Molecular characterization of *Rephiciphalusmicroplus* using sangersequencing COI&18SrRNA Markersfrom Chhatrapati Sambhajinagar, India

Abstract

Genus *Rhipicephalus* ticks, which infest cattle, are the main animal parasites that cause billions of rupees' worth of annual economic losses. Species identification is highly challenging because of the physical similarities among the members of the *Rhipicephalus (Boophilus*) genus. The adult of*R. microplus* ticks from Chh. Sambhajinagar (MS) India, were examined morphologically and molecularly in this work. The morphology of the *R. microplus* isolates was different from that of the real *R. microplus* clade A ticks.Out of 13 sample of species 2 species analysed and successful sequencing outcome aby using sanger sequencing COI&18SrRNA marker the database inserted into BLASTncbi database and result matches with *Rephicephalusmicroplus*. Our study looked into tick diversity using a molecular technique that included phylogenetic analysis, DNA sequencing, and PCR.

Keywords:*Rhipicephalus microplus*, Sanger sequencing, COI,18s rRNA.

Introduction

Parasitism is relationship between species where one organism the parasite, live on or inside another organism. The host causing it some harm and its adapted structurally to this way of life. parasitic organism reduces host fitness.Ectoparasites are mostly arthropods found in cattle that either cause diseases or act as vector transmitting other parasites and endoparasites are classified into intestinal, atrial may inhabit body tissue causing serious health problems(Manar M.S.EI-Tons,introductiontomedicalparasitology).From ancient world the cattle industry is one of the most important and profitable agribusiness activities in the world. Livestock play an important role in Indian economy near about 20.5 million people depends upon livestock for their live hood (Livestock census 2020) parasites cause negative impacts parasite have on health and welfare of animal can include Blood loss if Substantial can lead to anemia and death, reducing growth rate, reducing reproductive rate, reducing milk production.

“In Chh. Sambhajinagar district rural area depends upon livestock therefore aim to purpose of research to collect and identify, parasites on Cattles.In many parts of the world *Rhipicephalus* (*Boophilus*) *microplus*is known simply as the cattle tick. This is specially so where ithas spread from its origin in south east Asia to major cattle ranching areas like South America. This spread has been accidental, on commercial cattle transportations. In Africa this tick has established in much of southern and eastern Africa and it is widespread in Madagascar. *Rhipicephalus* (*Bo*.) *microplus*is more dangerous than *Rh*. (*Boophilus*) *decoloratus*because ittransmits both *Babesia bovis*and *Babesia bigemina*, incomparison to *Rh.* (*Bo.*) *decoloratus*which transmits only*Ba. bigemina*, the less pathogenic of the two protozoans. Forthis reason the ability to identify *Rh*. (*Bo*.) *microplus*is mostimportant as an aid to prevention of the spread of *Ba*. *bovis*.This tick

species and the others in the *Boophilus*sub-genus withinthe genus *Rhipicephalus* are so well known in their original classificationas members of the full genus *Boophilus*that the formernames are likely to remain in use for many years without confusion. The character states that have been used for this sub-genusare separate from the rest of the genus *Rhipicephalus" (Tamura et al., 2021).*

"Rhipicephalus (*Bo.*) *microplus*is a one-host tick with amonotropic type of behaviour. The time spent by the three stageson the host is about three weeks and egg laying can be completedin about four weeks. This is faster than *Rh.* (*Bo.*)*decoloratus*, moreover female *Rh.* (*Bo.*) *microplus*lay approximately500 eggs more than *Rh.* (*B.*) *decoloratus*females. Thesteady spread of *Rh.* (*Bo.*) *microplus*in Africa is assisted bythis higher reproductive potential which enables it to competesuccessfully against *Rh.* (*Bo.*) *decoloratus*where these ticksoccur together in climates that are most favourable to *Rh.* (*Bo.*)*microplus*..Large numbers of larvae are usually present on thevegetation in late spring, and successive generations of larvaethen occur through the summer and into the cooler autumn andearly winter month. According to BirurMallappa Amrutha et al (2023)When compared to the internal transcribed spacer 2 (ITS2), the mitochondrial cytochrome c oxidase I (COI) was found to be the preferred molecular marker. The interspecific differences between isolates of *R. annulatus* and *R.microplus* was 7.9% based on COI from South India. Furthermore, the *R. microplus* isolates showed greater intraspecific divergence (2.9%) compared to *R. annulatus* (1%), according to COI. The ITS2 sequences were unable to distinguish between *R. annulatus* and *R. microplus" (Tamura et al., 2021)* .

Patricetianget.al.(2024) were studiedon"molecular identificationof*Rephcephalus microplus.*Using phylogenetic analysis and the sequencing of the ITS2 region, 12S rRNA, and 16S rRNA genes, the identities of the *R. microplus* tick species were verified".Hence 18srRNA and COI markers useful to molecular identification of *R.microplus* species in this study.

Material and Methods

Study areas:This study was conducted in Chh. Sambhajinagar District M.S. India.Selection of cattle which are from different villages in ChhatrapatiSambhajinagar district.Farmers prefer to cattle farming as a side business along with farming. Generally climatic conditions are favourable for cattle but from last few years rising temperature and unpredictable weather patterns all have drastic impact on cattle health.

Image 1: Collection of ticks from various sites in Chh. Sambhajinagar district

Tick collection and identification: The Tick Specimens were collected from the infested cattle byhandpickmethod during of period 2023-24 from various areas of Chh. Sambhajinagar (MS) India.preservationofectoparasitein70%alcoholinwellstoppedglassvialswithlabellingparasites and Mounted with DPX on cavity slide. Presumptive Identification with help of Understereoscopicmicroscope.FinalIdentificationwillobserveundercompoundmicroscopeaccordin g toKeys& description.

Molecular studies

DNA extraction, polymerase chain reaction (PCR), and sequencing:

1. The DNA was extracted by TAKARANucleoSpin® Tissue Genomic DNAPurification Kit and quality checked on 1% agarose gelelectrophoresis.GelwasvisualizedusingUVTransilluminator (Himedia).
2. Fragment ofgeneCOI &18S rRNAwasamplified by1A and564R primers.
3. AsinglediscretePCRampliconband wasobservedwhenresolvedon1.2%Agarose gel.
4. ThePCRampliconwaspurifiedtoremovecontaminants.
5. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out withforwardprimer and reverseprimers usingBDTv3.1 Cycle sequencingkit on ABI3730xlGeneticAnalyzer.
6. ConsensussequenceofCOI&18SrRNAgenewasgeneratedfromforwardandreversesequenc edatausingBioeditsoftware.
7. The COI & 18S rRNAgene sequence was used to carry out BLAST with the database ofNCBIgenbankdatabase.
8. Basedonmaximumidentityscoreand alignmentsusingmultiplealignmentsoftwareprogramClustal W. Distance matrix was generated and the phylogenetic tree wasconstructed usingMEGA11.

Results and Discussion

SpeciesconfirmationusingCOI&18SrRNAmarker

Amplificationof COI&18SrRNA

TheCOI&18SrRNAregionwasamplifiedbyusingprimers(ForwardandReverseasdetailedinTable 1). AmplifiedPCR products werevisualizedon 1.2%agarosegel.

Table1. DetailsofPolymeraseChainReactioncomposition

Component	ComponentVolume
GoTaqGreenMastermix	25µL
Fprimer	3µL
RPrimer	3µL
TemplateDNA	6µL
Nuclease-FreeWater	13µL
Total reactionvolume	50µL

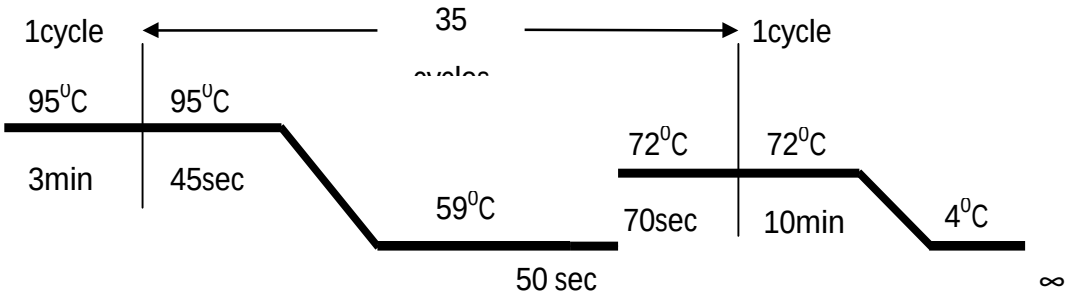


Figure1. COI&18SrRNAthermal cyclingconditionsused foramplification

2. Sequencing

PCR products were processed for cleanup to remove unincorporated nucleotide and residualprimersusingExonuclease-IandShrimpAlkalinephosphataseenzymefollowedbycyclesequencingreactionusingBigDye®Terminatorv.3.1CycleSequencingKit(AppliedBiosystems,Inc.). For Cycle sequencing same PCR primers were used. The thermal cyclerconditionswereaninitialdenaturationof2minat96^{0}Cand35cyclesof30secat96^{0}C,15secat 55^{0}C,

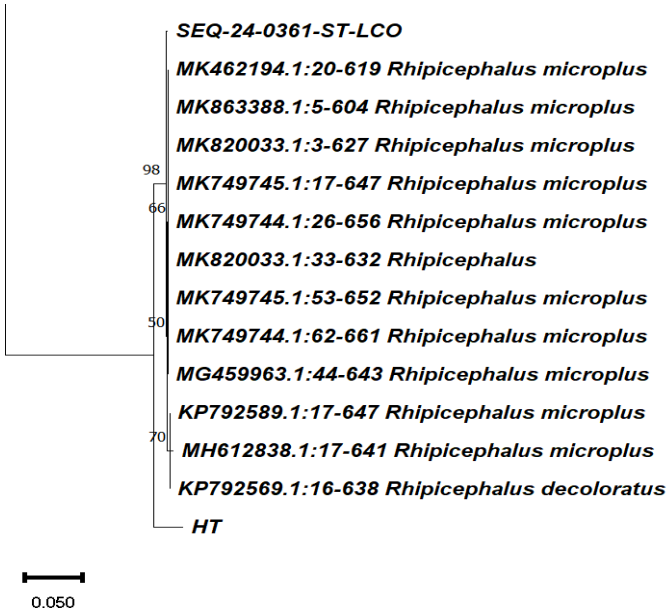
and 4 min at 60^0C.The Cycle sequencing is followed by sequencing cleanup by ethanolprecipitation followed bydissolvingtemplate in HiDi formamide and bidirectionally sequencedinABI3730 Geneticanalyzer.

Table 2. Sample IDs showing Similarity Searches in Sequence Alignment

PCRproducts werethenprocessedfor direct bi-directionallysequencingusingABIPRISM 3730× 1 Genetic Analyzer (Applied Biosystems, USA). The resulting DNA sequences were alignedusing CLUSTALW in MEGA 11, manually trimmed and edited to obtain complete sequences.Theconfirmation of species depends on thesequence similarityscore.

Homology searches were carried out using the BLAST program against the NCBI GenBankdatabase (https://blast.ncbi.nlm.nih.gov/Blast. cgi). Neighbor Joining tree was constructed usingMEGA 11 with all positions containing gaps and missing data were included for analysis. Cladesupportswere calculatedbased on 1,000 bootstrapresamplings.

Fig 2.Search resultin BLAST program against the NCBI GenBank



Evolutionaryrelationshipsof taxaCOIBased

SAMPLEID	Description	MaxScore	TotalScore	QueryCoverage	E-Value	Per Identity	AccessionNo.
HT	*Rhipicephalus microplus* Isolate 4 cytochrome oxidase subunit I (Cox1) gene partial cds , mitochondrial	1033	1033	97%	0.0	96.20%	KP792589
ST	*Rhipicephalusmicroplus*isolatePAK7 cytochromecoxidasesubunit1 (cox1)gene, partialcds; mitochondrial	1098	1098	100%	0.0	99.67%	MK462194

The evolutionary history was inferred using the Neighbor-Joiningmethod . The optimal tree is shown. The percentage of replicatetreesinwhichtheassociatedtaxaclusteredtogetherinthebootstrap test

(1000 replicates) are shown above the branches .The tree is drawn to scale, with branch lengths in the same units asthose of the evolutionary distances used to infer the phylogenetictree.Theevolutionarydistanceswerecomputedusingthep-distance method. and are in the units of the number of basedifferencespersite.Thisanalysisinvolved18nucleotidesequences.

Codon positionsincludedwere1st+2nd+3rd+Noncoding. All ambiguous positions were removedfor each sequence pair(pairwise deletion option). There were atotal of 651 positions in the final dataset. Evolutionary analyseswereconducted in MEGA11.

Our study utilized a molecular approach, incorporating PCR, DNA sequencing and phylogenetic analysis, to investigate the diversity of ticks.

Conclusion

The results of the present study confirmed the widespread existence of *R. microplus*in ChhatrapatiSambhajinagar district.The result highlighted genetic analysis to confirm true species statusof*R.microplus.*Out of two molecular markers, COIwas successful in resolving the phylogenetic relationship of *R. microplus*This study helpful for researcher to resolve diversity of tick species from genetical analysis.

Disclaimer(Artificial Intelligence)

Authors hereby declare that generative AI technologies such as QuillBot software has been used during the writing or editing of manuscripts for grammar checking and summarizing.

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