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Detection and molecular identification of scrub typhus vectors and pathogens from rice field rats, a traditional food item of Mizo tribes in Mizoram, Northeast India

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Abstract

Background & objectives *Orientia tsutsugamushi*, a bacterial pathogen of scrub typhus, is transmitted to humans through the bite of infected chigger mites, and rodents are the natural hosts of the disease vector. The traditional practices of the tribal ethnic groups (Mizo) of Mizoram state such as capturing and consumption of rat meat collected from the agricultural fields could be one source of vector-disease transmission route. The present study aimed to detect and identify the pathogen of scrub typhus from vectors collected from rice field rats which were captured by farmers for meat consumption purposes.

Methods One hundred and fifty-six freshly captured rice field rats were examined for ectoparasites. Detection and genotyping of *O. tsutsugamushi* from ectoparasites were done by real-time PCR and conventional PCR using species-specific primers. Rodents and ectoparasites were identified down to the species level using morphological and molecular techniques.

Results Rice field rats were identified as *Rattus tanezumi* and *Rattus nitidus*. A total of 7973 trombiculid mites collected from 156 rats were identified as *Leptotrombidium deliense, Leptotrombidium fletcheri,* and *Leptotrombidium chiangraiensis*. Of these, 26 pools of *L. deliense* and 15 pools of *L. fletcheri* tested positive for *O. tsutsugamushi,* and the *O. tsutsugamushi* detected belongs to Kato and Karp-related genotypes.

Interpretation & conclusion The present study reported the presence of *O. tsutsugamushi*-infected chigger mites in the captured rats (*R. tanezumi* and *R. nitidus*). Direct contact with the rats as a result of rat-eating habits may correspond to the high incidence rate of scrub typhus cases in Mizoram. Preventive measures are crucial for the control of scrub typhus disease in Mizoram.

Keywords Molecular detection, Rodent hosts, *Orientia tsutsugamushi*, Field rats, Mizo tribes

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Contributions to the literature

- This study is the first to confirm the presence of scrub typhus pathogens in the vector mites of rice field rats collected for meat consumption purposes by the tribal ethnic groups of Mizoram state, India.
- It highlights the very high chigger index among the rat species traditionally used by the tribal people of Mizoram state as a food item.
- It also highlights the possible role of rat eating habit of the Mizo tribes in the high incidence rate of scrub typhus disease among the farmers in the present study area.

Introduction

Scrub typhus is a vector-borne disease caused by *Orientia tsutsugamushi*, which is transmitted to humans from field mice and rats through the bite of infected chigger mites that live on the vertebrate host animals [1]. *Orientia tsutsugamushi* is a gram-negative, obligate intracellular bacterium. The disease of scrub typhus is characterized by fever, headache, muscle aches, and skin lesion called an eschar. Once inside the host cell, the bacteria replicate rapidly within the cytoplasm, and can also infect endothelial cells causing vascular damage leading to disseminated intravascular coagulation in severe cases [2, 3]. The vector mites belong to the genus *Leptotrombidium*. However, recent discoveries have reported new vector genera, such as *Schoengastiella* and *Euschoengastia,* capable of transmitting this agent [4, 5]. Knowledge of the host and vector species, their distribution, density, and habitats, is essential in understanding the epidemiology of scrub typhus in a particular region [6]. Rainfall, temperature, land use, and various socio-economic factors were reported to be involved in the activity of vector trombiculid mites, which eventually contribute to the extent of transmission of the disease [7, 8]. Chigger mites are small, hairy with a soft exoskeleton. Adults are usually 2 mm long, while larvae are 0.1 – 0.3 mm long with orange, red, or white in color. They are oval or round in shape with the larvae having three pairs of legs, while nymphs and adults are having four pairs of legs [9] (Brennan & Goff, 1977). Larvae are the only parasitic stage in trombiculid mites, while nymphs and adults are free-living and feed on arthropods [10]. Larvae are thus of significant medical importance due to their role in transmitting scrub typhus to humans.

Scrub typhus is prevalent in areas where scrub vegetation with small trees and bushes is encountered and in diverse habitats, including river banks, grassy lawns, rice fields, and kitchen gardens [11, 12]. Humans get infected when infected larval mites are picked up from the infested ground [13]. Rats and mice are the natural hosts for vectors of scrub typhus, while rodents and acarine hosts did not have symptoms of the disease [14]. Therefore, rodents and vector trombiculid mites are the reservoirs of the disease, and between the two, the infection is maintained by nature. Rodents are thus crucial for the survival of vectors and play an important role in the spread of the disease. The intensity of chigger infestations could vary considerably between rodent species [15]. Recognition of the primary host species in this region will help predict the risks to scrub typhus because the number of chiggers will increase when the principal rodent host species flourishes in agricultural fields and areas of human habitation. Therefore, knowledge of the diversity and prevalence of rodent host species will help in the prediction of human health outcomes [16].

Mizoram occupies 21,081 square kilometres and is located in the North-eastern part of India at the foot of the Himalayan range. It has two international borders, to the west with Bangladesh and to the east with Myanmar. It is located between 92°15' and 93°26'E longitude and 21°58' and 24°35'N latitude. In Mizoram, the first case of scrub typhus was recorded in 2012 [17]. Scrub typhus outbreaks and cases have significantly increased since 2018. Scrub typhus has become a serious public health concern, and in the last five years (2018–2022), there have been 19,651 cases reported in Mizoram, which is more than India's decadal (2010–2020) record (18,781) [18]. The rise of scrub typhus in the state may be connected to the alterations in the vegetation [19]. Shifting cultivation or *Jhum* system of cultivation is the primary system of agricultural cultivation practiced by all the ethnic groups of Mizoram throughout the state. The main crop in Mizoram is rice, which is farmed on a bigger area of land than other grains. Rodents are the major pest of rice in the state, causing both pre- and post-harvest losses. Therefore, destruction by rodent pests is one of the many difficulties faced by farmers to date. *Rattus argentiventer* is reported to be the most prevalent rat pest species in Southeast Asia [20]. One of the most common species of rodent pest in Mizoram is *Rattus tanezumi,* what is locally called 'Zupawl'. To date, there has not been a survey of the rodent species present within the rice fields in Mizoram. Farmers used to capture rice field rats during pre- and post-harvest periods, normally during September to January. This capturing and killing of rats is part of rodent pest management as well as for meat consumption purposes. A large number of people in Mizoram eat smoked rats, which are still in demand in the market of the capital city, Aizawl. However, only rat species (*Zupawl*) found in a rice field or some other agricultural lands, which have a prominent white-coloured dorsal side, were eaten. They have a clean and healthy look as

compared to the other species inhabiting peri-domestic areas. According to an elderly person, the habit of eating field rats was already seen during 'mautam' (the bamboo famine of the 1950s), when rodents multiplied rapidly in agricultural lands, especially in rice fields [21–23]. However, the consumption of rats' meat seemed to be an age-old tradition, and it may be assumed that it has begun since rice cultivation was started in Mizoram State. The present study aimed to detect and identify trombiculid mite vectors and pathogens of scrub typhus from rice field rats captured by farmers for meat consumption purposes. Infection of rodent hosts with scrub typhus pathogen was not be investigated in this study. The collection of specimens from farmers was conducted in different villages of Mizoram State, Northeast India.

Materials & methods

Study site and sample collection

In the present study, collection of ectoparasites from rice field rats was conducted during the month of November and December 2023 in 12 villages of Mizoram state, India, where a considerably high incidence rate of scrub typhus disease during the year 2018 to 2022 was reported with the highest incidence rate in Aizawl and Serchhip districts followed by Mamit, Lunglei, and Lawngtlai districts [18]. This is the post-harvest period of rice in shifting cultivation, and the heavy rat hunting season for farmers. Twelve different collection sites located in different corners of the state selected in the present study are shown in Fig. 1. Farmers caught rice field rats using different traditional trapping and catching techniques. Prior consent of the farmers was obtained to donate their freshly captured rice field rats (dead) for use in the collection of ectoparasites in the present study. A total of 24 rats (1 *R. tanezumi* and 1 *R. nitidus* each from 12 collection sites) were preserved in 70% ethanol for identification and museum specimen purposes. They were morphologically identified to species level using the taxonomical keys [24]. Liver and spleen tissue samples were also taken and preserved in 70% ethanol for molecular studies. The Institutional Animal Ethics Committee (IAEC) of Pachhunga University College, Aizawl, Mizoram, India (vide IAEC No. PUC-IAEC-2021-A09, dated 05-07-2021) approved the protocol for this study.

Collection and identification of ectoparasites

Ectoparasites were collected from the limbs, ears, and axillary region of the rats. They were then pooled based on the species and collection sites, and stored in 70% ethanol until further processing [25]. They were examined under a dissecting microscope for species identification following established identification keys and criteria [26, 27]. The mean abundance (M_A) was used to calculate the chigger mite index of the two rat species with the following formula:

$$M_A = M/H,$$

where, M = the individuals of chigger mites infested, H = the total of individuals of rats [28].

DNA extraction and PCR amplification

Following the manufacturer's instructions, genomic DNA was extracted from rodents' tissue and ectoparasite pools using the QIAamp DNA Mini Kit (Qiagen, GmBh, Germany). Approximately 50 ectoparasite specimens were used in each pool to retrieve enough DNA. At the temperature of -20℃, the extracted DNA was kept until further processing. PCR amplification of the *cytochrome c-oxidase subunit 1* (*COX1*) gene of rodents and ectoparasites was done following the published protocols [29, 30].

Real-time PCR detection of *Orientia tsutsugamushi*

O. tsutsugamushi was detected from ectoparasites using real-time PCR amplification of *47-kDa* gene. The PCR amplification was performed on a CFX Connect™ Real-Time System (Bio-Rad, USA). To summarize, the reaction mixture contained 12.5 µl of BioRad's 2X SYBR Green I Master Mix, 0.75 µl (10 pmol) of Otsu630 forward and Otsu747 reverse primers (Table 1), 9 µl of nuclease-free water, and 2 µl of DNA. Melt curve analysis from 65℃ to 95℃ follows the cycling conditions, which include initial denaturation at 95℃ for 7 min, 45 cycles at 95℃ for 10 s, and annealing and extension at 60℃ for 30 s. In every run, 2 µl of the *47-kDa* gene product (100 copies) of *O. tsutsugamushi* (Karp) was utilized as a positive control.

PCR amplification and sequence analysis of the 56-kDa TSA gene

From each of the *Orientia*-positive DNA extracts, a *56-kDa* nested PCR amplification was carried out using the previously published protocol [31]. Details of all the primers used are summarised in Table 1. PCR products were sequenced by Sanger's dideoxy method on an ABI 3730XL automated sequencer (AgriGenome Labs Pvt. Ltd., Smart City Kochi, Kerala, India) [32]. Sequences were edited using BioEdit Sequence Alignment Editor, ver. 7.0.5.3, and aligned using MEGA software (MEGA 7.0.26). To determine the identity of the sequences, blast search analysis was done using the NCBI's GenBank nucleotide database. Genetic distances were calculated using the Kimura two-parameter distance algorithm. Highly similar sequences were downloaded from GenBank, and a phylogenetic tree was generated using the maximum likelihood method in MEGA software and

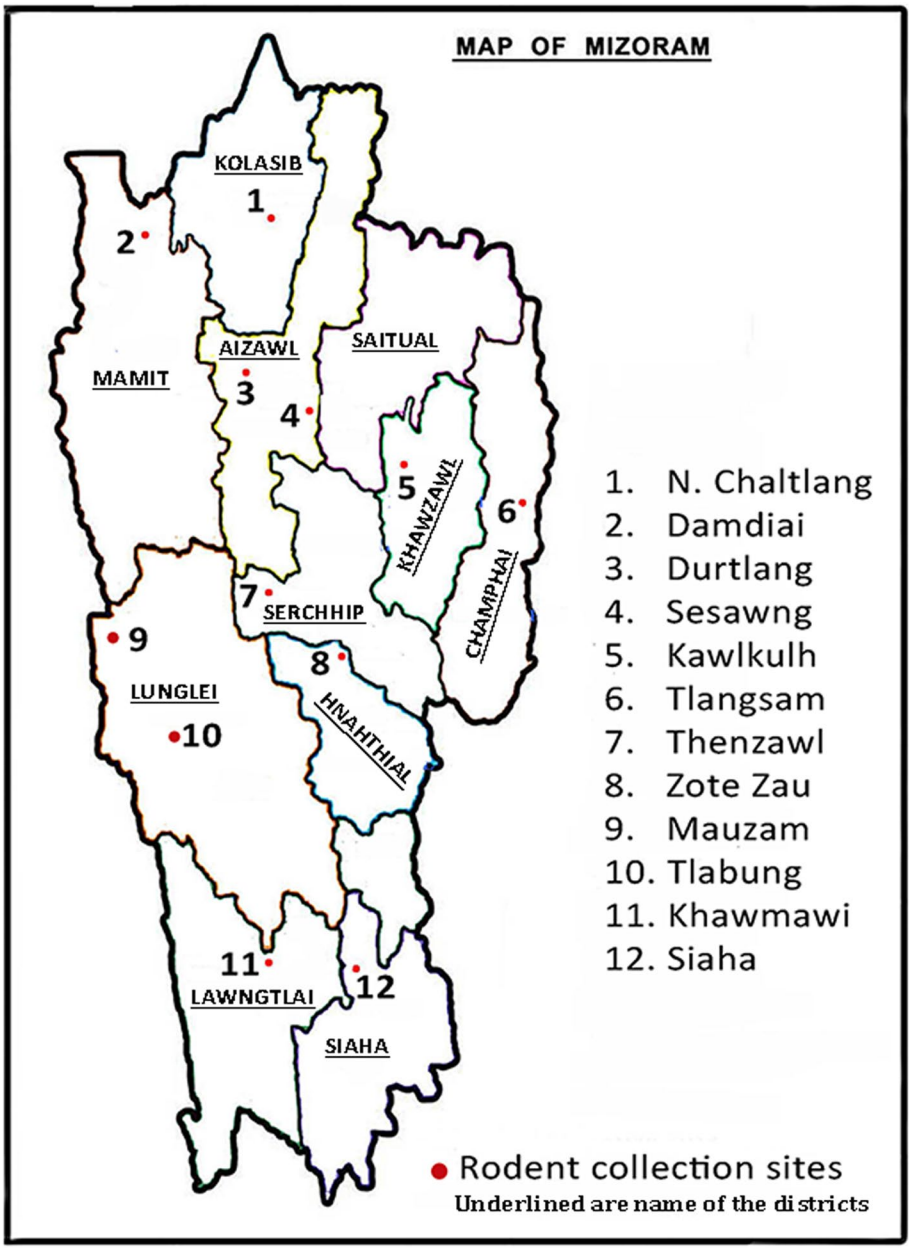

Fig. 1 A map showing sample collection sites at different corners of Mizoram state

was statistically supported by bootstrapping with 1000 replicates.

Results

One hundred and fifty-six newly captured rice field rats, which the farmers collected for meat consumption purposes (Fig. 2), were used for the present study. They were identified as *Rattus tanezumi* and *Rattus nitidus* by morphological and *COX1* gene sequence analyses. The *COX1* gene sequences of *Rattus tanezumi* and *Rattus nitidus* were deposited in the GenBank database with the accession numbers OQ061272 and OQ061273, respectively. A total of 96 *Rattus tanezumi* (8 from each village) and 60 *Rattus nitidus* (5 from each village) were examined. A total of 7973 trombiculid mites were collected from 156 rats. They were identified by morphological and molecular methods into three species: *Leptotrombidium deliense, Leptotrombidium fletcheri,* and *Leptotrombidium chiangraiensis*. One or more species of trombiculid mites infested nearly all of the captured rodents of both

Table 1 Details of primers used in the present study

Target gene	Primer name	Primer details	Product size
COX1	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	708 bp
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	
56-kDa TSA	Cycle 1		583 bp
	P34 (forward)	5′-TCAAGCTTATTGCTAGTGCAATGTCTGC-3′	
	P55 (reverse)	5′-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3′	
	Cycle 2		
	P10 (forward)	5′-GATCAAGCTTCCTCAGCCTACTATAATGCC-3′	
	P11 (reverse)	5′-CTAGGGATCCCGACAGATGCACTATTAGGC-3′	
47-kDa OMP	Otsu630	5′-AACTGATTTTATTCAAACTAATGCTGCT-3′	118 bp
	Otsu747	5′-TATGCCTGAGTAAGATACRTGAATRGAATT-3′	

bp base pairs

Fig. 2 Rice field rats used in the present study

species (87.8%). *Rattus tanezumi* had a higher infection rate (92.7%) and chigger index (55.7) as compared to infection rate (78.3%) and chigger index (43.6) of *Rattus nitidus* (Table 2). This mite burden on rats is in accordance with the findings reported previously where *Rattus tanezumi* and *Rattus nitidus* showed higher mite index when compared to the other rodent species [33]. Among the chigger mite species identified in the present study, *Leptotrombidium deliense* was the most common species comprising 51.6% of the total chiggers followed by *Leptotrombidium fletcheri* (39.5%), and *Leptotrombidium chiangraiensis* (8.9%).

Targeting the *47-kDa OMP* gene of *O. tsutsugamushi*, 153 pools of ectoparasites were used for the real-time PCR detection. The results showed that 26/76 pools (34.2%) of *Leptotrombidium deliense* and 15/63 pools (23.8%) of *Leptotrombidium fletcheri*, which were obtained from 107 rodent hosts (71.3%) of both the species, tested positive for the presence of *O. tsutsugamushi*. The amplification curve (A) and melt peaks (B) are also presented in Fig. 3. The topology of the 680 bp-long *COX1* gene tree of vector trombiculid mites in the present study, LD01 and LF01, belonged to *L. deliense* and *L. fletcheri,* respectively (Fig. 4). The representative *COX1* gene sequences of *L. deliense* and *L. fletcheri* deposited in the GenBank database were assigned accession numbers OQ283727 and OQ283729, respectively.

From the positive chigger mites, a *56-kDa TSA* gene of *O. tsutsugamushi* was amplified. The *56–kDa TSA* gene sequences displayed 99–100% identity with Karp and Kato-related genotypes from GenBank nucleotide database. Analysis of the 550 bp-long *56-kDa TSA* gene sequences showed that the pathogens from 32 pools of ectoparasites belonged to the Karp (HL01) and pathogens from 9 pools of ectoparasites belonged to Kato-related (HL08) genotypes of *O. tsutsugamushi* (Fig. 5). The DNA sequence of Karp and Kato-related genotypes were deposited in the NCBI GenBank database and the accession numbers were assigned as OQ055155 and OR039834 respectively.

Discussion

The present study used rice field rats, *Rattus tanezumi* and *Rattus nitidus* for the isolation of ectoparasites, and to detect and genotype *O. tsutsugamushi* from the vector mite species. Several chigger mites tested positive for *O. tsutsugamushi* by conventional (*56-kDa* TSA) and real-time (*47-kDa OMP*) PCR methods. This indicates that both species of these rodents captured by the tribal people of Mizoram for meat consumption purposes are important hosts for vectors of scrub typhus in the present study area. It has been reported that both the PCR detection methods targeting *56-kDa* and *47-kDa* genes showed no significant differences in their sensitivity and specificity [34]. In this study, conventional PCR of the *56-kDa* TSA gene was performed for detection, DNA sequencing and sequence analysis for molecular characterization

Table 2 Characterization of captured rats and chigger mites

Name of collection sites	*Rattus tanezumi*[a]		*Rattus nitidus*[a]	
	No. of infested rats (%)	No. of chiggers	No. of infested rats (%)	No. of chiggers
N. Chaltlang	6 (75%)	403	3 (60%)	182
Damdiai	8 (100%)	482	3 (60%)	236
Durtlang	8 (100%)	495	5 (100%)	245
Sesawng	8 (100%)	508	5 (100%)	247
Kawlkulh	7 (87.5%)	401	4 (80%)	191
Tlangsam	7 (87.5%)	372	3 (60%)	193
Thenzawl	8 (100%)	501	5 (100%)	232
Zote Zau	7 (87.5%)	396	4 (80%)	191
Mauzam	7 (87.5%)	487	3 (60%)	242
Tlabung	8 (100%)	490	5 (100%)	233
Khawmawi	8 (100%)	496	4 (80%)	251
Siaha	7 (87.5%)	321	3 (60%)	177
Total	**89 (92.7%)**[b]	**5353**	**47 (78.3%)**	**2620**
Chigger mite index	**55.7**[b]		**43.6**	

[a] A total of 8 *Rattus tanezumi* and 5 *Rattus nitidus* each were obtained from each collection site

[b] Statistical comparison (Student's *t*-test) was performed between rodent species using statistical software—OriginPro 8 SR0 version 8.0724 (OriginLab Corp., Northampton, MA, USA). The level of significance was set at $p < 0.05$

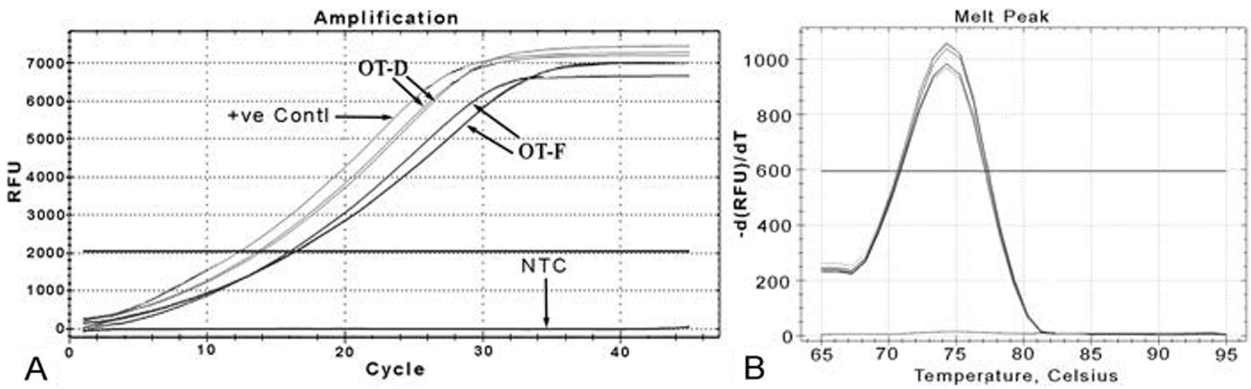

Fig. 3 Amplification (**A**) and melt curves (**B**) of *47-kDa OMP* gene of *O. tsutsugamushi* detected from *L. deliense* (OT-D) and *L. fletcheri* (OT-F) isolated from *R. tanezumi* and *R. nitidus* along with positive control (+ ve Contl) and no-template control (NTC)

of *O. tsutsugamushi*, while real-time PCR of the *47-kDa OMP* gene was done only for detection purposes. However, detection efficiency of these two PCR methods was not compared in this study. The most prevalent species of trombiculid mites in the current investigation was *L. deliense*, which was followed by *L. fletcheri*. The primary vector of *O. tsutsugamushi* is *L. deliense*, whose prevalence has also been noted in other regions of India and many countries in Southeast Asia [35, 36].

According to the earlier reports [18], there was a high incidence rate (3.54) of scrub typhus cases in Mizoram during the years 2018–2022. It has been reported that cases were significantly higher among the farmers when compared to the other occupational groups and it was also reported that a spike in scrub typhus cases was observed during July and January. Therefore, the traditional practice of hunting rice field rats for meat consumption purposes, leading to increased human contact with rodents' ectoparasites, especially trombiculid mites, vectors of scrub typhus, may correspond to the high incidence rate of scrub typhus cases in Mizoram [17, 18]. The present data also suggest the circulation of multiple genotypes of *O. tsutsugamushi* in vectors collected from rice field rats in the present study area. One of the limitations of the present study is that field collection of rodent specimens was done in limited number of villages, and only

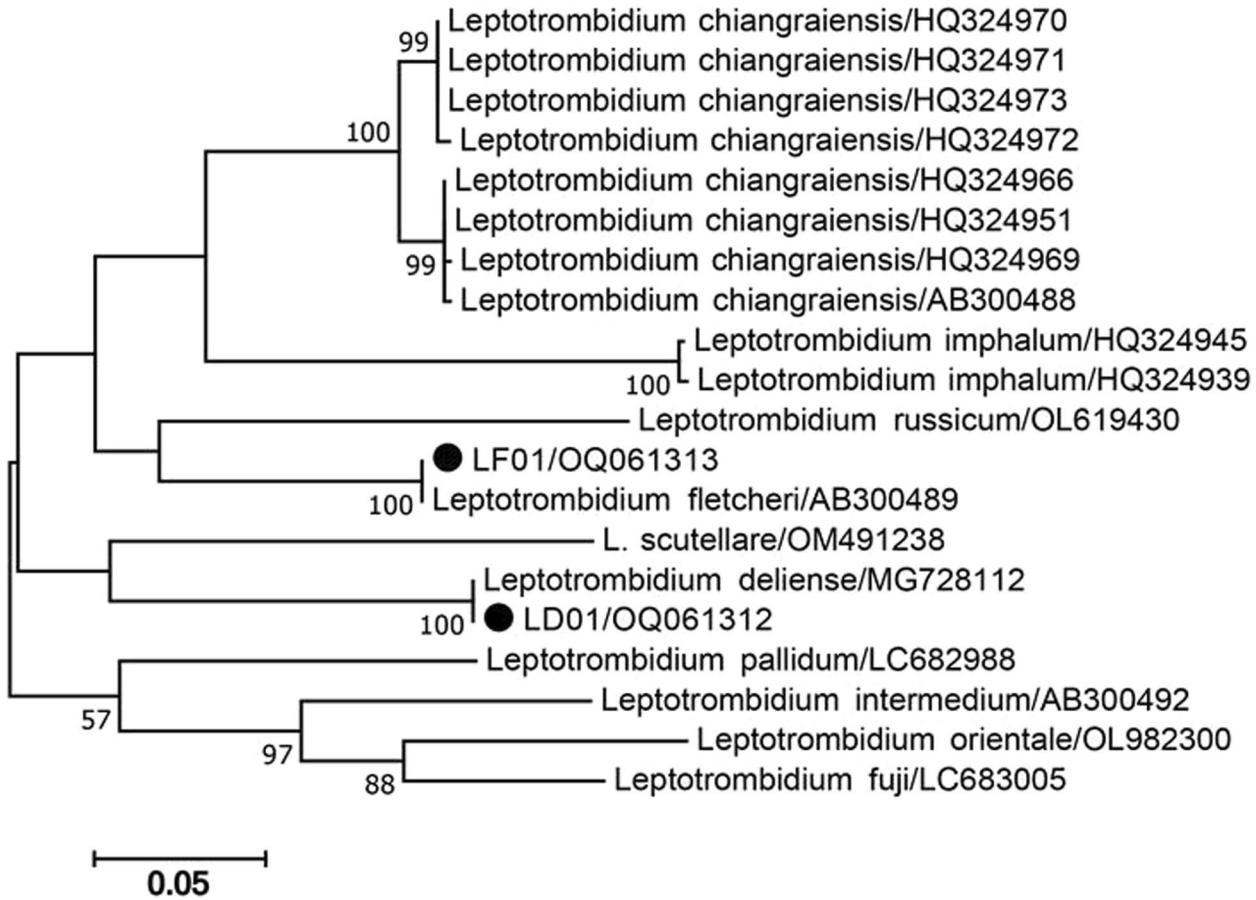

Fig. 4 Phylogenetic tree of trombiculid mites (*L. deliense*–LD01, *L. fletcheri*–LF01) based on the *COX1* gene fragment sequences along with 18 reference sequences. The tree was constructed in MEGA7 software using the maximum likelihood method. The scale bar represents a number of nucleotide substitutions per site

156 rats were examined. A wider range of *Orientia tsutsugamushi* genotypes might be possible if a similar study were done with more samples and study sites. There could be an unidentified vector trombiculid mite species and genotypes of *Orientia tsutsugamushi* in the present study area (Mizoram state, India). Another limitation of the present study is that although the pathogens of scrub typhus were detected and identified from the vector mites collected from rats which were in direct contact with the farmers, the exact role of rat hunting habits in the transmission dynamics of scrub typhus disease has not been investigated in this study. Therefore, investigation of the disease transmission dynamics is suggested for the focus of future research with a larger size of study populations.

Conclusion

In conclusion, we presented the first report on the detection and identification of *Orientia tsutsugamushi* in trombiculid mites collected from rice field rat species, *Rattus tanezumi* and *Rattus nitidus,* which have been captured by the farmers of tribal ethnic groups (Mizo) in Mizoram state, Northeast India, for meat consumption purposes. These two rat species harboured a large number of ectoparasites, of which *Leptotrombidium deliense* and *Leptotrombidium fletcheri* were found to be infected with *Orientia tsutsugamushi,* a bacterial pathogen of scrub typhus disease. The identified *Orientia tsutsugamushi* belonged to the Karp and Kato-related genotypes. The direct or indirect contact of farmers with rodent hosts in agricultural lands as a result of the rat-hunting practices among the tribal ethnic groups (Mizo) may correspond to the high incidence rate of scrub typhus cases in Mizoram state, Northeast India. Therefore, personal protective measures such as wearing protective clothing, use of insect repellant and avoiding areas with chiggers, environmental management such as reducing chiggers habitat through vegetation control, proper sanitation and managing rodent populations in areas where the disease is

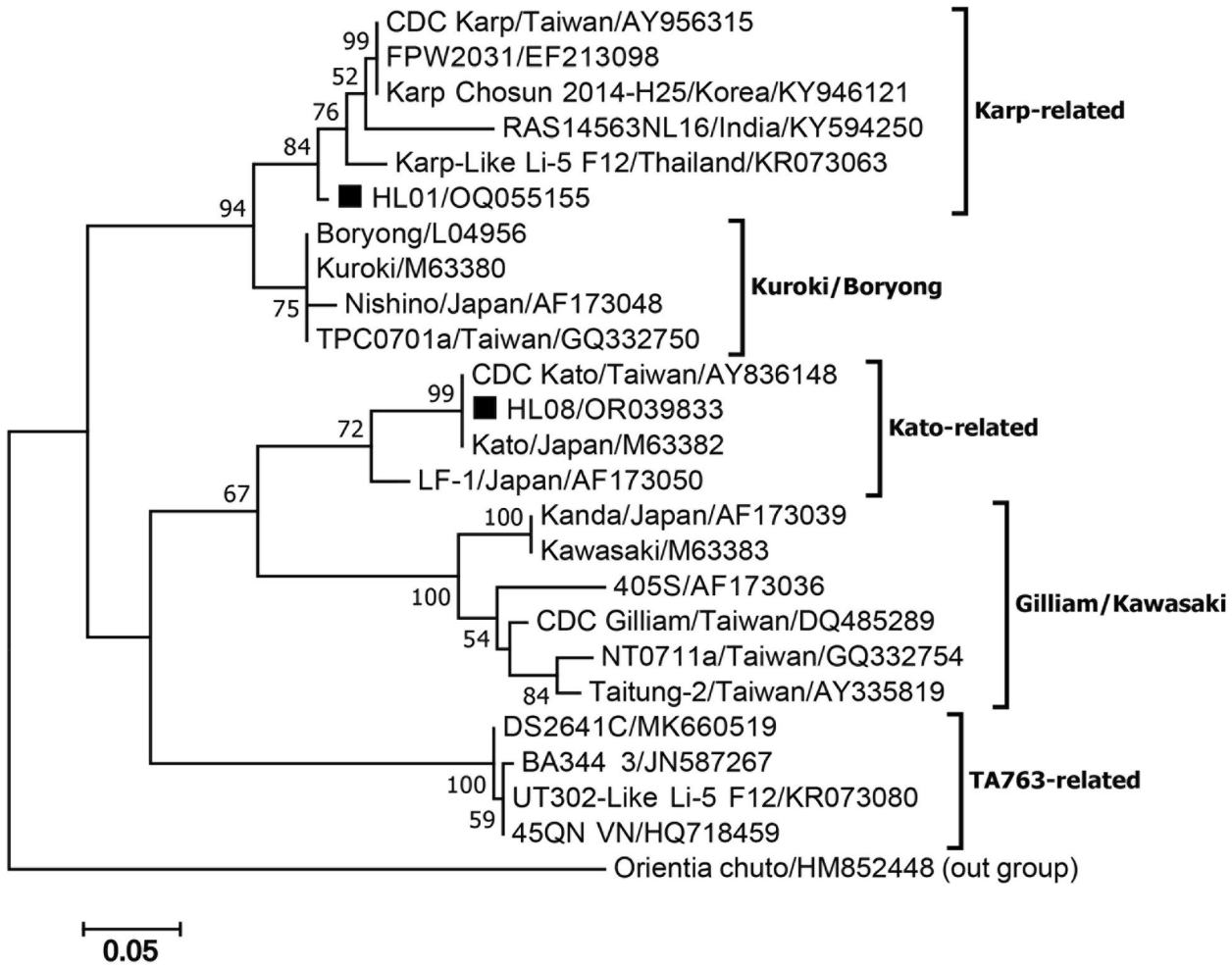

Fig. 5 Phylogenetic tree of *Orientia tsutsugamushi* (HL01, HL08) based on the *56-kDa TSA* gene fragment sequences along with 23 reference sequences. The tree was constructed in MEGA7 software using the maximum likelihood method. The scale bar represents a number of nucleotide substitutions per site

prevalent, and community awareness could be effective measures in the prevention of scrub typhus disease in Mizoram State, Northeast India.

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Authors' contributions
GR, HR and LP have drafted the manuscript and revised the final version. VR has designed methodology, supervised and undertaken data curation. GR, HR, LP, RV and ZR have undertaken the investigation and formal analysis of the work. All authors reviewed the final version of the manuscript.

Funding
Nil.

Data availability
The datasets used in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The present study protocol was reviewed by the Institutional Animal Ethics Committee (IAEC) of Pachhunga University College, Aizawl, Mizoram, India (vide IAEC No. PUC-IAEC-2021-A09, dated 05-07-2021), and approved for this study. Informed consent was also obtained from all the participants (Farmers) to donate their freshly captured (dead) rats for the present study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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