Infection, Genetics and Evolution 75 (2019) 103962

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Genetic characterization of *Theileria* species infecting bovines in India

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ARTICLE INFO

Keywords:
Theileria spp.
Bovines
Genetic characterization
18S rRNA
India

ABSTRACT

Genetic characterization of *Theileria* species infecting bovines in India was attempted targeting the 18S ribosomal RNA region of the parasite. Blood samples of bovines (n = 452), suspected for haemoprotozoan infections, from 9 different states of the country were microscopically examined for *Theileria* species infection. Four *Theileria* spp. positive blood samples from each state were randomly utilized for PCR amplification of the 18S rRNA gene (approx. 1529 bp) followed by cloning and sequencing. The sequence data analysis of all the 36 isolates revealed that 33 isolates had high sequence similarity with published sequences of *T. annulata,* whereas 3 isolates (MF287917, MF287924 and MF287928) showed close similarity with published sequences of *T. orientalis*. Sequence homology within the isolates ranged between 95.8 and 100% and variation in the length of targeted region was also noticed in different isolates (1527–1538 nt). Phylogenetic tree created for *T. annulata* sequences revealed that a total of 24 Indian isolates formed a major clade and grouped together with isolates originating from countries like China, Spain, Turkey and USA. Remaining 09 isolates clustered in a separate group and were closely related to the TA5 isolate of *T. annulata* (a new genotype) originating from India and also with the isolates from East Asian countries like Japan and Malaysia. All the three *T. orientalis* isolates had minimal intraspecific variation (99–100% homology) amongst themselves. Further, in the phylogenetic analysis *T. orientalis* Indian isolates were found to cluster away from other 14 isolates of *T. buffeli/sergenti/orientalis* originating from different countries (Australia, China, Indonesia and Spain). However, these 3 isolates clustered together with the *T. buffeli* Indian isolate (EF126184). Present study confirmed the circulation of different genotypes of *T. annulata* in India, along with *T. orientalis* isolates.

1. Introduction

Hemoprotozoan diseases of cattle are widely distributed, stretching from the polar circle to the equator due to the global occurrence of the vectors viz. ticks and blood sucking flies (Demessie and Samuel, 2015). Out of different hemoparasitic diseases of cattle; theileriosis, babesiosis and anaplasmosis are commonly known to occur in tropical and subtropical regions (Parthiban et al., 2010; Reetha et al., 2012; Maharana et al., 2016) with very high morbidity, mortality and huge economic losses (Shahnawaz et al., 2011). All the three diseases are transmitted by different genera and/or species of tick, which are most abundantly present in different agroclimatic zones of India. Recent trend of these diseases indicates that bovine tropical theileriosis has the highest prevalence in India followed by anaplasmosis and babesiosis (Velusamy et al., 2014).

Bovine tropical theileriosis caused by *Theileria annulata* is, an economically important disease in India and crossbred cattle (*Bos taurus*) are more susceptible in comparison to the Indian breeds of cattle (*Bos indicus*). Mild infection of *T. annulata* in cattle remains unnoticed and animals act as carriers but during the stress conditions (environmental, physical, nutritional), it may flare up into a clinical disease. Infected animals may present variable symptoms viz. fever, inappetence, anaemia, respiratory distress, swelling of pre scapular lymph nodes and reduded milk yield etc. Strategies to control tropical theileriosis include management of tick infestation, vaccination and treatment of infected animals (Akat et al., 2014). However, in the recent years, increased incidence of theileriosis has been reported from different parts of India (Kohli et al., 2014; Velusamy et al., 2014; Bhatnagar et al., 2015; Naik et al., 2016). Further, presentation of variable clinical signs and symptoms in animals are also observed by clinicians in the field. This

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https://doi.org/10.1016/j.meegid.2019.103962
Received 29 January 2019; Received in revised form 5 July 2019; Accepted 9 July 2019
Available online 11 July 2019

gives an indication of change in the genetic makeup of the parasite, as many reports highlights the circulation of several genotypes of *T. annulata* from different countries including India (Manuja et al., 2006; Liu et al., 2010; Aparna et al., 2011; Mans et al., 2011; George et al., 2015; Khan et al., 2013). Apart from this, *Theileria orientalis* infection is being increasingly reported from the country (Kakati et al., 2015). Various isolates of the parasite prevalent in endemic areas are also known to exhibit variable virulence in the susceptible cattle population (Darghouth et al., 1996; Taylor et al., 2003). Vaccination failures have been recorded in animals vaccinated with an attenuated cell culture vaccine, developed from local isolate and challenged with a heterologous strain collected from different geographical regions of India (Gill et al., 1980; Subramanian et al., 1986). Further, efficacy of an attenuated cell culture schizont vaccine under field conditions had showed that the vaccinated cattle were protected against the local homologous isolate only (Singh et al., 2001; Khatri et al., 2001).

In India, there is limited information on the genetic diversity amongst *T. annulata* isolates. The available reports are based on cloning and sequencing of 18S rRNA and Tams 1 gene of the parasite (Manuja et al., 2006; George et al., 2015; Roy et al., 2019) involving few isolates. Out of the above two, 18S rRNA gene has proved as an invaluable taxonomic marker to deduce phylogenetic relationship with other isolates already sequenced (Chaisi et al., 2011). This gene has both conserved and phylogenetically informative variable regions that are useful for determining relationships amongst the species (Hillis and Dixon, 1991). In order to address the issues related to increase incidence, variable clinical profile, protection conferred by available vaccine to homologous isolates and poor efficacy of theilericidal drugs, there is an urgent need to understand the epidemiology of parasite in different geographical areas. Therefore, present study was undertaken to establish the 18S rRNA based phylogenetic relationships of *Theileria* spp. infecting cattle population in India.

2. Materials and methods

2.1. Sample collection

A total of 452 blood samples (2–3 ml) of adult cattle (native/crossbred) suspected for haemoparasitic infections (fever, inappetance, anaemia and respiratory distress), were received from Veterinary hospitals/diagnostic labs of 9 different states of India including those in the north (Punjab, Uttar Pradesh, Uttarakhand and Haryana), east (Odisha and West Bengal), west (Gujarat), south (Tamil Nadu) and central part (Maharashtra) of the country Fig. 1. Detail of blood samples received/collected has been summarized in Table 1.

2.2. Blood smear examination

Thin blood smears, two from each sample, were prepared and were fixed with absolute methanol for one minute, air dried and stained with Giemsa stain (1,20) as per the standard method for detection of the haemoparasites. Screening of blood smears was done under oil immersion lens of a compound research microscope and the status of sample was recorded as positive or negative based on the examination of minimum 30–40 microscopic fields. *Theileria* spp. positive blood samples were labelled properly and preserved at 4 °C till further use.

2.3. Isolation of genomic DNA from blood samples

Genomic DNA was extracted from 200 µl of blood sample(s) of known *Theileria* positive cases using commercial kit (DNeasy blood kit, Qiagen, Germany) following the manufacturer's protocol. A total of 36 blood samples (4 *Theileria* positive samples by microscopy from each state) were utilized for isolation of genomic DNA. Concentration and purity of isolated DNA was measured using spectrophotometer and quality was checked in agarose gel electrophoresis (1.5% gel). Aliquots of extracted DNA were labelled properly and stored at −20 °C until further use.

2.4. Cloning and sequencing of 18S rRNA

Theileria specific primer pair (F1: GGC GGC GTT TAT TAG ACC and R2: CCT TGT TAC GAC TTC TCC) was selected from the published literature (Habibi, 2013) and used in the study to amplify hypervariable regions along with the 18S rRNA gene in a single reaction. PCR reaction was performed in 25 µl total volume containing 20–30 ng of template DNA, 2.5 µl of 10× PCR Green buffer (Puregene), 0.5 µl of 10 mM dNTP, 0.5 µl of each forward and reverse primer (20 pmol/µl), 0.2 µl Dream *Taq* DNA polymerase and nuclease free water to make the volume 25 µl. PCR amplification (primary) was performed using a S1000 thermal cycler (Bio-Rad, USA) under optimized conditions as follows: initial denaturation at 94 °C for 5 min, followed by 35 amplification cycles (94 °C for 45 s, 52 °C for 1 min and 72 °C for 90s) and a final extension step at 72 °C for 10 min. Known *T. annulata* genomic DNA was used as positive control, while nuclease free water (NFW) was used as negative control. The PCR products was electrophoresed in 1.5% agarose gel (Tris-borate-EDTA), stained with ethidium bromide and visualized under UV light. After ensuring specific amplification in primary PCR, a bulk PCR reaction of 50 µl volume was run for each one of the selected isolate. Amplified product was analysed in agarose gel electrophoresis as above and then the specific band was excised from the gel. Thereafter, PCR product was purified using the gel extraction kit (Qiagen) and concentration as well as purity of the each elute was analysed using the Nanodrop spectrophotometer. Finally, purified fragment of a total of 36 isolates (4 from one selected state) was then cloned in pTZ57R/T cloning vector using InsTA Clone PCR cloning Kit (Thermo Fisher Scientific, USA). Confirmation of recombinant clone(s) was made by colony PCR and plasmid PCR assay using the same set of PCR primers. Custom DNA sequencing services of Delhi, South campus, New Delhi (India) was availed for generation of nucleotide sequences of all the 36 isolates. Each isolate was sequenced in duplicate to eliminate any error in sequencing.

2.5. Sequence analysis

Nucleotide sequences ($n = 36$) generated in the study were primarily analysed using the BioEdit software and submitted to the GenBank. All the sequences were compared with the published sequences available in GenBank using BLAST program of the NCBI (http://blast.ncbi.nlm.nih.gov) and based on sequence homology all the isolates were categorized as *T. annulata* or *T. orientalis*. Nucleotide variations and length polymorphisms were also analysed.

2.6. Phylogenetic analysis

The nucleotide sequences generated in the study ($N = 36$) were used for constructing species specific phylogenetic tree based on sequence homology for *T. annulata* and *T. orientalis*. A total of 15 published sequences of *T. annulata* (Table 2) and 14 published sequences of *T. orientalis* (Table 3) originating from different countries were also retrieved and used for constructing the tree. Phylogenetic analysis was performed using Mega 6.0 with Neighbor Joining method and bootstrap values determined for 1000 replicates of the data sets (Tamura et al., 2013). The Tamura 3-parameter (T92 + G) Model was found to be the substitution model of choice using Mega 6.0. *Eimeria tenella* (KT184354) was used as outgroup member to root the tree.

3. Results

Microscopic examination of Giemsa stained blood smear ($n = 452$) revealed presence of *Theileria* spp. infection in a total of 102 cases. Overall prevalence of *Theileria* spp. infection in the study was 22.6%.

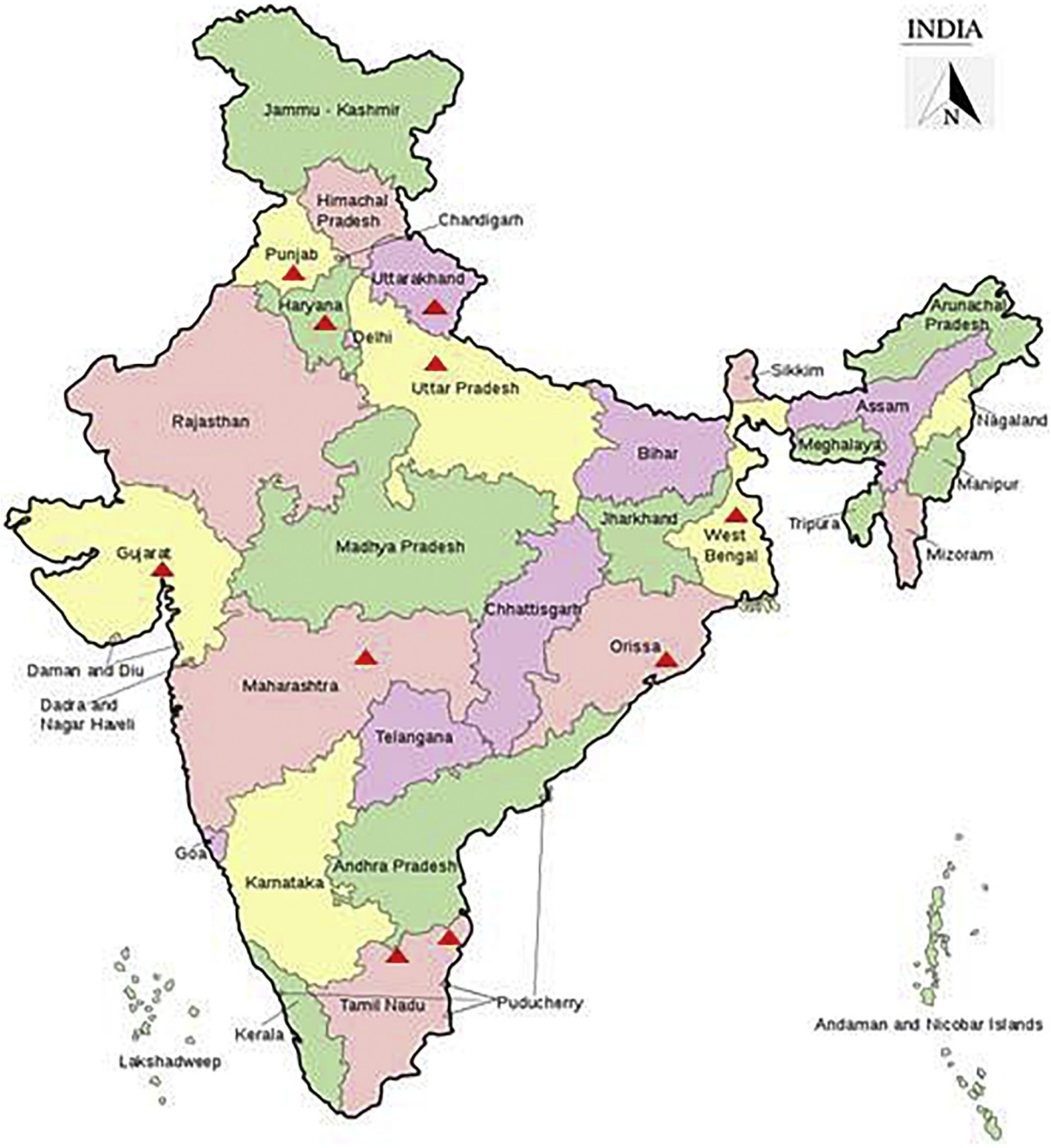

Fig. 1. Blood collection sites in different states of India.

Table 1
Places of blood sample collection from cattle.

S. No.	State	Place	No. of blood samples collected
1	Gujarat	Anand	10
2	Haryana	Hisar	26
3	Maharashtra	Akola	60
4	Odisha	Bhubaneshwar	22
5	Punjab	Ludhiana	21
6	Tamil Nadu	Chennai, Vellore	40
7	Uttarakhand	Udham Singh Nagar	07
8	Uttar Pradesh	Bareilly	250
9	West Bengal	Bhardhaman	16
	Total		452

In the PCR assay, specific amplification of approx. 1529 bp was obtained for all the 36 *Theileria* positive samples (Fig. 2). Analysis of nucleotide sequences of the cloned PCR products revealed length polymorphism (1527–1538 bp) amongst the isolates with few insertions and deletions at different locations (Supplementary file 1 and 2).

The species level confirmation was made by sequence analysis of all 36 targeted isolates. In the BLAST search analysis a total of 33 isolates (Table 2) showed highest sequence homology with *T. annulata* published sequences, whereas rest 3 isolates (Table 2) showed closeness with *T. orientalis* sequences. Further, sequence similarity searches in NCBI database indicated that a total of 24 isolates (out of 33) had 99–100% nucleotide similarity with the published sequences of *T. annulata* S15 Iran vaccine strain (KF429795) and also with 2 Indian isolates (KT367871 and KT367866). Remaining 9 *T. annulata* isolates (MF287948, MF287919, MF287920, MF287934, MF287949,

Table 2
GenBank accessions used in 18S rRNA gene based phylogenetic analysis of *T. annulata*.

S. No.	GenBank accession	Isolate	S. No.	GenBank accession	Isolate
1	MF287918[a]	Gujarat 2 India	26	MF287945[a]	Uttar Pradesh 1 India
2	MF287919[a]	Gujarat 3 India	27	MF287946[a]	Uttar Pradesh 2 India
3	MF287920[a]	Gujarat 4 India	28	MF287947[a]	Uttar Pradesh 3 India
4	MF287921[a]	Haryana 1 India	29	MF287948[a]	Uttar Pradesh 4 India
5	MF287922[a]	Haryana 2 India	30	MF287949[a]	West Bengal 1 India
6	MF287923[a]	Haryana 3 India	31	MF287950[a]	West Bengal 2 India
7	MF287925[a]	Maharashtra 1 India	32	MF287951[a]	West Bengal 3 India
8	MF287926[a]	Maharashtra 2 India	33	MF287952[a]	West Bengal 4 India
9	MF287927[a]	Maharashtra 3 India	34	KT367866	TA1 India
10	MF287929[a]	Odisha 1 India	35	KT367868	TA3 India
11	MF287930[a]	Odisha 2 India	36	KT367870	TA5 India
12	MF287931[a]	Odisha 3 India	37	KT367871	TA6 India
13	MF287932[a]	Odisha 4 India	38	KT736495	M1India
14	MF287933[a]	Punjab 1 India	39	KT736498	MR India
15	MF287934[a]	Punjab 2 India	40	KT736499	NE India
16	MF287935[a]	Punjab 3 India	41	KF559356	Ghansu China
17	MF287936[a]	Punjab 4 India	32	HM538216	Hubei China
18	MF287937[a]	Tamil Nadu 1 India	43	AB000271	Ikeda Japan
19	MF287938[a]	Tamil Nadu 2 India	44	AB000273	Ipoh Malaysia
20	MF287939[a]	Tamil Nadu 3 India	45	DQ287944	Madrid Spain
21	MF287940[a]	Tamil Nadu 4 India	46	AY524666	NA Turkey
22	MF287941[a]	Uttarakhand 1 India	47	M64243	Iowa USA
23	MF287942[a]	Uttarakhand 2 India	48	KF429795	S15 Iran
24	MF287943[a]	Uttarakhand 3 India	49	KT184354	*Eimeria tenella*[b]
25	MF287944[a]	Uttarakhand 4 India			

Note:
[a] Sequences generated in present study.
[b] Included as out group member.

MF287937, MF287939, MF287942 and MF287950) generated in the study showed 98–99% identity with the published sequences of *T. annulata* from North-East (KT736499) and Southern (KT367870) part of India. However, other 3 distinct isolates (MF287917, MF287924 and MF287928) showed closed similarity (>98% homology) with the sequences of *T. buffeli* Indian isolate (EF126184). Minimal intraspecific variation was also recorded amongst these isolates (98.9% identity).

Nucleotide sequence homology was recorded in the range of 95.4–100%, between the 33 newly generated isolates of *T. annulata* (present study) and sequences from India and other countries like China, Spain, Australia, Indonesia, USA, Japan and Malaysia. Most of the nucleotide variations in newly generated sequences were found to be occurring between four regions viz. nucleotide positions: 33–42, 78–87, 449–516 and 1126–1145 (Supplementary file 1). In the analysis, a total of 4 Indian isolates (MF287921, MF287933, MF287938 and MF287941) showed 100% identity with Iranian vaccine strain (KF429795) whereas, Gujarat isolate 3 (MF287919) showed minimum identity of 95.6% from the Iranian vaccine strain.

The phylogenetic tree constructed using the 33 newly generated sequences and 15 published isolates of *T. annulata* revealed that a total of 24 isolates (out of 33) clustered in a major clade with high bootstrap support (88), whereas remaining 09 isolates formed another clade with bootstrap support of 95 (Fig. 3). The major clade forming sequences of different isolates generated in study ($n = 24$) also exhibited close

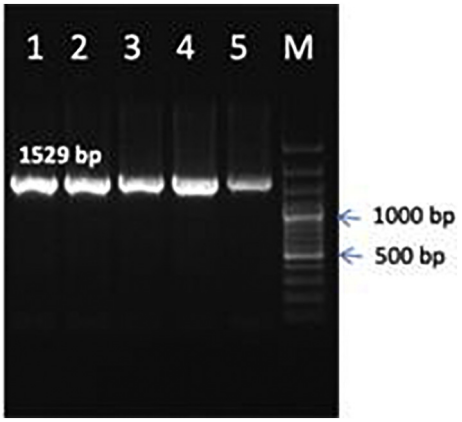

Fig. 2. PCR amplification of targeted gene (colony PCR).

Table 3
GenBank accessions used in 18S rRNA gene based phylogenetic analysis of *T. orientalis*.

S. No.	GenBank accession	Origin of isolate	S. No.	GenBank accession	Origin of isolate (*T. orientalis* group)
1	MF287917[a]	Gujarat 1 India	10	HM538209	Hubei China
2	MF287924[a]	Haryana 4 India	11	DQ104610	Hubei China
3	MF287928[a]	Maharashtra 4 India	12	AB000274	Medan Indonesia
4	EF126184	Uttar Pradesh India	13	AB520953	Kempsey Japan
5	AB000272	Warwick Australia	14	AF236094	Ikeda Japan
6	JF719834	Ghansu China	15	FJ225391	Madrid Spain
7	EU083802	Ghansu China	16	DQ287959	Madrid Spain
8	HM538195	Hubei China	17	AY661513	Texas USA
9	HM538197	Hubei China	18	KT184354	*Eimeria tenella*[b]

Note:
[a] Sequences generated in present study.
[b] Included as out group member.

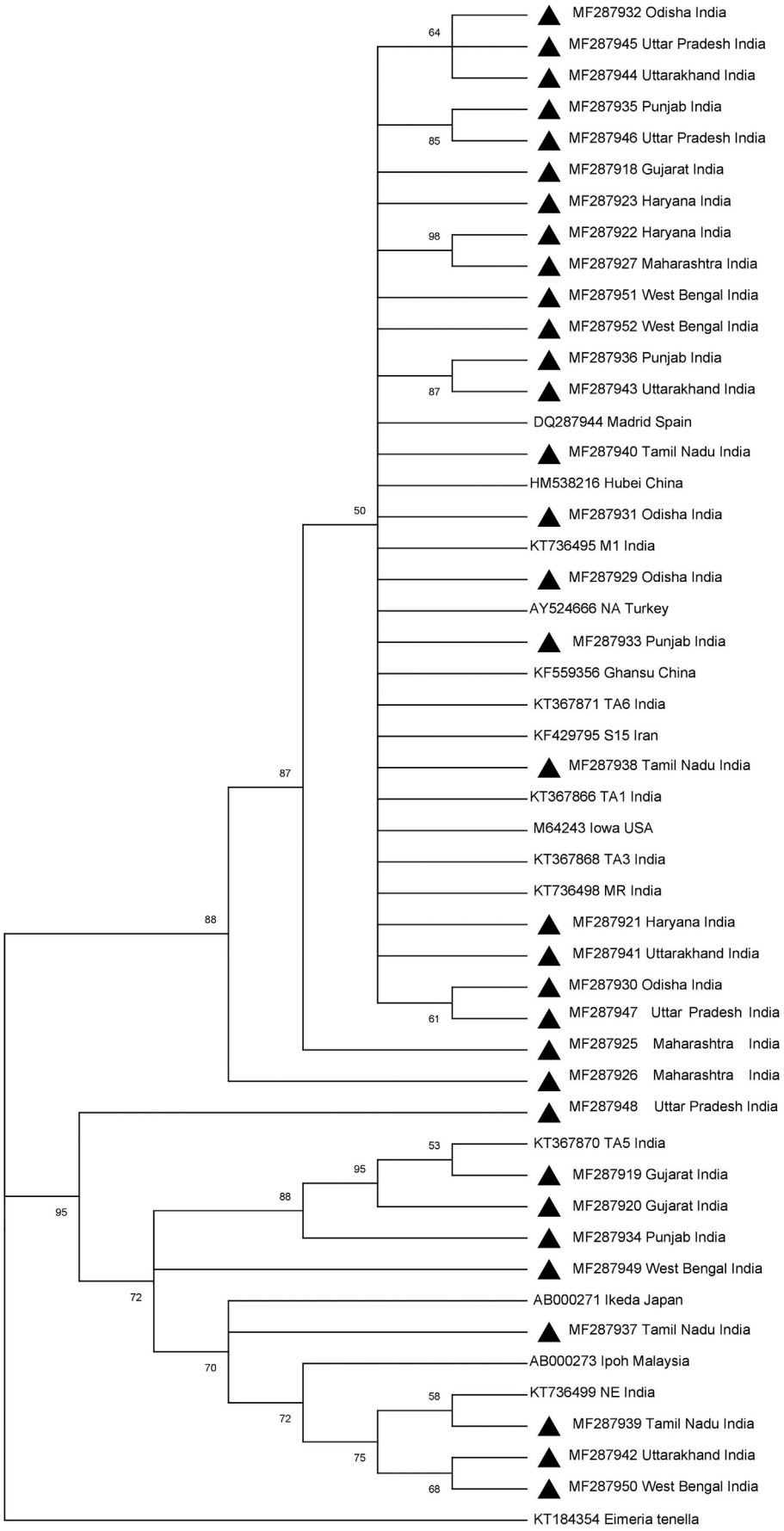

Fig. 3. Phylogenetic analysis of *T. annulata* (18S rRNA gene).

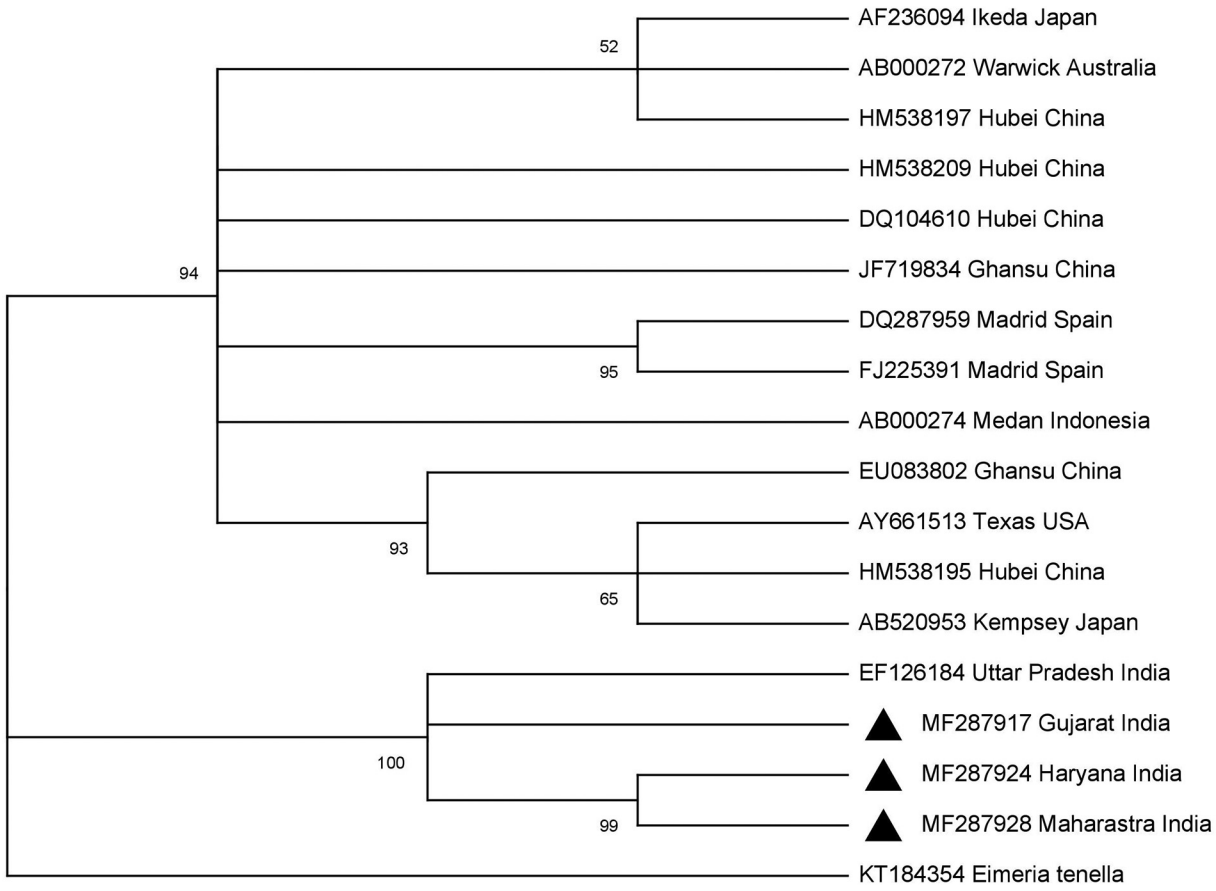

Fig. 4. Phylogenetic analysis of *T. orientalis* (18 S rRNA gene)

association with isolates of *T. annulata*, originating from other countries like Iran (KF429795), Spain (DQ287944), China (HM538216), USA (M64243) and Turkey (AY524666). However, sub group formation within the major clade was also recorded for a single isolate (MF287926- Maharashtra, India). Further, analysis revealed that a total of 9 new isolates formed a second clade involving the *T. annulata* isolates from East Asian countries like Japan (AB000271) and Malaysia (AB000273). In this clade, sister clade was formed by two Gujarat isolates (MF287919, MF287920) and by Uttarakhand (MF287942) and West Bengal (MF287950) isolates. However, a single isolate from Uttar Pradesh (MF287948) formed a subgroup within the clade.

The phylogenetic tree constructed using the 3 new isolates (having distant homology with *T. annulata*) and 14 published isolates representing the *T. orientalis/T. buffeli/T. sergenti* group was constructed using neighbor joining method with Tamura-3 parameter model for distance calculation, The 3 newly generated isolates formed a single clade with *T. buffeli* Indian isolate (EF126184) with high bootstrap value of 100 but was found closest to the Gujarat isolate 1 (MF287917) forming a sister group within the clade (Fig. 4). However, remaining 13 isolates belonging to *T. orientalis/T. buffeli/T. sergenti* group from different countries (Australia, China, Indonesia and Spain) clustered away from the Indian isolates and formed a separate clade (Fig. 4).

4. Discussion

In the present study, genetic characterization of *T. annulata* based on 18S rRNA gene was performed to understand the molecular epidemiology of this parasite in India. The specific amplification obtained (~1529 bp) in PCR assay for different isolates was further confirmed by sequencing of the target gene. In BLAST search analysis, 33 out of 36 isolates showed high level of sequence similarity with published sequences of *T. annulata,* whereas remaining 3 isolates showed closeness with published sequences of *T. buffeli* from India and formed sister clade with different isolates of *T. orientalis*.

Many reports on characterization of *T. annulata* targeting the 18S rRNA gene are available from different parts of the world (Chae et al., 1998; Gubbels et al., 2002; Inokuma et al., 2004; Liu et al., 2010; Habibi, 2012; Sivakumar et al., 2014; Adjou Moumouni et al., 2015). The role of 18S rRNA gene in genetic diversity studies of *Theileria* spp. holds importance due to the presence of conserved sequences as well as some hypervariable regions which are crucial and well-studied in order to determine the evolutionary patterns and similarity amongst *Theileria* species (Chae et al., 1998; Sivakumar et al., 2014). Of late, presence of *T. annulata* variants infecting cattle and buffalo from Pakistan targeting the 18S rRNA and ITS sequence based analysis has been reported by Khan et al. (2013) which support our results to a great extent.

Sequence data analysis of all the 36 isolates showed significant sequence variability both within and between the isolates. Maximum sequence heterogeneity recorded in the region 449 to 516 bp was quite obvious, as this region is representing the hyper variable (V4) region of the small sub unit ribosomal gene. Geographical specificity in different *T. annulata* isolates were not observed in the study, as almost similar nucleotide sequences were obtained from different areas. This might be due to panmictic population structure of the parasite (Gubbels et al., 2000). Further, sequence length polymorphisms in the 18S rRNA gene of different *T. annulata* isolates (1527–1538 bp), recorded in present study might be due to genetic variability in different isolates. This genetic variation was mostly contributed by the deletions, insertions and substitutions in the nucleotide sequences of different isolates. Very recently, based on some novel insertions and single nucleotide polymorphism (SNP's) in the 18S rRNA sequences, TA 5 isolate (KT367868) has been designated as a novel genotype of *T. annulata* in India (George

et al., 2015). Similar nucleotide insertions/deletions have been detected amongst different isolates, which is responsible for clustering of these isolates along with the novel genotype (TA5) in the phylogenetic tree. The nucleotide changes were appreciated maximum in this clade, in comparison to the clade holding Iranian vaccine strain (KF429795).

Multiple sequence alignment as well as the BLAST search analysis of 18S rRNA sequences generated in the study recorded presence of 3 distinct isolates having high sequence similarity with published sequences of *T. orientalis*. These 3 isolates were from Gujarat (MF287917), Haryana (MF287924) and Maharashtra (MF287928) and level of sequence homology between the isolates was 99–100%. The nucleotide sequences of these 3 isolates were found to be closely associated with published sequences of *T. buffeli* Indian isolate (EF126184), which indicates that the members of non-lymphoproliferative *Theileria* species (*T. orientalis/T. sergenti T. buffeli*), circulating in India, have genetic homogeneity. Lack of genetic diversity and/or polymorphism in the 18S rRNA locus of different *T. orientalis* isolates seems to be due to clonal expansion of a single strain within India. For genetic diversity in *Theileria* spp., recombination during sexual reproduction has been suggested to be a major mechanism (Henson et al., 2012; Katzer et al., 2006; Morzaria et al., 1993; Weir et al., 2007).

The results of this preliminary genetic characterization study confirmed that causative agents of both, bovine tropical theilerisosis (*T. annulata*) and benign theileriosis (*T. orientalis*) are circulating in different parts of India. Apart from that, several strains or genotypes of *T. annulata* are also in circulation and this might be the reason for variation in the degree of pathogenicity and response to treatment in the field cases. This finding might be useful in the planning and execution of managemental and control strategies against theileriosis, as selection of single diagnostic or immunological target may not be sufficient to achieve the goal. Further, a thorough molecular epidemiology and characterization of immunogenic genes of *T. annulata* is also required to understand the pattern and underlying mechanism of genetic diversity in *Theileria* species circulating in bovine population of the country.

Declaration of Competing Interest

This is to certify that all the authors of this article entitled "Genetic characterization of *Theileria* species infecting bovines in India" have no conflict of interest.

Acknowledgement

Authors are thankful to Director, ICAR-IVRI, Izatnagar for providing necessary facility for completing this research work. Financial assistance received from the NICRA project is dully acknowledged. National Innovations in Climate Resilient Agriculture (NICRA) Project of Indian Council of Agrcultural Research, New Delhi (India).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.103962.

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