



Molecular characterization and phylogenetic insight of cattle tick *Rhipicephalus microplus* from subtropical mountainous terrain of Meghalaya, India

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ABSTRACT

The present study intended to investigate the prevalence, molecular characterization, and phylogenetic perspectives of cattle tick *Rhipicephalus microplus* from Meghalaya's sub-tropical mountainous terrain. From April 2022 to December 2023, 539 ticks were collected from Holstein-Friesian (62) and Jersey (28) crossbred cattle of about one year age from Umling and Umsning blocks of Ri Bhoi district, Meghalaya. Ticks were identified morphologically, and haemoprotozoan parasites were detected by staining thin blood smears with Giemsa stain. The overall incidence of *R. microplus* tick infestation was 11.36% in crossbred cattle, 25.80% in Holstein Friesian crosses and 14.28% in Jersey crosses. Indigenous cattle breed unaffected by *R. microplus* tick infestation. *Babesia bigemina* infection was detected in merely 9.37% of Umiam's crossbred (Holstein Friesian) cattle. Tick mitochondrial DNA cytochrome c oxidase subunit I (COXI) genome amplification by Polymerase Chain Reaction (PCR) revealed 713bp band size. Sequencing and BLAST analysis of mitochondrial COX1 gene confirmed *R. microplus* isolate from Umiam, Meghalaya (OR830443.1). Mitochondrial COX1 gene of *R. microplus* isolates from Pakistan (OP379525.1), Bangladesh (MG459962.1), Myanmar (MG459964.1) and China (OQ547218.1, OR083275.1) shared 99.63 to 100% nucleotide homology with the mitochondrial COX1 gene of *R. microplus* isolate from Umiam, Meghalaya (OR830443.1). A close genetic relationship of *R. microplus* isolates from Meghalaya, North East India with those from the Northern state, Haryana and Southern state, Tamil Nadu and Kerala was observed. This report may be considered to be the first work on the genomic description and phylogenetic insight about *R. microplus* in cattle from sub-tropical mountainous terrain of Meghalaya, India which will be useful in developing strategies for the management and control of *R. microplus* tick in the region.

Keywords: COXI gene, Meghalaya, Molecular characterization, *Rhipicephalus microplus*

Ticks create a substantial threat to both human and animal well-being and result in substantial economic losses, especially in tropical and subtropical areas worldwide (Sultan *et al.* 2022). As stated by Walker *et al.* (2014), ticks represent a family of arachnids classified as ectoparasites and fed on blood. They are the members of the order Acari and the suborder Ixodida. According to Lew-Tabor and Valle (2016), tick infestation affects over 80% of the world's cattle population. The southern cattle tick, *Rhipicephalus microplus* (previously *Boophilus microplus*), is a significant tick species that feeds on cattle blood (Low *et al.* 2015). It has been found to transmit organisms such as *Anaplasma*, *Rickettsia*, *Theileria*, *Babesia*, and *Borrelia*. Ticks also cause anaemia, irritation, skin and epidermal damage, balding, hair loss, and decreased cattle productivity (Shemshade *et al.* 2011, Nnabuife *et al.* 2021). Furthermore, it is anticipated that *R. microplus* would result in yearly economic losses of 2.5

billion US dollars in tropical and subtropical areas (Lew-Tabor *et al.* 2014).

Cattle (192.49 million) play a significant role in India's economy, accounting for 35.93% of the overall livestock population. However, in Meghalaya, the total indigenous and crossbred cattle numbered 870165 and 33405, respectively, while in the Ri Bhoi district, the total number of indigenous and crossbred cattle totalled 38094 and 16468, respectively (Livestock census 2019). There are several areas in which *R. microplus* ticks may be found, including India, the countries of Southeast Asia, South America, Central America, Australia, African continent, the islands of Madagascar, Mascarene, New Caledonia, and French Polynesia (Estrada-Pena *et al.* 2006, Barre and Uilenberg 2010). Although there are various records reporting the prevalence of *R. microplus* ticks in cattle around the world, and just a few reports on their molecular characterization from Brazil (Csordas *et al.* 2016); Pakistan (Sultan *et al.* 2022, Zeb *et al.* 2020); Bangladesh, Pakistan and Myanmar (Roy *et al.* 2018); Punjab (Singh and Rath 2013), Kerala and Karnataka (Amrutha *et al.* 2023), Uttar Pradesh (Niranjan *et al.* 2022), Uttarakhand (Vatsya *et al.* 2008), Andhra Pradesh (Rajendran and Hafiez

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2003), Andaman and Nicobar Islands (De *et al.* 2022), Maharashtra (Shahardar *et al.* 1998), Assam (Mushahary *et al.* 2019) and Mizoram (Ghosh *et al.* 2019). However, comprehensive information about the prevalence and genomic characteristics of the *R. microplus* tick in cattle hailing from Meghalaya's subtropical mountainous terrain is still lacking. Thus, the current study aimed to investigate the incidence, genomic characterization, and evolution perspectives of the *R. microplus* tick in cattle from Meghalaya's subtropical mountain terrain.

MATERIALS AND METHODS

Study location and period: Meghalaya is a state in North Eastern India known as "the Abode of Clouds." The geographical coordinates of its location are between 25°02' to 26° 07' N latitude and 89°49' to 92°50' E longitude. This state is known for its hilly landscapes, which have undulating grasslands interrupted up by valleys and rivers. The study encompassed from April'2022 to December'2023 in six villages *viz.* Sarikuchi, Borkhatsari, Lalumpam in Umling block, and Umiam, Umsawkhwan, Umbir in Umsning block, respectively of Ri Bhoi district, Meghalaya (Fig. 1). The climate of the Umling block is characterized by hot and humid summers and pleasant, warm winters, with significant rainfall influenced by the monsoon. Temperatures can vary, reach up to 30-35°C in summer, while winters are milder (17°C). However, the climate of the Umsning block is generally influenced by the south-west monsoon, experiencing a temperate climate on the plateau and warmer tropical/sub-tropical pockets in the valley areas. Annual rainfall is significant, averaging around 2935 mm, with temperatures ranging from 10°C to 30°C. Since, *R. microplus* tick thrives in warm, humid tropical and subtropical climates with sufficient rainfall and can be found in various parts of India, particularly

during the warmer, wetter seasons. High humidity and temperatures are important environmental factors that are optimal for the tick's life cycle and reproduction, indicates the study area's environment is favourable for tick survival.

Sample collection: A total of 539 ticks were collected from Holstein-Friesian (62) and Jersey (28) crossbred cattle of about 1 year of age from the specified area. Ticks were gathered from multiple body regions, including the flanks, dewlap, and belly. Using a tiny forceps, they were carefully removed from the animals' body parts, being prudent not to break any appendages. Blood samples (1-2ml) from Holstein-Friesian crossbred (62), Jersey crossbred (28), and indigenous (86) cattle were drawn via jugular vein puncture and placed in appropriately marked vials containing ethylenediaminetetraacetic acid (EDTA). Ticks were collected and kept in 70 percent ethanol at about -20°C for further investigation.

Sample assessment: The collected ticks were morphologically identified using existing standard criteria (Walker *et al.* 2014). Blood samples were analysed on the same day, and those that were not evaluated on that particular day were stored at -4°C until their next use. Thin blood smears were made immediately after obtaining anticoagulated blood and stained with Giemsa stain to detect haemoprotozoan parasites. The blood smears are investigated under oil immersion (100x) using an Olympus BX51 microscope.

Genomic DNA extraction: Genomic DNA extraction was performed using morphologically identifiable *R. microplus* ticks collected from three separate locations: Umiam, Sarikuchi and Umsawkhwan. Before DNA extraction, *R. microplus* ticks were subjected to two washes in phosphate-buffered saline (PBS, pH 7.2), subsequently undergoing genomic DNA isolation with the DNeasy Blood and Tissue Kit (Qiagen) based on the instructions provided

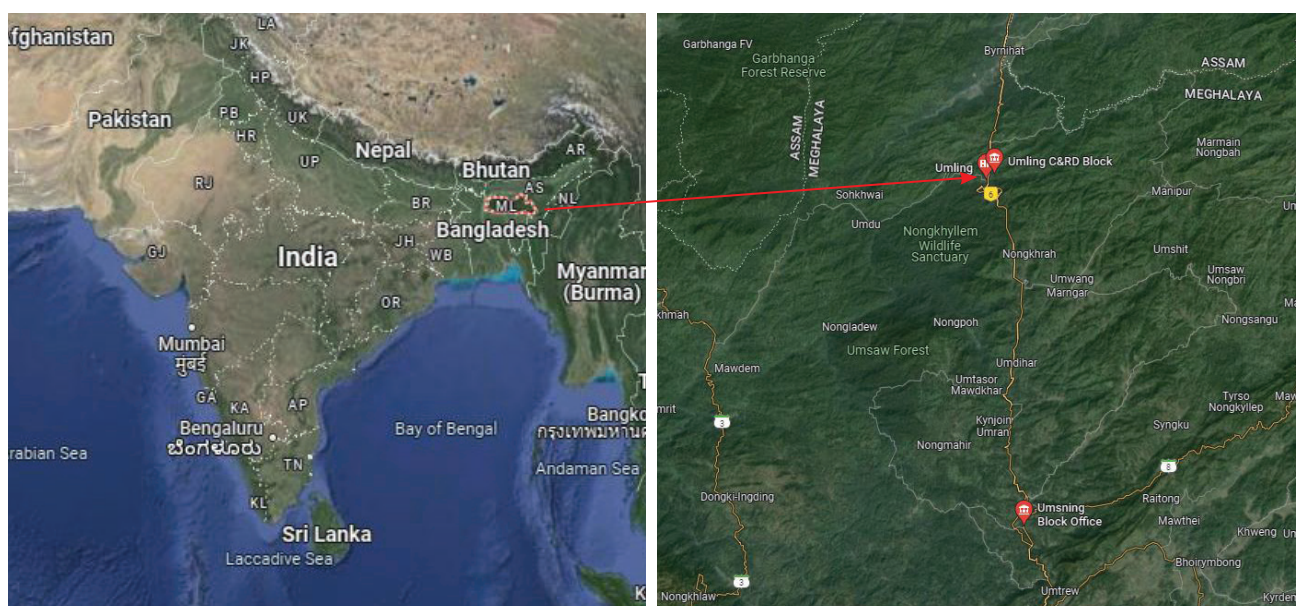


Fig.1. India Map depicting Meghalaya (ML) and study areas

(Source: <https://www.google.com/maps>)

by the manufacturer. Eventually, the genome became available in a volume of 100µL using EB buffer. The Nano Drop1000 UV-Vis Spectrophotometer, manufactured by Thermo-Fisher Scientific, was used to measure at 260nm and 280nm wavelengths, resulting in concentrations of about 200 ng/µL. The ratio of OD260/OD280 was found to be 1.81. Gel electrophoresis on a 0.8% agarose gel stained with ethidium bromide was used for additional verification.

Amplification and sequencing of mitochondria COXI gene: DNA from mitochondrial cytochrome C oxidase subunit I (COXI) gene of *R. microplus* was amplified using LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' primers, as previously narrated by Folmer *et al.* (1994). Amplification was conducted in a 25µL reaction volume consisting of 12.5µL of master mix (PCR Master Mix 2X), 5µL of genomic DNA template, 1µL of each primer, and 5.5µL of nuclease-free water. The amplification process has been carried out with a thermal cycler employing the following cycle conditions such as an initial denaturation period at 95°C for 5 minutes, subsequently followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C lasting 30 seconds, and extension at 72°C about 7 minutes. PCR results were analysed using submerged gel electrophoresis using 1.5% agarose gel, which was stained with the ethidium bromide dye and visualised with a UV transilluminator. The PCR result's value was assessed with a 1Kb DNA ladder. The PCR generated segments were sequenced using the Sanger DNA sequencing method.

Aligning sequences and analysis of phylogeny: The mitochondrial COX1 sequences from *R. microplus* have been obtained after sequencing and established by Basic Local Alignment Search Tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/>) with the database of NCBI GenBank. The sequences were then multi-aligned utilizing the CLUSTAL W algorithm (Thompson *et al.* 1994). The distance matrix was developed, and the phylogenetic tree has been created using the Molecular Evolutionary Genetic Analysis

(MEGA X) programme package. The evolutionary history of the species under consideration was determined using the Maximum Likelihood technique, with a bootstrap value of 1000 replicates (Felsenstein 1985).

RESULTS AND DISCUSSION

Prevalence of *R. microplus* tick in cattle of Ri Bhoi, Meghalaya: A total of 539 ticks were collected from Holstein Friesian cross (62) and Jersey cross (28) cattle. The percent prevalence of *R. microplus* ticks' infestation in crossbred Holstein Friesian cross (25.80%) and Jersey cross (14.28%), respectively. The overall prevalence of *R. microplus* tick in cattle of Ri Bhoi district, Meghalaya was 11.36% (Table 1). Maximum tick infestation was recorded in Umiam (28.12%) followed by Umsawkhwan (23.33%) and Sarkuchi (14.28%), respectively. The indigenous breed has been confirmed to be unaffected by *R. microplus* tick infestation. Examination of blood sample revealed *Babesia bigemina* infection in 9.37% crossbred (Holstein Friesian cross) cattle of Umiam only.

In the present study, the total incidence of *R. microplus* ticks in crossbred cattle was 11.36% which is in agreement with the previous studies by Sultan *et al.* (2022) and Niranjana *et al.* (2022). They have reported 44.5% and 61.89% *R. microplus* infestation in cattle from Pakistan and Uttar Pradesh, respectively. The discrepancy in percent incidence from the current study might be attributable to changes in the existing environmental circumstances that promote the growth of various tick species. Furthermore, according to Ali *et al.* (2021), aspects of the environment such as biotic (vegetation form, host accessibility, and management) and abiotic variables (precipitation, temperature variation) influence tick distributions. Rehman *et al.* (2017) previously noticed that *R. microplus* thrives in places with significant rainfall and low temperatures. Additional factors, especially farmer knowledge about managing ticks and host sensitivity, might be the primary source of widespread tick infestation (Pinheiro *et al.* 2010). Previous studies reported that indigenous cattle had lower

Table 1. Incidence of *R. microplus* tick among cattle of Ri Bhoi, Meghalaya

State (District)	Block	Name of village/ location	Breed of cattle	Animal screened (No.)	Tick collected (No.)	Tick positive (%)	Blood sample examined (No.)	Blood sample positive (%)
Meghalaya (Ri Bhoi)	Umling	Sarikuchi	Crossbred (Jersey cross)	28	160	4 (14.28)	28	-
		Borkhatsari	Indigenous	30	-	-	30	-
		Lalumpam	Indigenous	27	-	-	27	-
	Umsning	Umiam	Crossbred (Holstein Friesian cross)	32	253	9 (28.12)	32	3 (9.37)
		Umsawkhwan	Crossbred (Holstein Friesian cross)	30	126	7 (23.33)	30	-
		Umbir	Indigenous	29	-	-	29	-
Total		6	3	176	539	20 (11.36)	176	3 (1.70)

tick infestation than crossbred cattle (Niranjan *et al.* 2022, Nwachukwu *et al.* 2021) which is in accordance with the present findings. This might be pertaining to the indigenous breed's strong inherent immunity, which results in concomitant immunity owing to recurrent tick infestations (Khan *et al.* 2019).

Additionally, adapting indigenous breeds towards the climate of the area would increase their hardiness and resistance to infestation (Dorai-Raj 2014). However, Afzal (2008) claims that introducing exotic breeds of cattle to increase milk and meat output exposes these animals to tick infestation and tick-borne diseases. As stated by Hoogstraal (1985), *R. microplus* evolved in South and Southeast Asia before spreading to tropical and subtropical regions by introduction of tick-infested cattle by colonists or explorers. In the current investigation, *Babesia bigemina* (9.37%) infection was exclusively found in crossbred cattle, thus being in agreement with previous studies by Kumar *et al.* (2023), Kaur *et al.* (2021), Barman *et al.* (2018) and Bordoloi *et al.* (2021) from Tripura, Jammu, and Assam, respectively.

Amplification and sequencing of mitochondrial COX1 gene of R. microplus: Proper identification of species/ taxa within the *R. microplus* complex is critical for epidemiological studies, the evolution of *R. microplus* ticks, and the development of potential control strategies. COX1 is the preferred molecular marker for identifying different taxa within the *R. microplus* complex over internal transcribed spacer 2 (ITS2), 12S rRNA, or 16S rRNA due to its high variability in distinguishing different

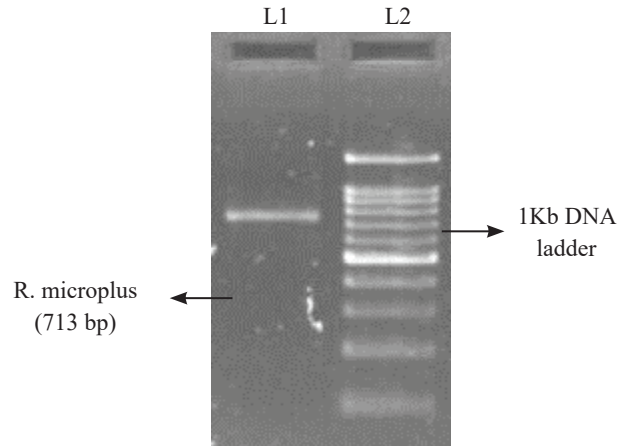


Fig. 2. PCR product separation on agarose gel (1.5%). L1: *R. microplus* (713 bp), L2: 1Kb DNA ladder

R. microplus assemblages (Roy *et al.* 2018, Low *et al.* 2015, Burger *et al.* 2014). The current study validated the presence of *R. microplus* ticks in cattle by amplifying the mitochondrial COX1 gene from isolated genomic DNA using species-specific primers. The predicted size of 713bp (*R. microplus*) PCR result was observed employing agarose gel electrophoresis (Fig. 2). The amplicon encompassing the mitochondria COX1 genome of *R. microplus* isolates of Umiam, Meghalaya was sequenced and submitted into the GenBank database of the National Centre for Biotechnology Information (NCBI). BLAST (Basic Local Alignment Search Tool) analysis revealed that the sequence was from the mitochondrial COX1 gene of *R. microplus* (Accession

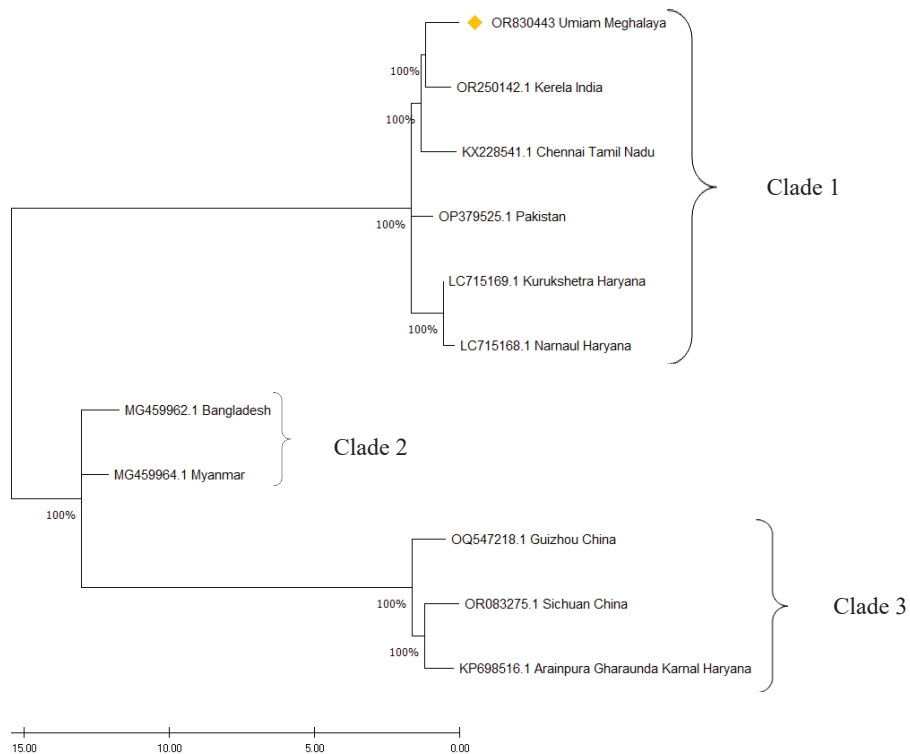


Fig. 3. Evolutionary relationship between *R. microplus* Umiam, Meghalaya isolate with other isolates

No. OR830443.1) isolates from Umiam, Meghalaya.

Aligned sequences and analysis of phylogeny: Partial fragment (713bp) of the mitochondrial COX1 gene of *R. microplus* isolate of Umiam, Meghalaya, has been sequenced and submitted in the National Centre for Biotechnology Information (NCBI) GenBank database. The sequence was confirmed to emanate from the mitochondrial COX1 genome of *R. microplus*, Umiam, Meghalaya isolate (Accession No. OR830443.1) by employing the Basic Local Alignment Search Tool (BLAST). Mitochondrial COX1 gene of *R. microplus* isolates from Pakistan (OP379525.1), Bangladesh (MG459962.1), Myanmar (MG459964.1) and China (OQ547218.1, OR083275.1) shared 99.63 to 100% nucleotide homology with the mitochondrial COX1 gene of *R. microplus* isolate from Umiam, Meghalaya, India (OR830443.1).

Earlier, Roy *et al.* (2018) reported an equivalent percentage of sequence identity with respect to the COX1 gene of *R. microplus* isolates from Bangladesh, Pakistan, Myanmar, and China, which is in accordance with the findings of the current investigation. In the present study, an analogous sequence identity is also shared with the mitochondrial COX1 gene of *R. microplus* isolates from the Northern and Southern states of India, *viz.*, 99.63% Narnaul, Haryana (LC715168.1), 99.81% Kurukshetra, Haryana (LC715169.1), 99.81% Karnal, Haryana (KP698516.1) and 100% Kerala (OR250142.1), 100% Chennai, Tamil Nadu (KX228541.1), respectively which is in accordance with the findings of Krishnamoorthy *et al.* (2021) and Amrutha *et al.* (2023).

Maximum Likelihood based phylogenetic tree revealed 3 different clades with *R. microplus* Indian isolates *viz.*, Umiam Meghalaya (OR830443.1), Kerala (OR250142.1), Chennai Tamil Nadu (KX228541.1), Pakistan (OP379525.1), Narnaul Haryana (LC715168.1) and Kurukshetra Haryana (LC715169.1) isolates clustered in clade 1. Bangladesh (MG459962.1) and Myanmar (MG459964.1) isolates in clade 2 while China isolates (OQ547218.1, OR083275.1) and Karnal, Haryana (KP698516.1) isolates in clade 3,

respectively (Fig. 3). Additionally, monophyletic node is observed between the clade 1 and clade 2, which indicates a shared ancestry between the two. Prior investigations have also revealed close genetic relatedness between *R. microplus* ticks of the Indian isolates with the neighbouring nations, namely, Bangladesh, Pakistan, and Myanmar (Roy *et al.* 2018, Krishnamoorthy *et al.* 2021, Amrutha *et al.* 2023). Notably, the Chinese and Indian ticks in the current study were found to be divided into different clades, indicating genetic disparity. Earlier, Burger *et al.* (2014) conducted a phylogenetic study using the mitochondrial COX1 gene and discovered two distinct clades for the China isolate: *R. microplus* clade A, which included isolates from Brazil and Cambodia, and *R. microplus* clade B, which contained isolates from China. Similarly, Low *et al.* (2015) constructed a phylogenetic tree based on the COX1 gene that revealed three clades: *R. microplus* clade A, which includes *R. microplus* ticks from Brazil, Cambodia, and Malaysia; *R. microplus* clade B, which includes *R. microplus* ticks from China; and *R. microplus* clade C, which includes *R. microplus* ticks from Chennai, India, and Malaysia. However, a study on the genetic divergence analysis of eleven isolates found that the Umiam, Meghalaya (OR830443.1) isolate had a close relationship with the Kerala (OR250142.1) isolate for the COX1 gene. On the other hand, the remaining isolates were found to have a distant connection to the Umiam, Meghalaya (OR830443.1) branch (Fig. 4).

The current study concluded that *R. microplus* ticks prevailed in crossbred cattle in Ri Bhoi district, Meghalaya, and had spread *Babesia bigemina* infection in cattle. Amplification, sequencing, and BLAST analysis of the mitochondrial COX1 gene revealed *R. microplus* isolate from Umiam, Meghalaya (OR830443.1) with sequence similarity of 99.63 to 100% to sequences in the NCBI database. This report may be considered the first investigation on the genomic characterization and phylogenetic insight of *R. microplus* in cattle from the mountainous terrain of Meghalaya, India, and might be

Table 2. Genetic distance (FST) of COX1 gene of *R. microplus* between different isolates

	Accession number	1	2	3	4	5	6	7	8	9	10	11
1. Umiam, Meghalaya	OR830443.1											
2. Chennai, Tamil Nadu	KX228541.1	11.38										
3. Pakistan	OP379525.1	7.68	7.93									
4. Bangladesh	MG459962.1	0.00	0.00	0.00								
5. Sichuan, China	OR083275.1	0.00	0.00	0.00	0.00							
6. Guizhou, China	OQ547218.1	0.00	14.67	0.00	11.96	15.39						
7. Kurukshetra, Haryana	LC715169.1	9.30	12.92	7.93	24.92	0.00	28.02					
8. Kerala, India	OR250142.1	5.98	7.14	9.50	13.98	0.00	0.00	6.33				
9. Myanmar	MG459964.1	7.57	12.53	15.39	6.54	8.89	0.00	0.00	0.00			
10. Narnaul, Haryana	LC715168.1	21.26	0.00	7.99	10.87	0.00	12.23	0.75	0.00	38.99		
11. Karnal, Haryana	KP698516.1	2.49	0.00	0.00	14.87	9.00	10.77	16.43	19.65	22.14	9.68	0.00

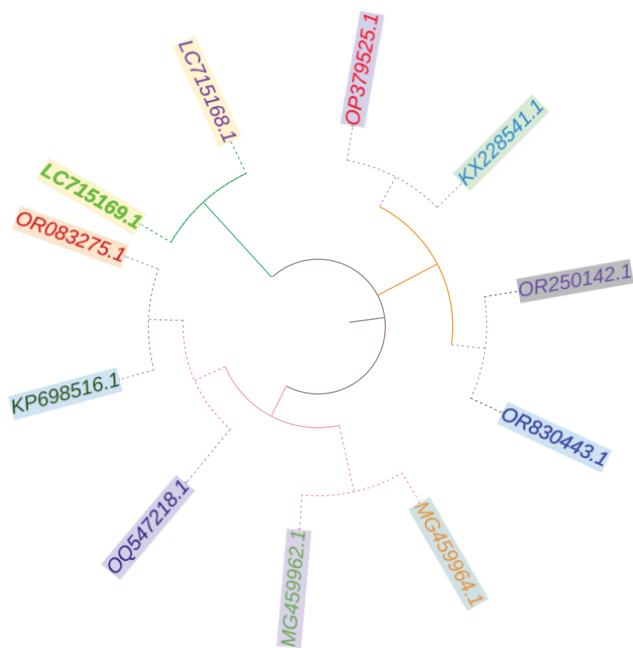


Fig. 4. Evolutionary relationship of *R. microplus* isolates

useful for future research as well as developing a control plan for the management of *R. microplus* ticks in the region.

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