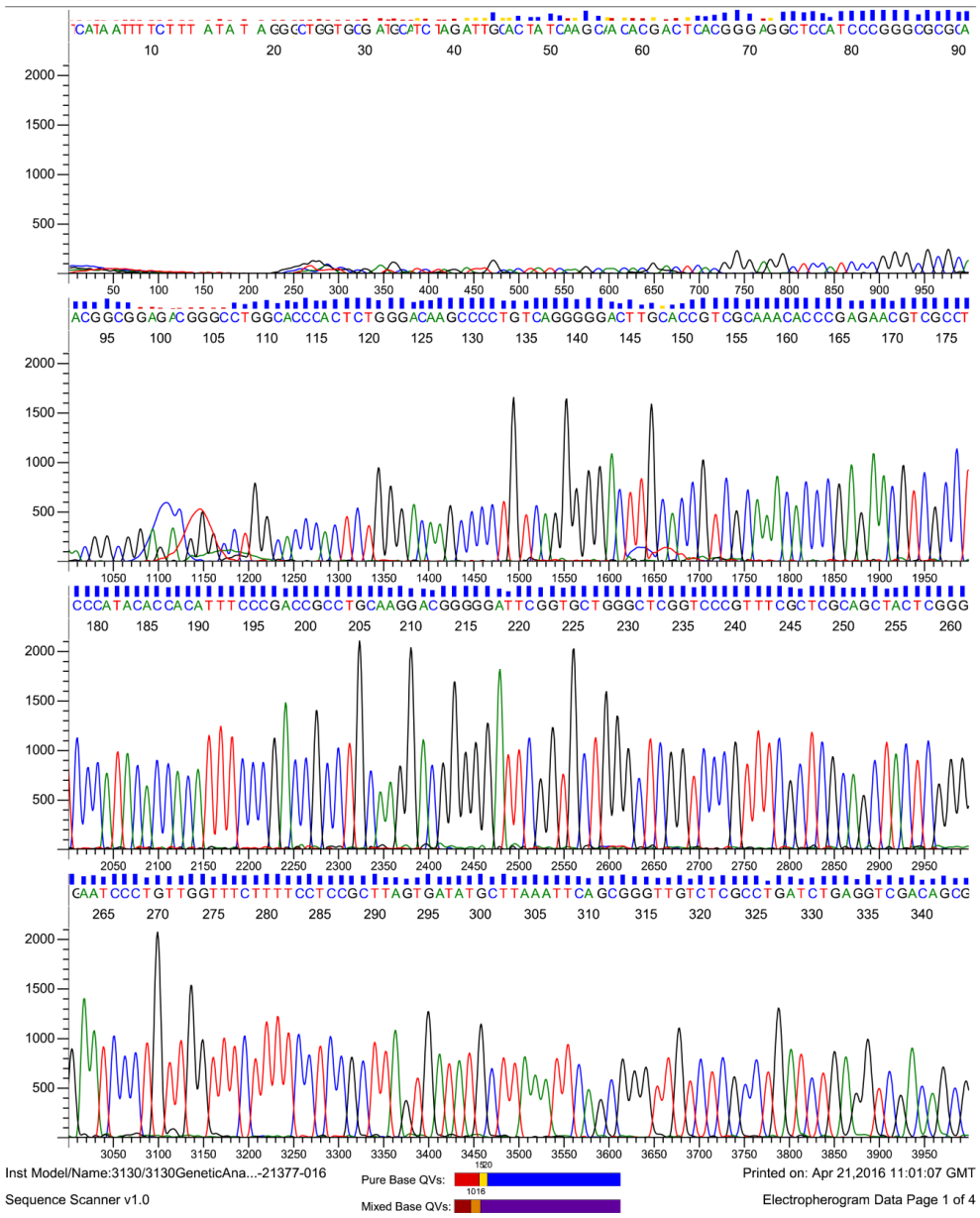


Figure 2.7 Chromatogram showing peaks corresponding to bases of ITS2 sequence from *R.* (*B*) *microplus* after Sanger sequencing.

a

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Rhipicephalus microplus (JX974347)   TGCTGTAGTATTTTGACTATACAAAGGTATTGAAATAAGGTTTTAATTGAATGCTAAAAGAATGGAATATCAAAGAATAACTTTCTTAAAATTAAAAATT
Rhipicephalus microplus (KC853421)   TGCTGTAGTATTTTGACTATACAAAGGTATTGAAATAAGGTTTTAATTGAATGCTAAAAGAATGGAATATCAAAGAATAACTTTCTTAAAATTAAAAATT
Rhipicephalus microplus (KC953868)   TGCTGTAGTATTTTGACTATACAAAGGTATTGAAATAAGGTTTTAATTGAATGCTAAAAGAATGGAATATCAAAGAATAACTTTCTTAAAATTAAAAATT
Consensus                            tgctgtagtattttgactatacaaaggtattgaaataaggttttaattgaatgctaaaagaatggaatatcaaagaataactttcttaaaattaaaaatt

Rhipicephalus microplus (JX974347)   GAAATTTTTTTAATTTGTGTAAAAACAATTATATAAATTAAAGACAAGAAGACCCTAAGAATTTTTAAAATTTAAATTAACACATTTTTGTTTTAATTAA
Rhipicephalus microplus (KC853421)   GAAATTTTTTTAATTTGTGTAAAAACAATTATATAAATTAAAGACAAGAAGACCCTAAGAATTTTTAAAATTTAAATTAACACATTTTTGTTTTAATTAA
Rhipicephalus microplus (KC953868)   GAAATTTTTTTAATTTGTGTAAAAACAATTATATAAATTAAAGACAAGAAGACCCTAAGAATTTTTAAAATTTAAATTAACACATTTTTGTTTTAATTAA
Consensus                            gaaatttttttaatttgtgtaaaaacaattatataaattaaagacaagaagaccctaagaattttaaaatttaaattaacacatttttgttttaattaa

Rhipicephalus microplus (JX974347)   ATTTTAACTGGGGCGGTTAAAAAATATTAAAAACTTTTTAATTTTGAAAATGACCCATTATTAATGAAAATATGATAAAATACTCTAGGGATAACAGCGT
Rhipicephalus microplus (KC853421)   ATTTTAACTGGGGCGGTTAAAAAATATTAAAAACTTTTTAATTTTGAAAATGACCCATTATTAATGAAAATATGATAAAATACTCTAGGGATAACAGCGT
Rhipicephalus microplus (KC953868)   ATTTTAACTGGGGCGGTTAAAAAATATTAAAAACTTTTTAATTTTGAAAATGACCCATTATTAATGAAAATATGATAAAATACTCTAGGGATAACAGCGT
Consensus                            attttaactggggcggttaaaaaatattaaaaacttttttaattttgaaaatgacccattattaatgaaaatatgataaaatactctagggataacagcgt

Rhipicephalus microplus (JX974347)   TATATTTTTTGATAGATCATATTGACAAAAAAGTTTGCGACCTCGATGTTGGATTAGGATACTTTTTTAATGAAGATATTAAAATAAGAAGTTTGTTCAA
Rhipicephalus microplus (KC853421)   TATATTTTTTGATAGATCATATTGACAAAAAAGTTTGCGACCTCGATGTTGGATTAGGATACTTTTTTAATGAAGATATTAAAATAAGAAGTTTGTTCAA
Rhipicephalus microplus (KC953868)   TATATTTTTTGATAGATCATATTGACAAAAAAGTTTGCGACCTCGATGTTGGATTAGGATACTTTTTTAATGAAGATATTAAAATAAGAAGTTTGTTCAA
Consensus                            tatatttttgatagatcatattgacaaaaaagtttgcgacctcgatgttggattaggatacttttttaatgaagatattaaaataagaagtttgttcaa

Rhipicephalus microplus (JX974347)   CTTTTAAATTCCTACTTGATCTGAGTTC
Rhipicephalus microplus (KC853421)   CTTTTAAATTCCTACTTGATCTGAGTTC
Rhipicephalus microplus (KC953868)   CTTTTAAATTCCTACTTGATCTGAGTTC
Consensus                            cttttaaattcctacttgatctgagttc
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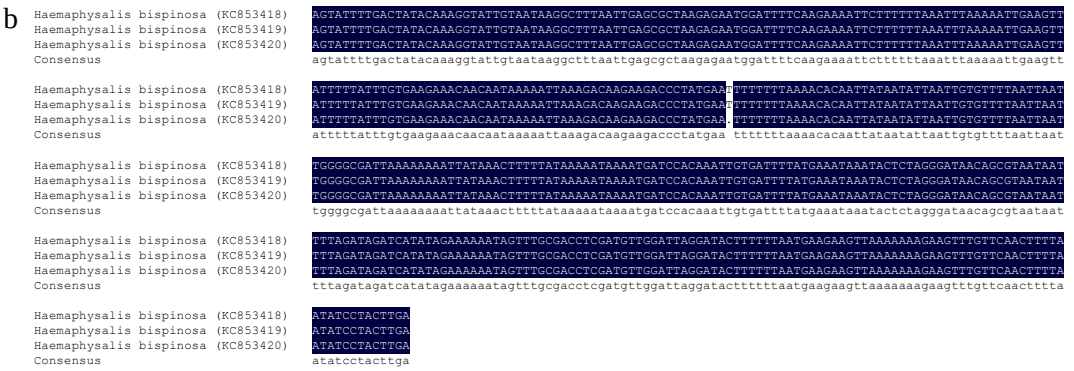
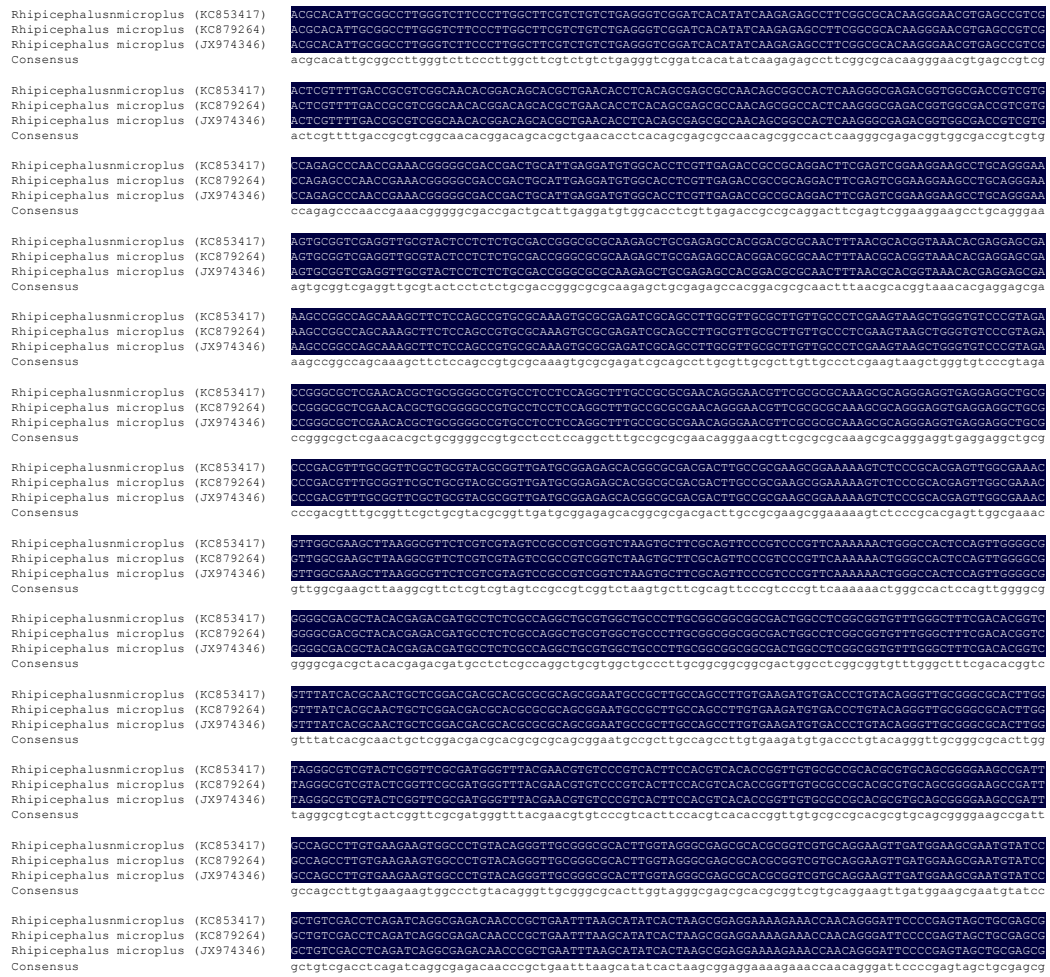


Figure 2.8 Nucleotide sequence alignment of 16S rDNA of (a) *R.* (*B*) *microplus* and (b) *H. bispinosa*.



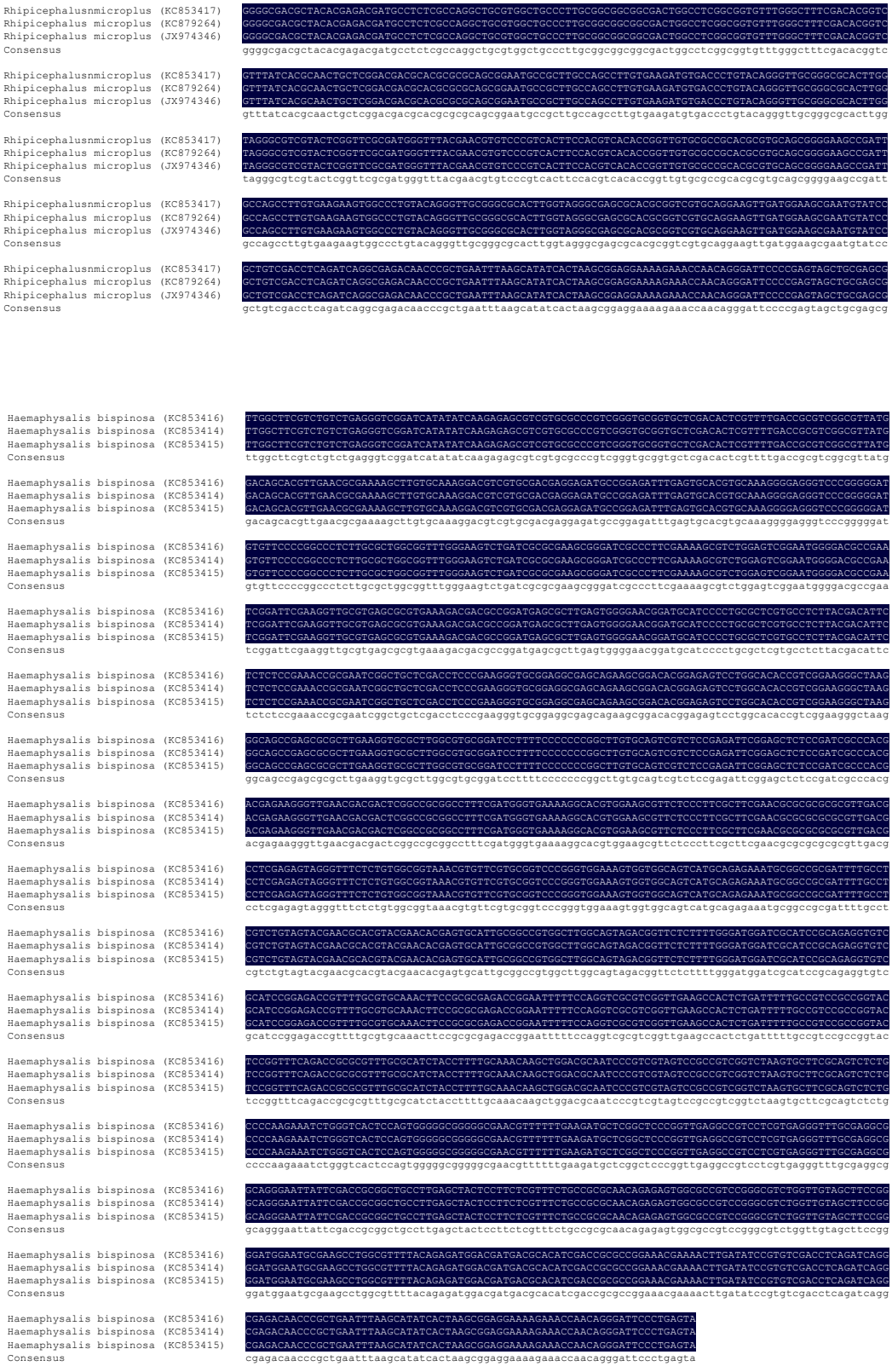


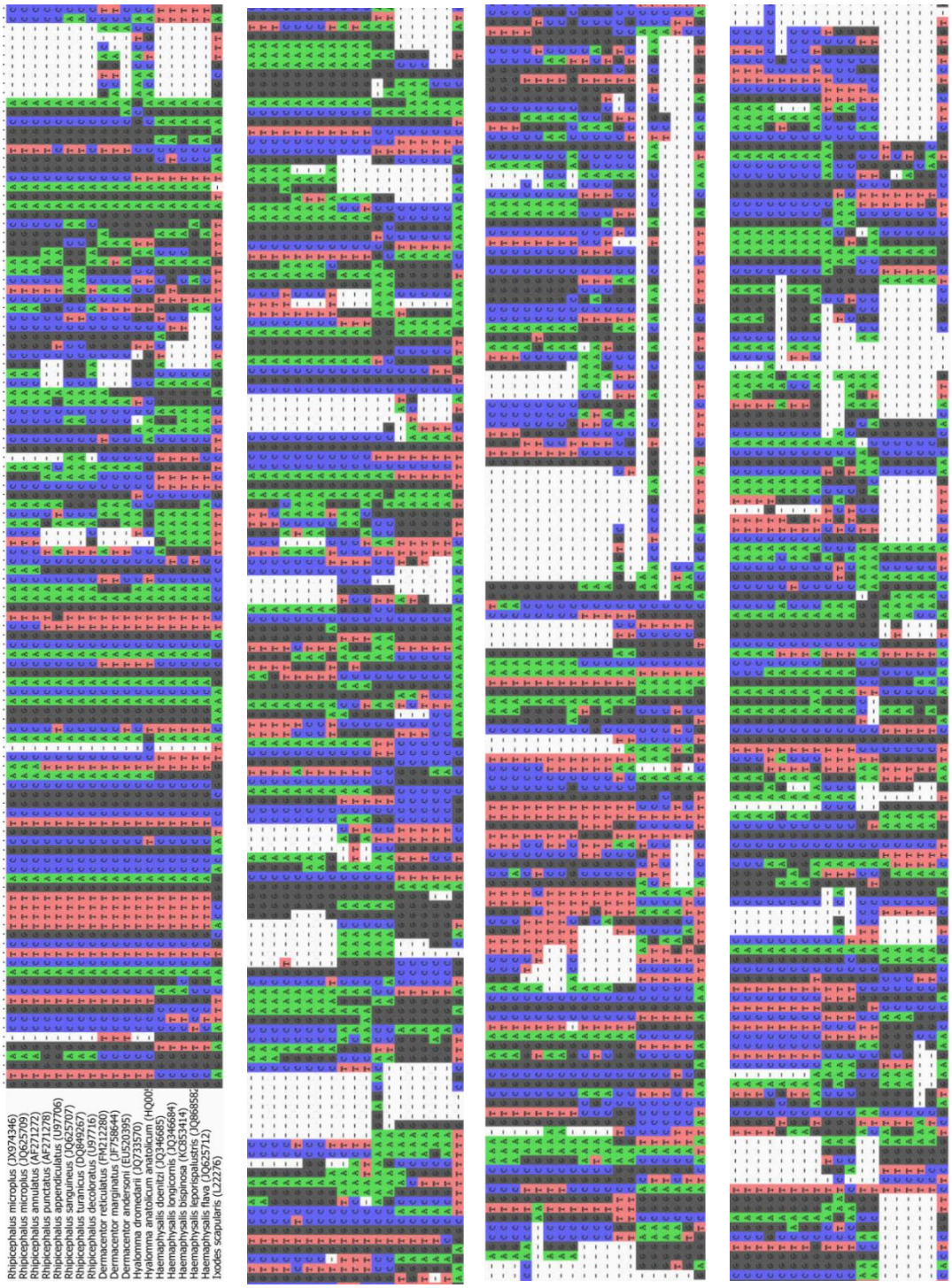
Figure 2.9 Nucleotide sequence alignment of ITS2 of (a) *R.* (*B*) *microplus* and (b) *H. bispinosa.*

2.3.4 Phylogenetic analysis

Phylogenetic relationships based on the alignment of ITS2 and 16S rDNA sequences were performed to analyze the evolutionary status of the two tick species in this study. The partial ITS2 sequences of *R.* (*B*) *microplus* and *H. bispinosa* were aligned to those of 17 tick species available in the nucleotide database at the NCBI (Fig. 2.10). The NJ tree was constructed based on alignment of ITS2 sequences using *Ixodes scapularis* (GU319067.1) as outgroup. Phylogenetically the *Rhipicephalus* tick in the present study is *R.* (*B*) *microplus* (Fig. 2.12, 2.13), which validates its identity concluded by morphological characterization. The tree based on ITS2 showed that *H. bispinosa* and *H. longicornis* are closely related as they were clustered together (Fig. 2.12). However, NJ tree constructed based on alignment of 16S rDNA sequences (Fig. 2.13) using *Dermanyssus gallinae* (L34326.1) as outgroup showed that *H. longicornis*, and *H. doenitzi* were clustered together with a bootstrap value of 62, while *H. bispinosa* branched out from these two suggesting that they may be closely related species. Pairwise distance analysis of the *Haemaphysalis* ticks showed that *H. bispinosa* is genetically closest to *H. longicornis* (Table 2.4). When the ITS2 sequences of *H. bispinosa* and *H. longicornis* were aligned, it was found that a nucleotide fragment of 236 bp was missing from the sequence of *H. bispinosa* (Fig. 2.14).

Table 2.4 Pairwise distance of ITS2 of *Haemaphysalis bispinosa* with 5 *Haemaphysalis* and 1 *Ixodes* ticks.

	Species (accession number)	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	*Haemaphysalis bispinosa* (KC853414)	-						
2	*Haemaphysalis longicornis* (JQ346684)	0.104	-					
3	*Haemaphysalis doenitzi* (JQ346685)	0.210	0.245	-				
4	*Haemaphysalis qinghaiensis* (HQ005302)	0.253	0.272	0.270	-			
5	*Haemaphysali sflava* (JQ625712)	0.257	0.274	0.273	0.021	-		
6	*Haemaphysalis leporispalustris* (JQ868582)	0.263	0.281	0.274	0.135	0.146	-	
7	*Ixodes scapularis* (GU319067)	1.264	1.336	1.395	1.267	1.294	1.310	-


Rhipicephalus microplus (JX974346)
Rhipicephalus microplus (JQ625709)
Rhipicephalus annulatus (AF271272)
Rhipicephalus punctatus (AF271278)
Rhipicephalus appendiculatus (U97706)
Rhipicephalus sanguineus (JQ625707)
Rhipicephalus turanicus (DQ849267)
Rhipicephalus decoloratus (U97716)
Dermacentor reticulatus (FM212280)
Dermacentor marginatus (JF758644)
Dermacentor andersoni (EU520395)
Hyalomma dromedarii (JQ733570)
Haemaphysalis doenitzi (JQ346685)
Haemaphysalis longicornis (JQ346694)
Haemaphysalis bispinosa (KC853414)
Haemaphysalis leporispalustris (JQ868582)
Haemaphysalis flava (JQ625712)
Ixodes scapularis (L22276)

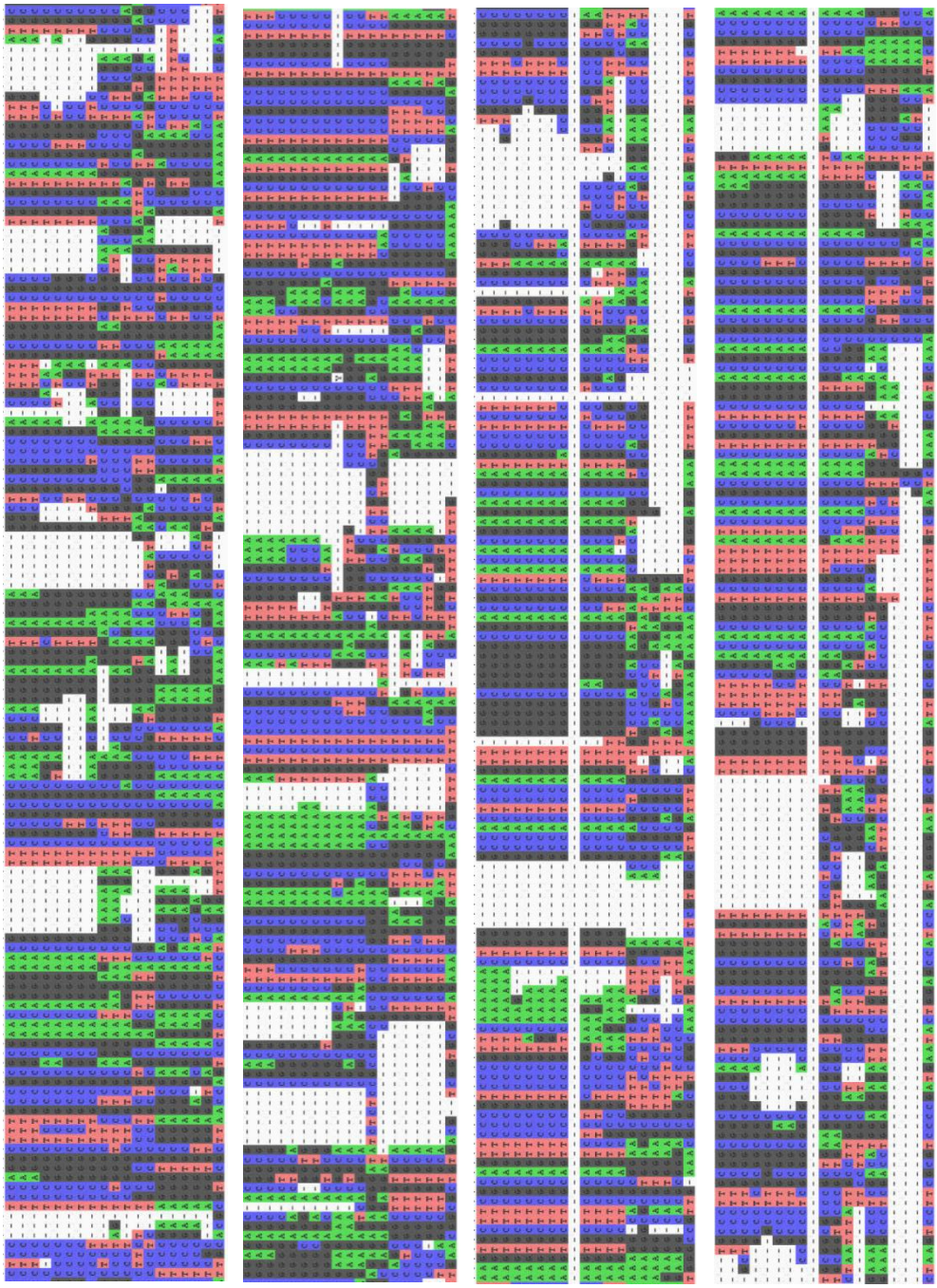


Figure 2.10 Nucleotide sequence alignment of ITS2 of *H. bispinosa* performed using ClustalW and viewed on AliView.

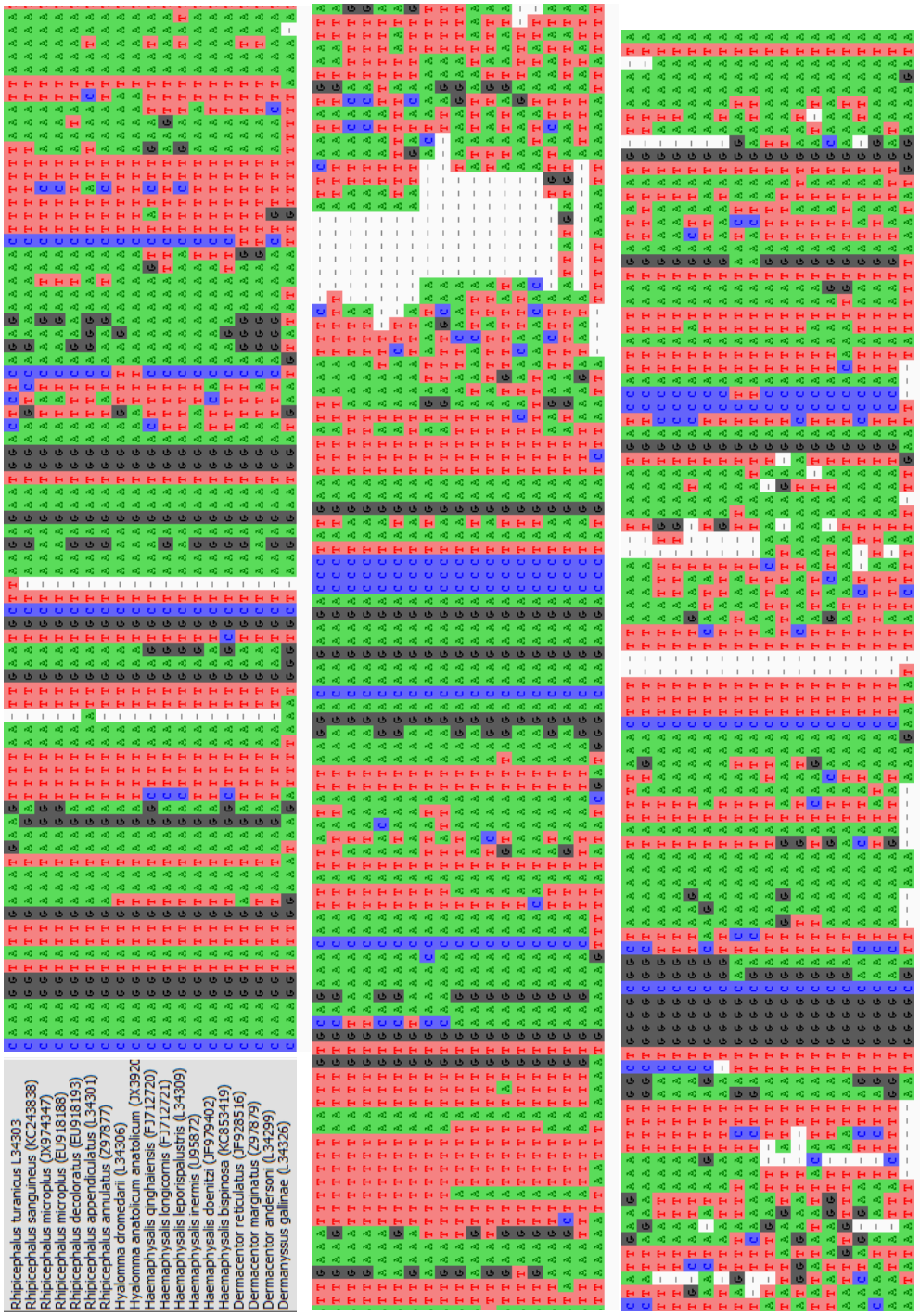


Figure 2.11 Nucleotide sequence alignment of 16S rDNA performed using ClustalW and viewed on AliView.

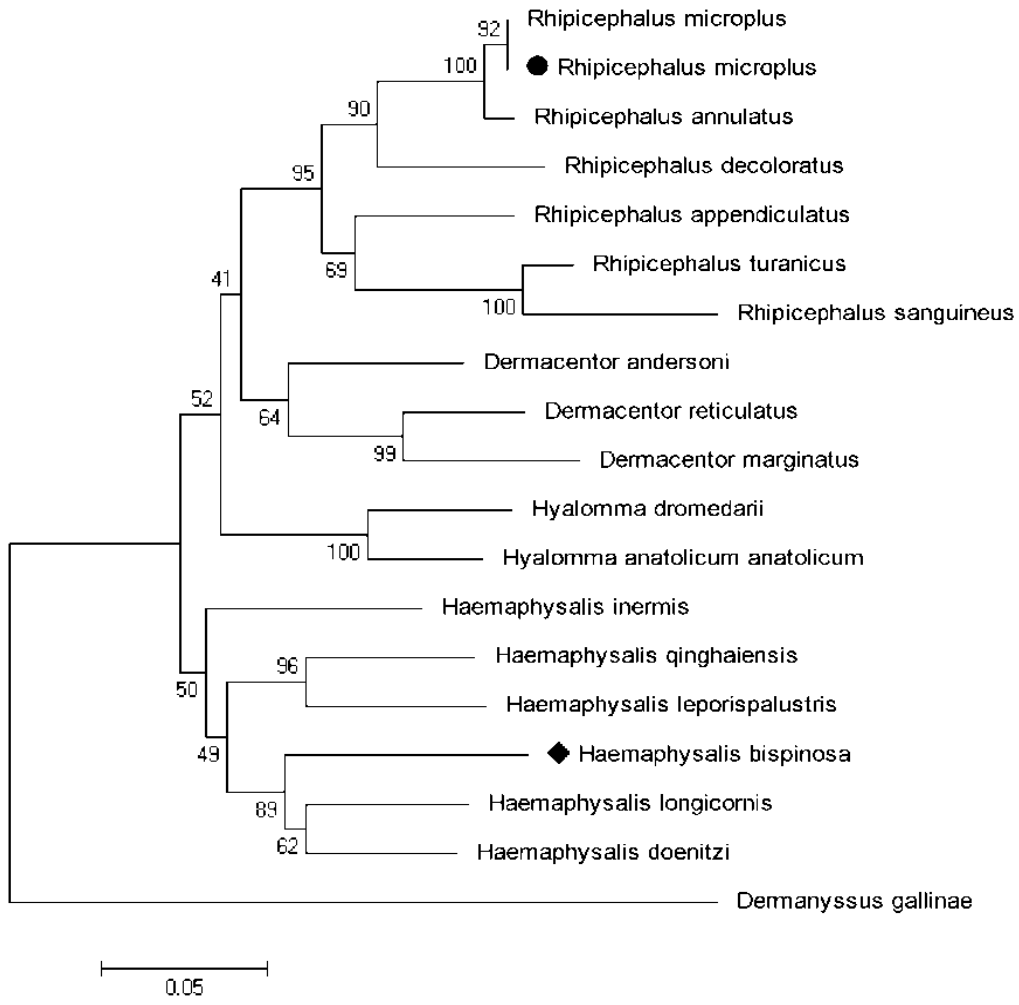


Figure 2.12 Neighbor-Joining tree constructed based on sequence alignment of ITS2 sequences with 1,000 bootstraps. ITS2 sequences from database are: *R. microplus* (JQ625709.1), *R. decoloratus* (U97716.1), *R. appendiculatus* (U97706.1), *R. zambeziensis* (DQ849261.1), *R. turanicus* (DQ849267.1), *R. sanguineus* (JQ625707.1), *R. punctatus* (AF271278.1), *D. andersoni* (EU520395.1), *D. reticulatus* (FM212280.1), *D. occidentalis* (DQ248056.1), *D. marginatus* (JF758644.1), *Hyalomma dromedarii* (JQ733570.1), *Hy. anatolicum anatolicum* (HQ005303.1), *Haemaphysalis flava* (JQ625712.1), *H. doenitzi* (JQ346685.1), *H. longicornis* (JQ346684.1), *H. qinghaiensis* (HQ005302.1), *H. humerosa* (AF199115.1), *H. leporispalustris* (JQ868582.1), and *I. scapularis* (GU319067.1).

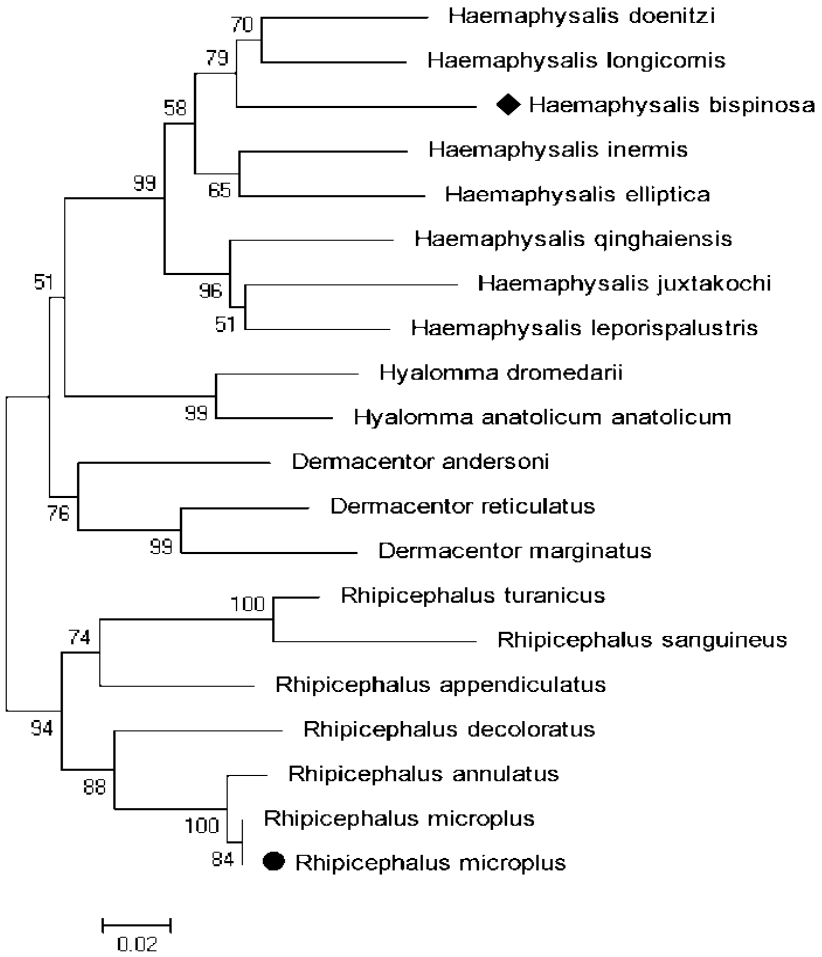


Figure 2.13 Neighbor-Joining tree constructed based on sequence alignment of 16S rDNA sequences with 1,000 bootstraps. 16S rDNA sequences are *R. microplus* (EU918188.1), *R. decoloratus* (EU918193.1), *R. annulatus* (Z97877.1), *R. appendiculatus* (L34301.1), *R. turanicus* (L34303.1), *R. sanguineus* (KC243838.1), *D. andersoni* (L34299.1), *D. reticulatus* (JF928516.1), *D. marginatus* (Z97879.1), *Hy. dromedarii* (L34306.1), *Hy. anatolicum anatolicum* (JX392003.1), *H. doenitzi* (JF979402.2), *H. longicornis* (FJ712721.1), *H. qinghaiensis* (FJ712720.1), *H. leporispalustris* (L34309.1), *H. elliptica* (HM068961.1), *H. juxtakochi* (AY762324.1), and *H. inermis* (U95872.1).

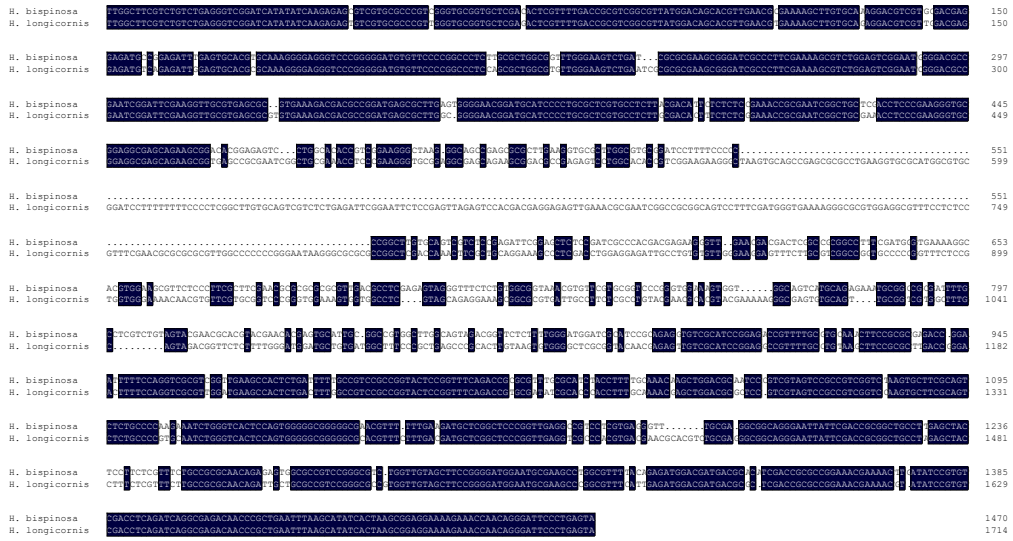


Figure 2.14 Nucleic acid sequence alignment of ITS2 sequences of *H. bispinosa* and *H. longicornis* using DNAMAN software. Similar bases are shaded and dots represent missing bases.

2.3.5 PCR-RFLP analysis of ITS2

Restriction maps analyzed using online tools revealed *Hind*III to be suitable enzyme for distinguishing the two species in the present study, as ITS2 sequence of *R.* (*B*) *microplus* has three and *H. bispinosa* has two *Hind*III restriction sites (Fig. 2.15). The results of PCR-RFLP assay could differentiate between the two ticks based on the sequence differences of ITS2. Digestion of ITS2 of *R.* (*B*) *microplus* by *Hind*III resulted into three bands (around 700 bp, 500 bp and 300 bp), whereas that of *H. bispinosa* was digested into two bands (around 1500 and 200 bp) (Fig. 2.16). The PCR-RFLP assay had identical profiles for the two developmental stages of *H. bispinosa* (Fig. 2.16; Lane 3 and 4 represents nymphal ticks, Lane 5 and 6 represents adult ticks).

Name: Haemaphysalis bispinosa ITS2

Conformation: linear

Enzymes: HindIII

Noncutters:

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
HindIII	AAGCTT	6	five_prime	1	121

Name: Rhipicephalus microplus ITS2

Conformation: linear

Enzymes: HindIII

Noncutters:

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
HindIII	AAGCTT	6	five_prime	2	512, 806

Figure 2.15 Restriction sties were mapped using online tool RestrictionMapper.

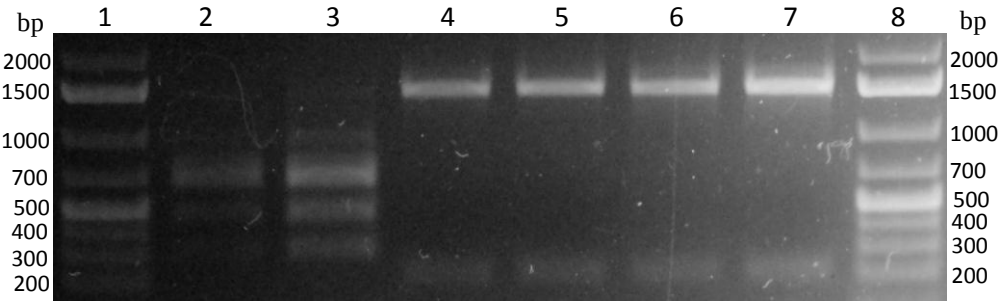


Figure 2.16 PCR-RFLP analysis of ITS2 gene using restriction enzyme *Hind*III. Lanes 2 and 3: *R. microplus*; lanes 4-7: *H. bispinosa* samples; lane 1 and 8 represents 1 kb plus DNA molecular weight marker.

2.4 Discussions

Ticks are obligate hematophagous ectoparasites of terrestrial vertebrates, comprising of three families, the Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae. These blood-feeding parasites acquire blood meal from their host; soft ticks feed for few hours whereas hard ticks feed for several days, continuously deteriorating the animal health leading to decrease in productivity such as milk production. Moreover, they are serious vectors of many disease-causing viruses and bacteria. For example, they transmit several arboviruses (e.g., tick-borne encephalitis virus, several Reoviridae, Bunyaviridae, and Iridoviridae), protista (*Babesia* and *Theileria*), and bacteria (*Rickettsia, Ehrlichia, Borrelia*) [83]. Hence, ticks and tick-borne diseases (TTBDs) are of immense economic importance. TTBDs affect 80% of the cattle throughout the globe, particularly tropical and subtropical countries, including India [86]. India share nearly 57% of world's buffaloes, 16.5% cattle, 16.3% goats, and 5.7% sheep (FAO 2004) and 70% of the population depend on agriculture for their livelihood. TTBDs cause very large damage to livestock in India (cost of TTBDs estimated to be US$ 498.7 million) [87], affecting the livelihood of a large fraction of the population [88]. 106 tick species have been reported in the Indian subcontinent, of which the genera *Rhipicephalus* and *Hyalomma* are the most widespread [233]. Tick belonging to *Amblyomma* (including *Aponomma*), *Haemaphysalis*, *Dermacentor*, *Rhipicephalus* (including *Boophilus*), *Hyalomma*,

Ixodes, *Nosomma*, *Argas*, *Ornithodoros*, and *Otobius* genera are found across the various states [89], among which *Amblyomma*, *Haemaphysalis*, *Dermacentor*, *Rhipicephalus*, *Hyalomma*, and *Ixodes* ticks are found in the state of Assam [88].

Quick and reliable identification of ticks is therefore important to control the spread of ticks and tick-borne diseases. Besides that ticks are rich source of anti-hemostatic molecules which are potential targets for drug development against cardiovascular diseases. Morphological characterization based on phenotypic characters is laborious, often imprecise and may lead to misidentification. For instance, differentiation of ticks of closely related species of ticks at the larval and nymphal stage is difficult and often inaccurate [234]. Morphological keys used to distinguish between species tend to overlap between species and sometimes vary within species and according to age and size, which makes it difficult to identify the ticks. In addition to that morphological identification of damaged specimens due to handling and preservation is often inaccurate. Ticks like *Rhipicephalus* and *Hyalomma species* are difficult to identify morphologically even by using the nomenclature [235]. Closely related species *Hyalomma anatolicum excavatum* and *Hyalomma anatolicum anatolicum* are difficult to differentiate using morphological traits which are very weak [236]. Moreover morphological identification of ticks can be misleading because of intraspecific variations and morphological changes of the tick's body during feeding [234].

The limitations of morphological identification and characterization of ticks can be overcome by using molecular markers. Molecular markers like mitochondrial 12S/16S rDNA, cytochrome oxidase subunit I (COI) and nuclear ribosomal ITS2 have been successfully used to study the evolution and phylogenies of mites and ticks. ITS2 has proved to be useful in inferring phylogenies since it contains little intra-specific variation but considerable inter-specific difference [237,238]. Mitochondrial DNA sequences may also be suitable for genetic analysis of closely related species because they evolve rapidly and are inherited maternally [239,240]. Based on ITS2 sequence the genetic diversity at both inter- and intra-species levels of various Ixodidae ticks as well as their phylogenetic relationship have been successfully analyzed [237,241,242]. Mitochondrial 16S rDNA has also been widely used to analyze the phylogeny and systematic evolution of ticks [242-247]. Moreover ITS1

and ITS2 has been successfully used to differentiate ticks within individuals/population, between individuals of different geographic locations within a species, and between species of *Ixodes dammini*, *I. scapularis* and *I. pacificus* [242]. Similarly 16S rDNA has been used to assess the phylogenetic relationships of various economically important tick species [227,240,244,248]. Mitochondrial DNA sequences are suitable for genetic analysis of closely related species because they evolve rapidly and are inherited maternally.

In the present study, two tick species, *Rhipicephalus* (*B*) *microplus* and *Haemaphysalis bispinosa*, were identified and characterized using both morphological and genetic tools. These were found to be two most abundant species that parasitize cattle and other domestic animals in this region. *R.* (*B*) *microplus* is primarily a cattle tick, while *H. bispinosa* was found to parasitize both cattle and goats. *H. bispinosa* is the most common species under the genus *Haemaphysalis* and is reported to be prevalent in different parts of India [89,216]. The fully-engorged ticks of both the species were difficult to be distinguished with naked eyes due to their appearance and colour. The most distinct feature between the two species is the basis capitulum which was observed to be hexagonal for the *Rhipicephaline* tick, while rectangular for the *Haemaphysaline* tick. Well-developed cornua was absent in the later differentiating it from the former. The male *Rhipicephaline* ticks had caudal appendages which distinguished it from its female counterpart and the other tick species in the study. These features were visible only under microscope and though the adult ticks could be differentiated visually, fully-engorged ticks were hard to differentiate. Both the ticks when full-engorged appeared dark in color, similar in size and festoons were not apparent.

R. (*B*) *microplus* ITS2 sequence in the present investigation showed maximum similarity with that of *R.* (*B*) *microplus* (JQ625709.1) with nucleotide divergence of only 0.1%. Phylogenetic analysis supported the sequence alignment and distance analysis results. The *R.* (*B*) *microplus* ITS2 sequence (JX974346.1) showed more than 98% homology with that of *R. microplus* (JQ625709.1), containing 14 transversions, 4 transitions, 1 deletion and 3 additions. While ITS2 sequence of *H. bispinosa* was found to be 64.53% and 62.75% similar to that of *H. longicornis* and *H. doenitzi*, respectively. The partial 16S rDNA of *R. microplus* showed 100% similarity to *R.*

microplus (EU918188.1) with no deletion or addition of nucleotide bases. The 16S rDNA of *H. bispinosa* showed 88.11% similarity to *H. longicornis* (FJ712721.1) and 87.79% similarity to *H. doenitzi* (JF979402.1). There is no earlier report of characterization or phylogenetic analyses of *H. bispinosa* based on 16S rDNA or ITS2 sequences. Hence, this is the first report of such study. The mitochondrial 16S rDNA of both the tick species was found to be AT-rich (about 77% A+T). This is in accordance with the A+T content of the 16S rDNA sequences of other hard ticks available in the database (74-79% A+T). Phylogenetic tree based on 16S rDNA and ITS2 showed *H. bispinosa* was closest to *H. longicornis.* However, sequence alignment of ITS2 sequence showed that a segment of 236 bp is missing from *H. bispinosa* as compared to *H. longicornis* (Fig. 2.14). Such deletion or addition of DNA segment in ITS2 sequence is observed during its evolution which resulted in increase or decrease of the length of stems of the secondary structure [249]. Analysis of the ITS2 sequence revealed that increase in size might be because of replication slippage which generated larger repeats [249]. Comparison of the ITS2 sequence of *H. bispinosa* and *H. longicornis* showed that 236 bp is missing in *H. bispinosa.* Hence, *H. bispinosa* might have evolved from *H. longicornis* by decrease in length of stems of the secondary structure or the later may have evolved from the former by increase in the same. The G+C contents of the ITS2 sequences of the two ticks were also in accordance with those of ticks from the Metastriata lineage (61-62%) [249].

Molecular tool has been successfully used to differentiate between closely related tick species which differs marginally when morphological characters are considered [229,247,250]. To establish the species-specific profiles and to discriminate the distant or closely related ticks, a PCR-RFLP of ITS2 sequence was performed. The PCR-RFLP profile of ITS2 of *R. (B) microplus* and *H. bispinosa* showed clear distinction in the digestion pattern of the ITS2 sequence. The ITS2 genes of the two tick species differed in their nucleotide base-pairs such that the restriction patterns of the two were different. The ITS2 of *R. (B) microplus* had two *Hind*III restriction sites, while that of *H. bispinosa* had one site. Hence this PCR based tool could be used to quickly differentiate the tick species prevalent in this region which differs marginally when fully fed. Hence the morphological characters along with the genetic markers can be combined for distinguish between closely

related species. This is the first report of morphological and molecular characterization of tick species prevalent in this region. This technique can be used to map the diversity of the tick species prevalent in this region and can be helpful in immunization of cattle and goat against the pathogen carried by the ticks.