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Genetic Similarity within Isolates of Population of the Cattle Tick, *Rhipicephalus microplus* Based on Mitochondrial Cytochrome Oxidase Subunit-I Gene Sequences

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Abstract: Molecular phylogenetic analysis was carried out using mitochondrial nucleotide sequences (COI) to provide evidence in genetic relatedness among various isolates of cattle tick *Rhipicephalus microplus* collected from different locations in Chennai, Tamil Nadu, India. The mitochondrial COI gene region of ticks was amplified and sequenced. A multiple sequence alignment using edited sequences of various isolates of ticks was carried out and a phylogenetic tree was constructed. The results on multiple sequence alignment and phylogram revealed that COI gene sequence of the eight isolates of ticks collected had more than 99.90 % similarity. It confirmed that COI gene was most conserved in the mitochondrial DNA of the collected cattle tick, *R. microplus.* Subsequently, the sequences were used to predict the secondary structure models of COI which also revealed similar such close relationships. These results in the present study reflect a single monophyletic group with no functional diversity at the mitochondrial COI gene region either in the sequence or structures was observed at various isolates of cattle tick *R. microplus*. The indication of absence of genetic variation within isolates of ticks was revealed in the present.

Key words: Arachinida · Ixodidae · Molecular Phylogeny · Cattle Vector · Isolate · Chennai

INTRODUCTION

Ticks are considered as vectors of many infectious diseases in cattle and human around the world. It has been reported that 80% of 1200 million cattle are at risk for ticks and tick-borne diseases causing a global annual loss of US$ 7000 million [1]. *Rhipicephalus microplus* is an important pest of cattle in subtropical and tropical countries of the world [2]. Control measures of *R. microplus* in these countries are usually accomplished by regular applications of chemical acaricides [3]. The long term use of these chemical compounds was known to cause harmful effects [4]. Some of these synthetic compounds have residual effects and accumulate in environment, which induce resistance in various strains of ticks. It has been known for a long period of time that the tropical cattle tick can also develop resistant strains against the synthetic acaricides [5, 6]. Further, their use also caused great concern in society and government, by harming the animals themselves and humans who consume the products from these animals [7]. A basic understanding on the genetic variability of tick population is actually helpful in recognizing the susceptibility or resistance nature of ticks for effective control [8]. The molecular techniques and related tools are significantly advanced the knowledge on control of ticks and diagnosis of tick-borne diseases. Studies on genomic variations within a species have great impact on understanding the pathogen transmission, the epidemiology of the illness and its control [9]. Hence, an attempt was made in the present investigation to identify genetic similarity in the populations of tick species *R. microplus* collected from various locations in and around the city of Chennai, Tamil Nadu, India using the most conserved mitochondrial cytochrome oxidase subunit-I (COI) gene sequences.

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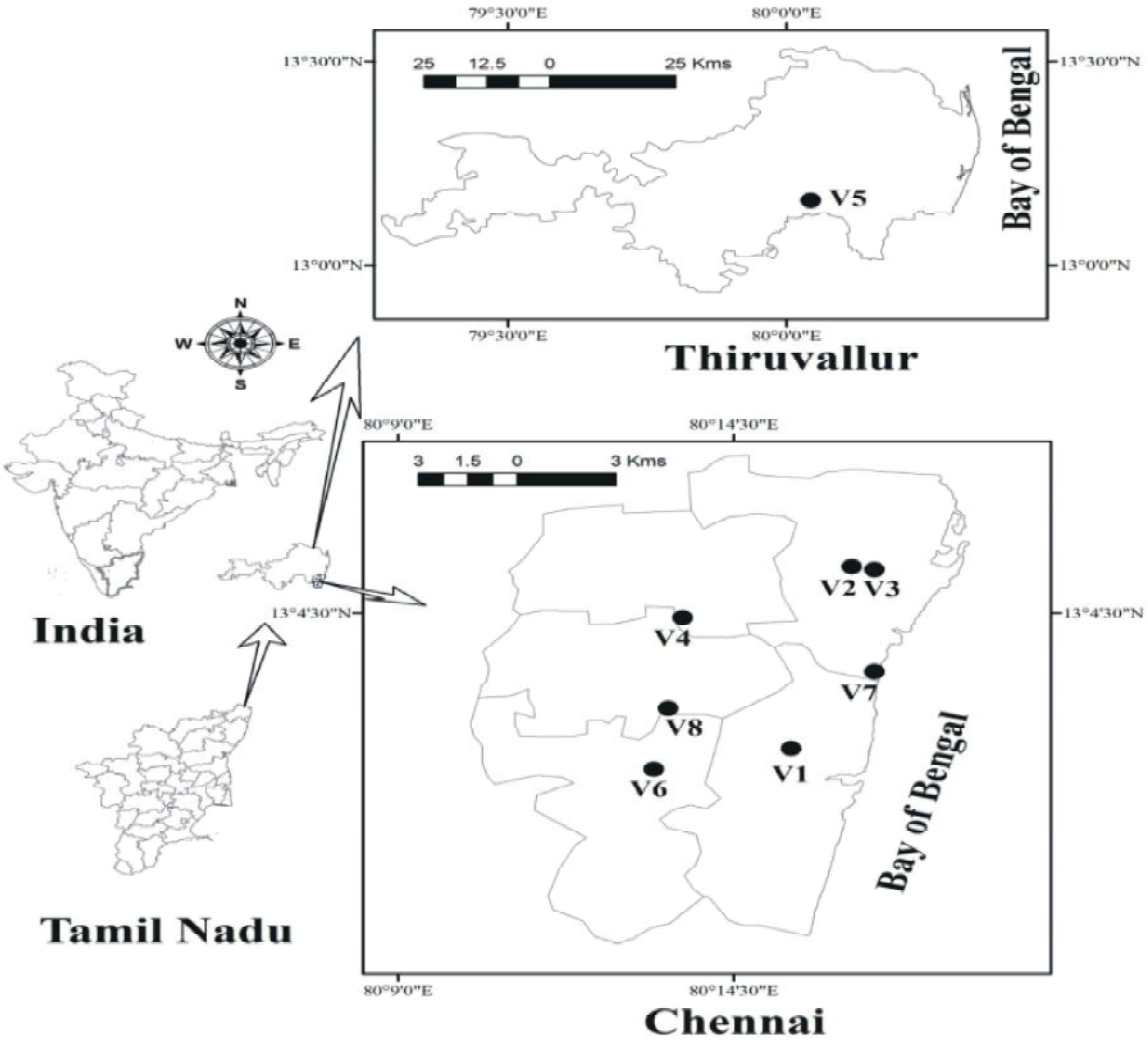


Fig. 1: Collection sites of cattle tick, *Rhipicephalus microplus* in and around the district of Chennai, Tamil Nadu. v1 to v8 are eight collection sites namely, v1- Mandaveli; v2 - Basin bridge; v3 - Sowcarpet; v4 - Aminjikarai; v5 - Pallavedu pettai; v6 - Saidapet; v7 - Triplicane and v8 - West Mambalam

MATERIALS AND METHODS

Adult ticks of *Rhipicephalus* (=*Boophilus*) *microplus* (Arachinida: Ixodidae) were collected from naturally infested cattle in different places in and around Chennai (Fig. 1). They were collected from each selected site from the body of cows by the aid of blunt pointed forceps and brush to avoid any harm to ticks and host. The collected ticks were put in vials and brought to the laboratory. Adult ticks were identified and classified using their morphological characteristics according to the standard descriptions provided by Walker *et al.* [10]. The whole specimens of *Rhipicephalus* (=*Boophilus*) *microplus* were subjected to scanning electron microscope (Hitachi S-3400 scanning electron microscope at 15.0•kV) from the Centre for Advanced Studies in Botany, University of Madras, Chennai, India. The specimens were mounted on holders, examined and photographs were taken.

Total DNA was extracted using phenol-chloroform method [11]. The gut was collected from single adult female ticks and gut contents were removed. It was washed with sterile distilled water. It was then ground with pre-chilled mortar and pestle to fine powder using liquid nitrogen and suspended in 1ml digestionbuffer (100 mM Tris-HCl, pH 8.0;10 mM EDTA pH 8.0; 1.4 M NaCl; 1% SDS; 0.2% mercaptoethanol with 100 µg/ml proteinase K). The sample was incubated with occasional

shaking, in tightly capped micro tubes for 60 minutes at 50°C. The sample was then extracted with equal volume of phenol-chloroform and was centrifuged for 10 minutes at 10000 rpm. The top aqueous layer was transferred to a new tube. To the supernatant, 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 100% ice cold ethanol were added to precipitate the DNA. It was centrifuged for 2 minutes at 4000 rpm. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer (pH 8.0). DNA concentration was measured spectrophotometrically at 260 nm in a spectrophotometer. Aliquots of the DNA (100 ng) were then used for polymerase chain reaction.

The COI region of mitochondrial DNA was amplified from the total genomic DNA by PCR using conserved primers [12]. C1-N-2191 (5'-CCC GGT AAA ATT AAA ATA TAA ACT TC-3') and C1-J-1718 (5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3') were used to amplify the region of COI of mitochondrial DNA. PCR was carried out in 25 µl reaction volume in a Long Gene MG25+ Thermal Cycler. It was mixed well and centrifuged briefly. A control reaction was prepared without template DNA (negative control). The tubes were then placed in a thermal cycler. It was preheated at 94°C for 4 minutes followed by 35 cycles of 1 min at 94°C, 1 min at 56°C,

1 min at 72 °C and final extension of 72°C for 5 minutes. The amplified product (5µl) was mixed with sample buffer (1µl) and checked for the amplification using 1.2 % agarose gel electrophoresis [11]. The amplified DNA products were purified using gel purification kit purchased from Chromous Biotech, Bangalore, India. The purification was done as per the protocol provided by the kit method. The purified PCR fragments were sequenced using DNA Sequencing Services with Xcelris Laboratory Ltd., Ahmedabad, India. Nucleotide sequences were then edited using BioEdit programme [13]. The gene sequences were aligned in FASTA format and then analyzed using ClustalW [14]. The program was then used to calculate the best match for the selected sequences, similarities and differences through viewing the phylograms based on Neighbour-Joining (N-J) phylogeny. The individual edited sequences were then analyzed for the secondary structures of COI [15].

RESULTS

The fully-engorged ticks collected from cows were identified based on the morphological characters like capitulum, scutum, ventre and legs. The ticks were identified to species level using whole specimens by stereomicroscope and scanning electron micrographs based on the characteristic morphological characters (Fig. 2 & 3). The identified ticks were belonged to *Rhipicephalus* (=*Boophilus*) *microplus*. The species of genus *Rhipicephalus* was confirmed to be *R. microplus* based on the characteristic features like hypostomal dentition and absence of bristle bearing protuberance on the internal margin of the palpal segment. Coxa 1 spurs were distinct and genital aperture at posterior lip was U-shaped in female *R. microplus*. *Rhipicephalus* (=*Boophilus*) *microplus* is very similar to *R.* (=*Boophilus*) *annulatus*. Both species are without a protuberance bearing a seta on the inner margin of the first palp articles. The internal margin of the first palp article of *R. microplus* is short and deeply concave but in the case of *R. annulatus* this margin is long and slightly concave. In addition, the spurs and cleft between the spurs on the first coxa of *R. annulatus* female are less distinct than those of female of *R. microplus*. The second coxa of *R. annulatus* females is without a spur but there is a small spur on the second coxa of *R. microplus* females. The male of *R. annulatus* lacks a caudal appendage but male of *R. microplus* has a caudal appendage.

The molecular phylogenetic relationships of eight different isolates of the population of ectoparasitic tick, *R. microplus* collected from cattle was drawn based on the most conserved mitochondrial cytochrome oxidase subunit-I (COI) gene sequences. PCR amplification of partial COI gene from the total genomic DNA of various isolates of the cattle tick, *R. microplus* yielded a fragment of DNA in agarose gel electrophoresis of all the samples at a region around 500 bp (Fig. 4). The nucleotide sequencing of purified COI fragment of various tick samples after edition was observed as 447 bp in length (Table 1). The nucleotide base composition revealed an increased adenine and thymine (AT) content in all the COI sequences of various tick samples than the guanine and cytosine (GC) content. It was between 68.01 % and 68.23 % in the AT content of different tick isolates of *R. microplus* than the GC content that recorded a value between 31.77 % and 31.99 % (Table 1).

The COI specific nucleotide sequences obtained for various isolates were subjected to NCBI-BLAST and these sequences were found to match with the available database sequences of *Rhipicephalus microplus*. Then the sequences were analyzed under multiple sequence alignment programme to observe the sequence homology among various isolates of ticks (Fig. 5). They were then subjected to BOOTS TRAP- Neighbour-Joining tree with

Table 1: Mitochondrial COI nucleotide sequence information of various isolates of cattle tick, *Rhipicephalus microplus*

			Molecular weight		Nucleotides									
Isolate of ticks	DDBJ GenBank accession number	Size of COI gene (bp) (edited)	Single stranded (Da)	Double stranded (Da)	A (bp)	Mol %	T (bp)	Mol %	AT %	C(bp)	Mol %	G (bp)	Mol %	GC %
V1	AB896714	447	134524	270398	113	25.28	191	42.73	68.01	79	17.67	64	14.32	31.99
V2	AB896812	447	134483	270381	113	25.28	192	42.95	68.23	78	17.45	64	14.32	31.77
V3	AB896813	447	134524	270398	113	25.28	191	42.73	68.01	79	17.67	64	14.32	31.99
V4	AB896814	447	134483	270381	113	25.28	192	42.95	68.23	78	17.45	64	14.32	31.77
V5	AB896815	447	134524	270398	113	25.28	191	42.73	68.01	79	17.67	64	14.32	31.99
V6	AB896816	447	134483	270381	113	25.28	192	42.95	68.23	78	17.45	64	14.32	31.77
V7	AB896817	447	134524	270398	113	25.28	191	42.73	68.01	79	17.67	64	14.32	31.99
V8	AB896818	447	134524	270398	113	25.28	191	42.73	68.01	79	17.67	64	14.32	31.99

Guanine (G), Adenine (A), Thymine (T), Cytosine (C), Mol % - Molar percent, bp – base pair

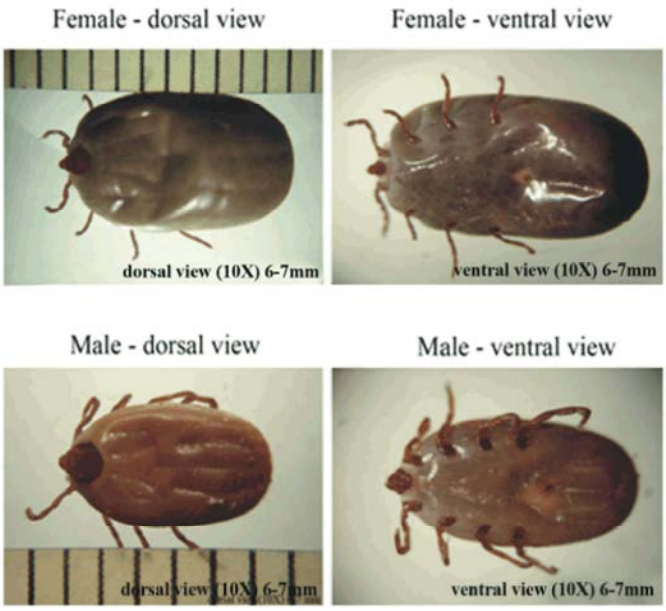


Fig. 2: Photomicrographs of cattle tick, *Rhipicephalus microplus*

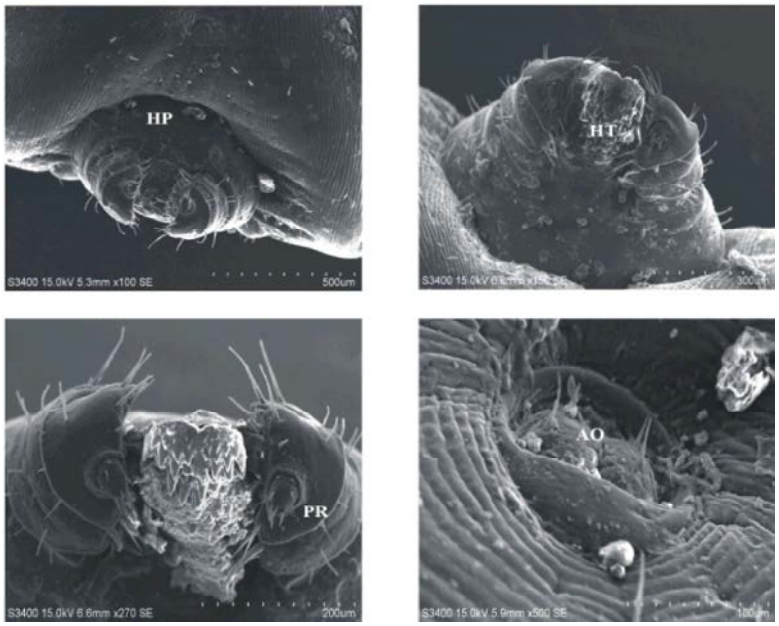
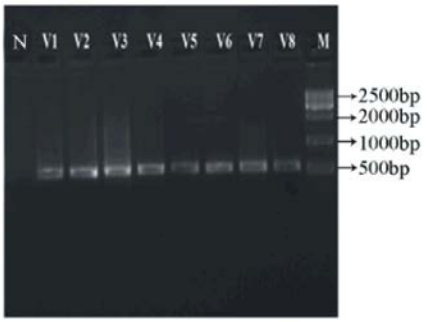


Fig. 3: Scanning electron photomicrographs showing frontal view of the head and ventral view of cattle tick, *Rhipicephalus microplus*. HP - hexagonal plate; HT - hypostomal teeth; PR - palpi ridged; AO - anal orifice.



V1 - Mandaveli;V2 - Basin bridge; V3 - Sowcarpet; V4 - Aminjikarai; V5 - Palavedu pettai;
V4 - Saidapet; V7 - Triplicane; V8 - West Mambalam; N - Negative control; M - Molecular Marker

Fig. 4: Agarose gel electrophoresis of mitochondrial COI specific amplified fragment from the genomic DNA of eight different isolates of cattle tick, *Rhipicephalus microplus*

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V7   1 AATATAAGATTTTGA----------TAGCTCA---CCCTGAAGTTTATATTTTATAA 447
V8   1 AATATAAGATTTTGA----------TAGCTCA---CCCTGAAGTTTATATTTTATAA 447
V5   1 AATATAAGATTTTGA----------TAGCTCA---CCCTGAAGTTTATATTTTATAA 447
V3   1 AATATAAGATTTTGA----------TAGCTCA---CCCTGAAGTTTATATTTTATAA 447
V1   1 AATATAAGATTTTGA----------TAGCTCA---CCCTGAAGTTTATATTTTATAA 447
V2   1 AATATAAGATTTTGA----------TAGTTCA---CCCTGAAGTTTATATTTTATAA 447
V4   1 AATATAAGATTTTGA----------TAGTTCA---CCCTGAAGTTTATATTTTATAA 447
V6   1 AATATAAGATTTTGA----------TAGTTCA---CCCTGAAGTTTATATTTTATAA 447
       ******************************* ***************************
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Note: Variation in the nucleotide base at 357th position in the edited COI sequences is indicated in a box.

Fig. 5: Multiple sequence alignment of edited mitochondrial COI nucleotide sequences of eight different isolates of cattle tick, *Rhipicephalus microplus* (ClustalW 2.1)

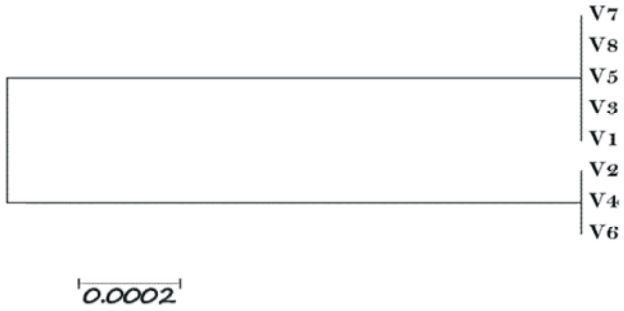

Fig. 6: Neighbour-Joining (NJ) TREE with UPGMA for nucleotide sequences of mitochondrial COI gene from different isolates of cattle tick, *Rhipicephalus microplus*

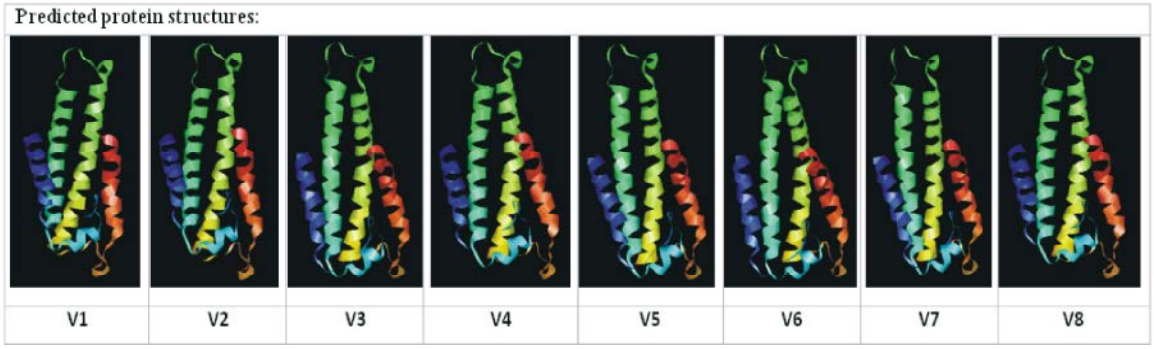

Fig. 7: Protein secondary structure predicted for the edited nucleotide sequences of mitochondrial COI gene from different isolates of cattle tick, *Rhipicephalus microplus*

UPGMA that revealed sequence identity matrix among various tick isolates between values of 0.000 and 0.0002 (Fig. 6). There was a variation in the nucleotide base at one point in the edited sequences of COI region among various isolates of ticks which contributed formation of isolates v2, v4 and v6 in a subcluster in the major cluster comprised of isolates v1, v3, v5, v7 and v8. This indicated occurrence of almost no genetic variation among different isolates of cattle tick, *R. microplus* based on nucleotide sequences of mitochondrial cytochrome oxidase subunit-I. Later, the individual COI amino acid sequences of various isolates of ticks were used to predict their secondary structures. There was a perfect structural homology predicted for various COI based amino acid sequences of different isolates of ticks without any significant variation among various secondary structures (Fig. 7: v1 to v8).

DISCUSSION

Ticks are obligatory blood feeding ectoparasites of terrestrial mammals. These blood feeding parasites acquire blood meal from their mammalian hosts. Among various families of ticks, the hard ticks belonging to Ixodidae feed on their hosts for several days persistently weakening the health of animals. Furthermore, they are serious vectors of many diseases in mammals [16]. Hence, ticks and tick-borne diseases are of immense economic importance. Tick-borne diseases affect 80 % of the cattle throughout the globe, particularly tropical and subtropical countries, including India [17]. Control measures of *R. microplus* are usually accomplished by regular applications of chemical acaricides [3]. The long term use of these chemical compounds was known to cause harmful effects [4]. Further, their use also caused injury to the animals themselves and humans who consume their products [7].

A basic awareness on genetic diversity of tick population is useful in understanding the nature of ticks for their effective control [8]. Studies on genomic variations within a species of tick have great impact on understanding the vector transmission and its control. Several molecular tools that are specifically used in genetic analyses helped to improve the knowledge on control of ticks [9]. Among them, mitochondrial DNA sequences are considered suitable for genetic analysis of closely related species as they evolve rapidly and inherited maternally [18]. An attempt was made in the present study to analyze the genetic similarity that exist at intra-population level with reference to various isolates of cattle tick, *R. microplus* using mitochondrial COI nucleotide sequences. The results in the present study indicated an incidence of no genetic variation among different isolates of cattle tick, *R. microplus* in the mitochondrial COI gene sequence based multiple alignments of these nucleotide bases. The literature consequently reported that sequence alignments, the basis for phylogenetic comparison, that do not account for structural homology may not reflect true evolutionary relationships. In DNA, genotype may be considered as the sequence of nucleotides, whereas the phenotype is the structure that may be formed by that sequence [19].

Therefore, an attempt was made in the study to construct secondary structure for all the isolates of cattle tick *R. microplus* using mitochondrial cytochrome oxidase subunit-I amino acid sequences. In similarity to the results on multiple sequence alignment, no variations were observed in the COI secondary structures of various isolates of *R. microplus* in the isolates of cattle ticks. As reported earlier, features of secondary structure themselves can be treated as evolving characters and phylogenetic connections may be traced by changes in structural character status [20]. The types of variation that might be observable thus include single base substitutions, insertions and deletions, base-pair substitutions within a conserved region; and insertion and deletion of entire secondary structure elements [21, 22]. As a result, genetic similarity observed in the secondary structures of COI amino acid sequences of various isolates of tick, *R. microplus* could be related to the fact that all the isolates were genetically identical i.e., they were in one monophyletic group. This kind of conservation in genetic elements within isolates of a single tick population is most important in terms of their influence on the nature of vector potential.

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