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Molecular Phylogenetic Affinities of Endangered *Trombidium Grandissim* Using Mitochondrial *16S RDNA* Sequence.

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ABSTRACT

Molecular phylogenetic analysis has become a valuable tool for inferring speciesevolutionary relationship amidst the taxa. The majority mites of the family Trombidiidae are ectoparasites in the larval phase and free-living predators in the deutonymphal and adult phases on a variety of arthropods. We have collected sample *Trombidium grandissimum* from Andhra Pradesh and built a molecular phylogeny of Trombidiidae and Acari taxa from across its distribution using 16s ribosomal rDNA gene to infer the phylogenetic affinities among the subclass Acari, whether the sample from Andhra Pradesh are nested and also perceive the *T. grandissimum*is more closely related to the conspecifics from the Indian subcontinent or otherwise. Our consequences show the family Trombidiidae have formed cluster with the monophyletic families namely Demodicidae also indicating that the Trombidiidae families are closely related to the representatives of Demodicidae families as descend under the subclass Acari.

Keywords: *T.grandissimum,* 16s rDNA, MEGA v6.0, RaXmal, Acari, Phylogeny analysis.

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INTRODUCION

The *Trombidiidae* and related group mites are the red velvet mites (Zhang 1998) are seasonally available and commonly found during monsoon rainy season in open dry soils and garden areas**.** *T.grandissimum* (Fig 1) is a striking mite among all other arthropods, because of its brilliant red or orange color (Hingley 1993). These mites belong to the prostigmatid suborder Parasitengona; evolved a complex life cycle with few exceptions. The larvae are parasitic on insects and other invertebrates and are morphologically different from other free – living post larval stages, which are predators of small arthropods. Because their prey and hosts include insect and mite pests of economic importance these mites are considered to have potential as biological control agents (Eickwort 1983; Welbourn 1983; Zhang 1998). There are some previous review on the general biology and ecology of members of *Trombidiidae* (Robaux 1974; Welbourn 1983, Zhang 1991) and also on the medicinal properties of *T.grandissimum* like paralysis, urinary disorders, premature ejaculation, typhoid, malaria and many other diseases since they have immunological, analgesic, anti-bacterial, diuretic, anti-rheumatic and anesthetic properties (Lighty George et al, 2010, Yamakawa 1998).

The subfamilies of *Trombidiidae sensu* (Thor 1935) united to class in the Trombididoidea (Zhang 1998) with the concept of taxa and classification at the family level. The superfamily Trombidioidea was united and given higher status (Trombidia) and further divided by Feider (1959). Feider (1979) classification distinguished 22 families in 11 superfamilies. Welbourn (1991), raised *Trombidioide* a*sensulato*to the cohort Trombidina consisting of ten families in four superfamilies the Trombidiidae (Trombidiinae, Podothrombiinae and Allothrombiinae) was positioned in *Trombidioidea sensu* and *stricto* along with the Microtrombidiidae, Eutrombidiidae and Neothrombiidae. Hypotheses about the phylogenetic relationships and structure have been predicted by Welbourn (1991) at the superfamily level, by Welbourn (1984) at the family level within the *Trombidioidea* and by Zhang (1994, 1995) at the generic level in the *Trombidiidae* and *Neothrombiidae*. In this article we concerns about Trombidiidae with referring to other genetically related families are made to expose the general developments in *Trombidioid* mites. So we aimed at to analyze molecular phylogenetic affinities to emphasize the diverse nature of *Trombidiidae* using partial sequences of 16s rDNA gene of Indian representatives namely *Trombidium grandissimum* and also with the available NCBI database.

MATERIAL AND METHODS

Collection of samples:

The adult *T.grandissimum*sized about 1.5 cm to 3 cm were collected from red soil fields of Nuzvid, Andhra Pradesh(16.7850° N, 80.8488° E) during the period of monsoon(June to July). The samples were stored at room temperature in the red soil collected from field till further use.

Extraction of DNA:

T.grandissimum genomic DNA was isolated according to the protocol described by Dellaporta (1983). Prior to DNA extraction, *T.grandissimum*was freeze dried at -20^{O} C for 120 min, then the samples were washed with 95% ethyl alcohol and crushed using mortar and pestle. The crushed tissue was mixed in 3 ml of lysis buffer (50 mMTris-HCl, 10 mM Na_2EDTA,100 mMNaCl, 1% SDS) and 10 µl of proteinase K (10 mg/ml) in 50 ml capacity polypropylene centrifuge tubes and incubated at 65 ºC in a water bath for 1 h with gentle swirling. The lysed samples were thoroughly mixed with phenol: chloroform: isoamyl alcohol (25:24:1). The contents were centrifuged and the aqueous phase was transferred to a fresh centrifuge tube and mixed well with double volume of Isoproponal and 3 M sodium acetate (100µl)and mixture was kept at -20 ºC for 60 min to allow complete precipitation of DNA by gently inverting the tube at least six times. Then the mixture was centrifuged at 10,000 rpm for 15 min to pellet the DNA. The supernatant was decanted; pellet was washed with wash buffer (70 v/v ethanol) and air dried. The DNA was dissolved in 200 µl T E buffer (10 mM Tris-HCl and 1 mM Na_2EDTA, pH 8.0) and the contents were transferred to 2 ml microfuge tubes. The dissolved DNA was treated with 10 mg/ml RNase A at 37 ºC for 1 h, stored at -20 ºC for further use.

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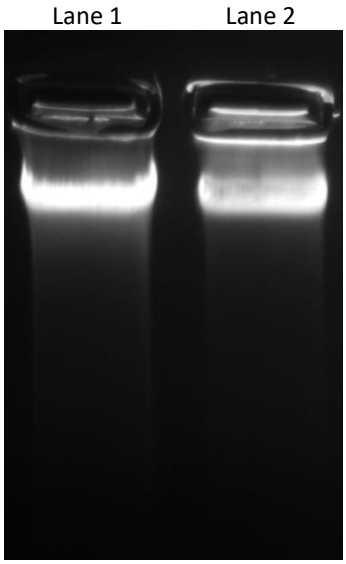
Fig 1: *Trombidiumgrandissimum* collected from Nuzvid, Andra Pradesh.

Quantification of DNA

The genomic DNA was quantified using UV-spectrophotometer. The quantity of DNA were measured by obtaining the absorbance reading at 260 nm and the purity of DNA were estimated by calculating the ratio of absorbance reading at 260nm and 280nm.The quality of genomic DNA extracted was evaluated by horizontal electrophoresis (Medox-Bio) using 0.8 % agarose gel (Fig 2) with 1.5 ul of 10ug/ml EtBr in 1.0 X TBE. 5ul of sample mixed with 2ul of 6X loading dye was run at 100 V and 100mA for 25 min. The gel was photographed under Gel Doc system (Mediccare™, Chennai, India).

Fig 2: Genomic DNA extracted from Trombidium



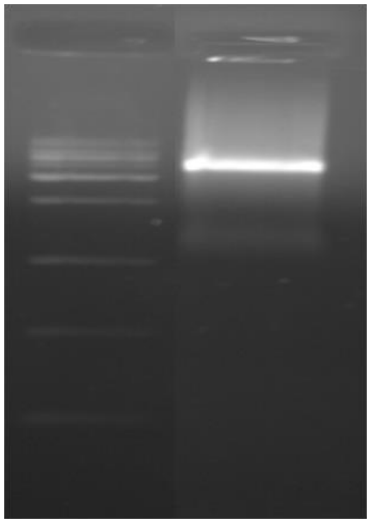
Lane 1 – DNA of *T.grandissimum* RNase A treatment.
Lane 2 - DNA of *T.grandissimum* RNase A treatment (Duplicate)

PCR analysis of *T.grandissimum*:

Amplification of 16s rDNA was performed using the universal 16s primers (Palumbi *et al*., 1991) and PCR reaction mix was prepared for 25 ul volume with components of DNA (1ul), 0.5 ul of Taq(100u/ul), Taqbuffer with $MgCl_2$(1ul), 2ul of 10pM primers, 1ul of dNTP's and make up the volume with MiliQ water and performed in Thermal Cycler (Agilent, USA/ Eppendorf, USA). The reaction volume for all PCR reactions was set to 25 µl (All the reagents required for PCR studies were purchased from (HiMedia, Mumbai, India). PCR amplifications were carried out starting with an initial denaturation at 94 ºC for 5 min, denaturation at 94 ºC for 30 seconds, annealing at 58 ºC for 45 seconds and extension at 72 ºC for 1 min. These steps were repeated for 35 cycles followed by a final extension for 10 min at 72 ºC. The reaction mixture without a template was

run as a negative control. Amplified DNA fragment was separated by gel electrophoresis in 1.2% agarose gel and images documented using Gel Doc system shown in Fig.3 (Mediccare™, Chennai, India).

Fig 3: Amplification of 16s rDNA



Lane 1 -100 bp (Genomic DNA ladder)
Lane 2 – 515 bp amplification

The PCR reactions were performed in duplicates with 100bp molecular marker. Purified PCR product was sequenced bidirectionally with the same primers used for PCR amplification by M/S Eurofins, Bangalore. The sequence information obtained from both the strands was checked for complimentary matching and verified by using BLAST. Mitochondrial genes were identified by sequence comparison using BLAST searches at NCBI with the BlastN algorithm. The sequences obtained were submitted to NCBI andthe given accession number is KP297891.

Phylogenetic analysis

The DNA sequence alignment of subclass Acari species was performed with default parameters in MUSCLE (Edgar RC, 2004b) using the program MEGA v5.2 (Tamura K, 2013). The alignments of chosen species having more than 90% identity in blast were shown (**"*"**) in the fig.4 indicates 100% nucleotide base of Trombidium identical with other selected species. Maximum likelihood (ML) algorithm was used to estimate phylogenies adopting RAxML software tool (Silvestro and Michalak, 2012). For executing ML analysis, we selected 39 taxa along with three outgroups to build the phylogenetic affinities. We used the GTR+ Γ DNA substitution model and analyzed independently in RAxML. The ML phylogenetic analysis involving the unpartitioned nucleotide sequences were analyzed through raxmlIGUI v1.3 software package. The constructed phylogenetic tree nodal support values (Figure 3) were obtained by performing with 1000 bootstrapping replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

T.grandissimum is an exotic invertebrate commonly found in Indian subcontinent and other countries. Though many researchers have reported classification and morphological identification, very little information is available about its genetic relatedness. The development of molecular genetics tools over the last two decades has allowed the construction of species phylogenies based on their genetic relatedness at the molecular level, enlightening evolutionary studies. Phylogenetic trees have been used to compare ecologically-related taxa enabling their level of co-evolution or reciprocal adaptation through time to be studied, such as symbioses (Itino *et al.*, 2001). To understand the phylogenetic relationship of T.*grandissimum,* we have utilized 16s rDNA sequence analysis and comparison with other families of arthropods. The DNA was extracted using modified Phenol: Chloroform method. The concentration of DNA was found to be 12.15ug/ml and the yield obtained was 1.215 ug from 0.5 freeze dried sample. The integrity of DNA was verified by running in agarose gel shown in figure 2 (Sambrook*et al.,* 1989).

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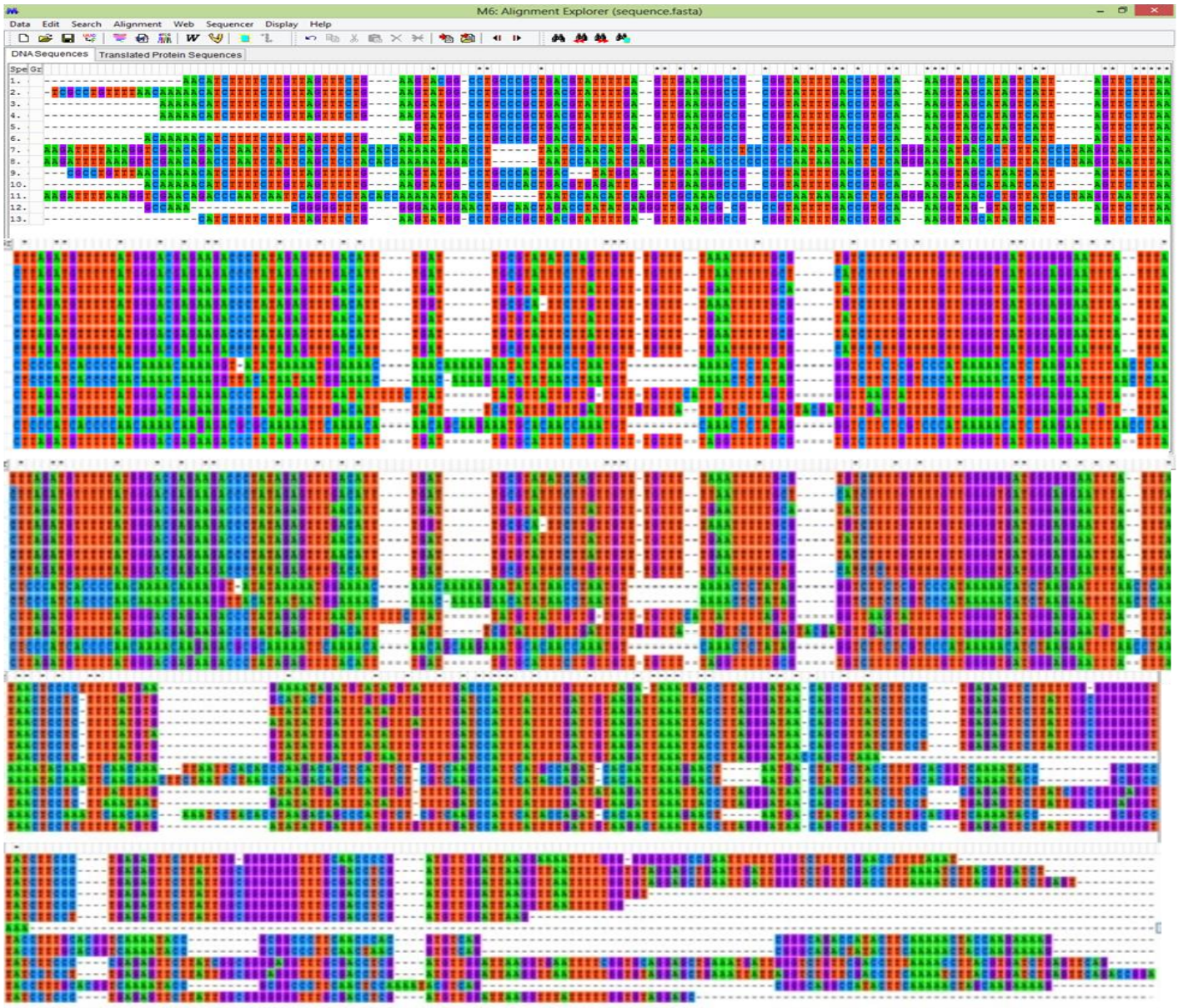
Fig 4: Alignment of total 13 sequences having maximum identity in BLAST result



1.Trombidium grandissimum, 2.Odontotermes longignathus, 3.Odontotermes hainanensis, 4.Odontotermes malaccensis voucher, 5.Odontotermes hainanensis, 6.Odontotermes hainanensis, 7.Odontotermes horni, 8.Odontotermes montanus, 9.Odontotermes amanuensis, 10.Globitermes sulphureus, 11.Sphaerotermes sphaerothorax, 12.Odontotermes zambesiensis, 13.Odontotermes javanicus

(*) indicates, 100% nucleotide base of Trombidium matching with other above mention species.

Mitochondrial genome is a convenient method for understanding for the phylogenetic analysis of animals because of its maternal inheritance. Hence in the present study we have explored the genetic relatedness of T.grandissimum by 16s rDNA sequencing analysis. The partial sequence of 16s rDNA gene (Fig 3) of *T.grandissimum* (515bp) was compared with the sequences of the selected Termitidae, Eriophyidae, Demodicidae species falling respectively under class Insecta and Arachnida which had sequence identity in NCBI (BLAST). Maximum likelihood (ML) phylogenetic analysis of 39 genera belonging to subclass Acari was shown in Fig.5 and this tree was rooted on the taxa namely Coptotermes sjoestedti, Coptotermes curvignathus (Rhinotermitidae) corresponding to the Acari taxonomy (Dabert, 1999). However, our focal taxon is shown to

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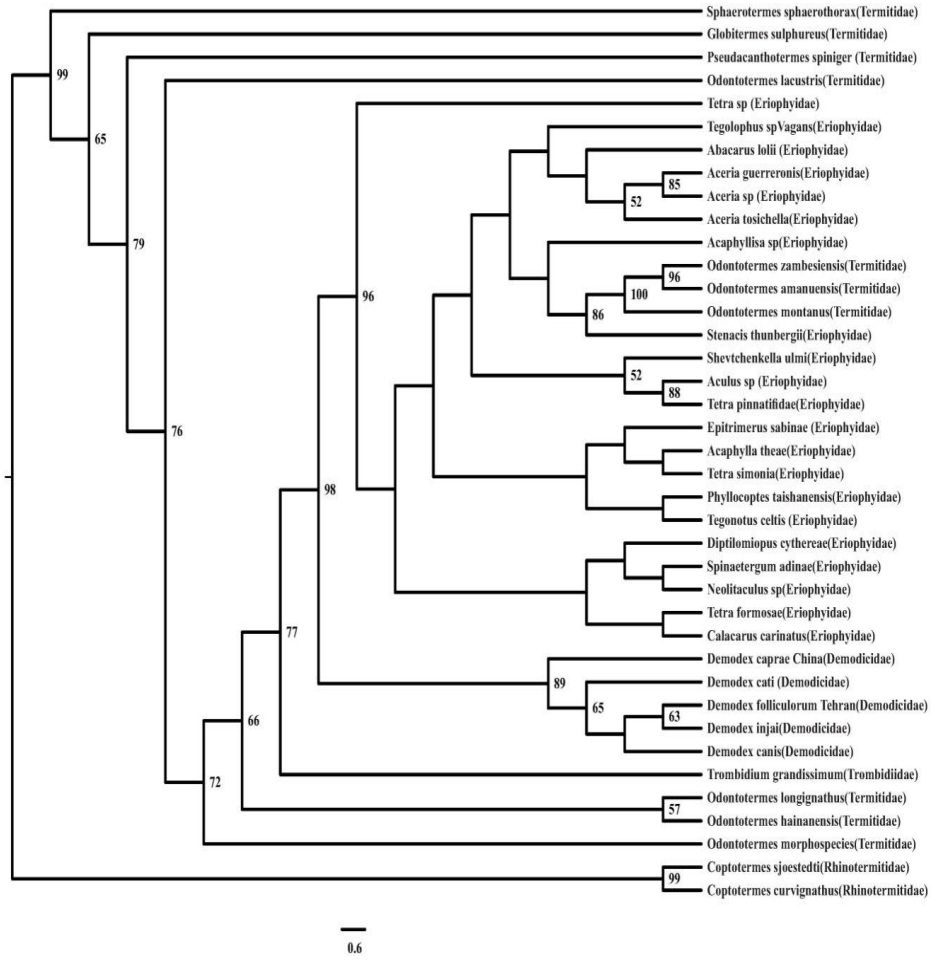
be formed a primary cluster with family Demodicidae amid very high bootstrap support. The molecular phylogenetic analysis of *T.grandissimum* revealed that the family Trombidiidae has more genetic proximity towards Demodicidae family members by 16s rDNA partial sequence analysis. In addition, the cladogram also indicates that the Trombidiidae possibly embedded from the family Demodicidae.

Fig 5: Molecular Phylogenetic analysis of *Trombidiumgrandissimum* by Maximum Likelihood method



CONCLUSION

This study concludes that *T.grandissimum* (Andhra Pradesh) is more closely related to the conspecifics form the Indian subcontinent and our result shows a strong support to the genetic nearness towards Demodicidae family members by 16s rDNA partial sequence analysis. Moreover the existing genetic database doesn't suffice to investigate further. Hence further evidences and phylogenetic studies are needed to support the present work.

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