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Payal Pal
Department of Zoology, Immunology and Regenerative Medicine Research Laboratory, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, West Bengal, India

Ena Ray Banerjee
Professor, Department of Zoology, Immunology and Regenerative Medicine Research Laboratory, University of Calcutta, Kolkata, West Bengal, India

Corresponding Author:
Ena Ray Banerjee
Professor, Department of Zoology, Immunology and Regenerative Medicine Research Laboratory, University of Calcutta, Kolkata, West Bengal, India

DNA barcoding-technological advancement to bridge taxonomic identification and molecular systematics in species delineation of Indian spider

Payal Pal and Ena Ray Banerjee

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Abstract
In current years, DNA barcoding has come to be an effective tool in molecular identification of species because it is a simple, quick and specific method. Advances in the molecular branch of biology over the last few decades has reshaped systematic biology and taxonomy. The biological effects of worldwide climate led to increase in the need to identify emerging organisms in order to conserve different species as increasingly their habitats continue to be destroyed.
In this study, through isolation of genomic DNA and amplification of certain mitochondrial region (COI), we have identified a new cryptic species of spider found in West Bengal, India. Although our collected spiders diverged genetically, they did not translate to change in their ecology, morphology or behavior under different environmental conditions which indicates that this technological framework for quick and precise species identification is DNA barcoding.

Keywords: Species identification, mitochondrial cytochrome c oxidase subunit I, COI, Cryptic diversity, Sequencing, Mitochondrial DNA

Introduction
Spiders are an ancient group of arthropods, which have fossil records (Shear, 2011) [25] from the Devonian period [approximately 400 million years ago] (Penney, 2013) [17]. Spiders are a diverse invertebrate group with above 40000 extant species (Turnbull, 1973) [27]. As a result of their very large quantity and high diversity of species, spiders have been cultivated as model organisms in the field of ecology, developmental science, evolutionary science and behavioural science (Hilbrant *et al*., 2012; McGregor *et al*., 2008; Pruitt & Riechert, 2012; Uetz *et al*., 2016) [14, 15, 20, 27]. Theraphosidae [tarantulas] is a spider family that includes nearly 1000 tropical and subtropical species that are among the largest terrestrial arthropods [World Spider Catalog, 2016]. Because of their striking visual appeal and abundance in densely populated areas, they are inextricably linked to human culture. They are important natural predators and they inhabit almost all terrestrial ecosystems (Adis & Harvey, 2000; Shear, 2011) [1, 25]. They are very significant to IPM [Integrated Pest Management], and in the field of horticulture and agriculture ecosystems (Michalko *et al*., 2019; Yang *et al*., 2017) [16, 31]. Spiders act as indicator species and sometimes have been used as biological control-actors for different diseases. Approximately 1686 species of spiders have been currently recorded from India as compared to the global diversity. It can be difficult to identify spiders based on their morphological characteristics, particularly when it comes to juveniles, polymorphism, and sexual dimorphism (Coddington & Levi, 1991) [6]. The adoption of extra technologies that can aid in quick spider identification and clearing up any taxonomic or morphologic ambiguity is crucial in light of these obstacles (Barrett & Hebert, 2005; Raso *et al*., 2014) [2, 21]. Two or more species qualify as cryptic/sibling/hidden species in our eyes when they have superficially morphologically indistinguishable characters. According to some authors, the characteristics of cryptic species should be recent divergence, only recognizable by molecular information, or exist in sympatry, or be isolated reproductively.
There are millions of different animal species, making the animal kingdom a difficult topic for taxonomy. Given this high level of diversity, it is crucial to assess techniques for identifying species and defining species borders (Blaxter, 2004; Godfray, 2002) [3]. Tools based on genetics and DNA are enabling the identification of cryptic species complexes, allowing for more precise and thorough analyses of biodiversity levels, both within and between species.

In order to identify specimens and uncover new species, one of these techniques, DNA barcoding, relies on sequencing and comparison of a standardized section of the genome (Hebert *et al*., 2003; Ratnasingham & Hebert, 2016) [12, 22].

The use of a single gene or genetic area, commonly the cytochrome c oxidase I (coxI) gene of the mitochondrial genome in animals, is referred to as DNA barcoding or DNA taxonomy (Valentini *et al*., 2009) [29]. In summary, it may be said that DNA barcoding or DNA taxonomy represents a spartan approach to the taxonomic business. The DNA barcoding technique now represents the biggest attempt to catalogue biodiversity using molecular methods. The fundamental tenet of DNA barcoding is that highly conserved DNA sequences, whether coding or non-coding areas, only slightly change over the course of a species' evolution. The mitochondrial coxI gene, composed of around 650 base pairs, provides the basis for the DNA barcoding identification method (Hebert *et al*., 2003) [12]. Taxonomists can quickly classify specimens using DNA barcoding by identifying divergent taxa that could be new species (Barrett & Hebert, 2005; Hebert & Gregory, 2005) [2, 13]. The DNA barcode works with fragments, is applicable to all life stages, reveals species that resemble one another, and lessens ambiguity. DNA barcoding uses expertise to aid the quick identification of new species as well as the expedited identification of established organisms. In modern molecular biology, one of the most frequent processes is the extraction of DNA from cells. To release the DNA from the cell into the extraction buffer, the cell membranes must first be damaged. Successful DNA profiling of unknown samples is largely dependent on the quality and the amount of DNA that is recovered from the sample. PCR is efficient because it multiplies the DNA exponentially for each cycle. A PCR cycle takes some minutes and each segment of DNA that is made can serve as a template for new ones. Ancient DNA analysis may also make use of PCR (Cheng *et al*., 1994) [5]. The variety of theraphosids is still poorly understood, and our knowledge of their phylogeny is very primitive. The purpose of this work was to demonstrate that DNA barcoding technology is a simple and useful approach for identifying spider specimens and discovering cryptic species of Indian spiders.

Material and Methods

Specimen collection and their taxonomic identification: In this study, 7 spider specimens were collected from various locations across West Bengal, especially from Belda, Debra, Boral, Howrah and Durgapur. Different collection methods like sweep netting, hand picking, pitfall traps and vegetation beating were used in this study during 2016 to 2018 [Figure 1, Table 1]. The spider specimens were preserved in 90% molecular grade ethanol (Merck) and stored at -20°C in our laboratory. The morphological investigation of all the 7 specimens was done by using an Olympus SZX7 stereomicroscope. Investigations were also conducted on the photographs of the cephalothorax, chelicerae, sternum, genitalia, maxillae, spinneret, palp, labium and claw of suspected cryptic species and potential species complexes [Table 1]. Through the use of previously published morphological keys, data, measurements, and literature, the specimens' enlarged photos were recognised (Sen *et al*., 2012) [24].

Fig 1: Spider specimens were collected from various locations across West Bengal

Table 1: Collection areas of our spider specimens

Spider specimens no.	Date of collection	Location of collection	Latitude and longitude coordinates
T1	25.05.2016(Dead)-[Male]	Belda, West Bengal	22.070757, 87.338417
T2	20.05.2016 (Dead)-[Male]	Debra, West Bengal	22.390611, 87.567306
T3	04.06.2018 (Dead)-[Female]	Boral (Rishi Rajnarayan Pally), West Bengal	22.44774, 88.3652072
T4	07.06.2018 (Dead)-[Male]	Howrah (Bagnan), West Bengal	22.595770, 88.263641
T5	09.06.2018 (Dead)-[Male]	Durgapur (near IQ city), West Bengal	23.533440, 87.321930
T6	09.06.2018 (Live) and 09.07.2018 (Dead)-[Male]	Durgapur (DPL township), West Bengal	23.533440, 87.321930
T7	15.06.2018 (Dead)-[Female]	Howrah (Bagnan), West Bengal	22.595770, 88.263641

Isolation of DNA: The initial step in gathering genetic information suitable for the analysis was total DNA extraction, using the protocol of an extraction kit (Invitrogen). At first, about 20 mg of samples [one leg of each sample was used] were taken and the ethanol was evaporated at room temperature. Then, 20µL Proteinase K and 180µL PureLink Genomic Digestion Buffer was added. The solution was then thoroughly blended using a vortex before being incubated at 50 °C overnight. To eliminate any particle materials, the lysate was centrifuged at maximum speed for 3 minutes at room temperature, and then transferred to a fresh, sterile microcentrifuge tube. Following a quick vortex, 20µL of RNase A was added to the lysate, which was then let to sit at room temperature for 2 minutes. PureLink Genomic Lysis/Binding Buffer (200µL) was then added and mixed well by vortexing. The lysate was then mixed thoroughly by vortexing for 5 seconds with 200 µL of 96-100% ethanol to produce a homogeneous solution. Genomic Lysis/Binding Buffer, ethanol, and the lysate [~640 µl] were all added to the PureLink Spin Column. At room temperature, the column was centrifuged for one minute at 10,000×*g*. The spin column was put into a clean PureLink collection tube that was included with the kit, and the collection tube was discarded. After adding 500µL of Wash Buffer 1, the column was centrifuged at 10,000×*g* for one minute at room temperature. The spin column was put into a fresh PureLink collection tube once and the collection tube had been discarded once more. At room temperature, the column was centrifuged at maximum speed for three minutes after receiving 500µL of Wash Buffer 2 and ethanol. The spin column was put in a sterile 1.5 ml microcentrifuge tube, the collection tube was discarded, and 50 µL of nuclease-free water was added to the column. For one minute, the tube was left at room temperature. The column was then removed after being centrifuged for one minute at its top speed at room temperature. The tube contained the purified genomic DNA, which was then stored at -20 °C.

DNA amplification by PCR: The extracted DNA, 5 U/µl Taq DNA polymerase, 50 mM $MgCl_2$, 10X PCR buffer, 10 mM dNTP mix, 10 µM forward primer [InvCOIF: 5'-GGTCAACAAATCATAAAGATATTGG-3'], 10 µM reverse primer [InvCOIR: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'], and nuclease-free water were combined to create the PCR reaction mixture. The DNA was amplified in a thermal cycler (Applied Biosciences), using the following thermal profile: initial denaturation for 1 min at 94 °C, followed by 1 cycle of 30 s at 95 °C, subsequent annealing for 30 s at 50 °C, and subsequent extension for 35 cycles of 45 sec at 72 °C, and final extension for 10 min at 72 °C.

Gel electrophoresis analysis of the PCR product: The PCR product was visualized by agarose gel electrophoresis, using 1% agarose (Himedia). The gel was run for approximately for 30 minutes at 120V. Finally, the gel was observed using UV transillumination and photographed using photodocumentary system (Bio Rad). The bands were compared with a 100 bp marker (Himedia). The amplified PCR products were purified using the QIAquick Gel Extraction Kit [Qiagen, Valencia, CA] in accordance with the manufacturer's instructions after being checked on 1% agarose gel.

Sequencing: The GENET BIO DNA purification kit was used to clean up the PCR products, and the Big Dye Terminator ver. 3.1 Cycle Sequencing Kit of Applied Biosystems, Inc. was used to perform bi-directional sequencing on the results. The sequencing lab at the Bose Institute in Kolkata, India, sequenced the PCR products. On the ABI Multiplex Thermal Cycler, 3.2 picomoles of both forward and reverse PCR primer pairs were used with the following thermal profile: 95 °C for 1 minute, followed by 25 cycles of 95 °C for 10 seconds, 50 °C for 5 seconds, and a final extension at 60 °C for 4 minutes. Applied Biosystems Inc.'s Big Dye X-terminator kit was used to clean the cycle sequencing products, and the 24 capillary ABI 3500 XL Genetic Analyzer was used to sequence the results. The SeqScape software version 2.7 of Applied Biosystems Inc. was used to check both the forward and reverse chromatograms in order to acquire the consensus sequences.

Data Analysis for Species identification: Sequences were viewed, trimmed, contiged and aligned using the MEGA7 software and the identity of each of the resulting sequences were checked using BLAST program [https://blast.ncbi.nlm.nih.gov] in the NCBI website. The sequences were also run through an ORF finder [https://www.ncbi.nlm.nih.gov/orffinder/] to check for and confirm the lack of nucleotide gaps, indel [insertion/deletions] and stop codons. The sequences of the same gene isolated from different species were downloaded, which showed to be the most closely associated of the two samples under study, as revealed by BLAST from the NCBI website. Multiple sequence alignment of all the samples was performed using MUSCLE program in MEGA 10 software. Neighbor joining and Maximum Likelihood tree based phylogenetic analysis were conducted as these have greatest potential for computationally intensive analysis based on large datasets (Elias *et al*., 2007) [7]. All the analysis was performed considering bootstrap value of 1000. To obtain accession numbers, the produced sequences were uploaded to GenBank using the Bankit submission programme [https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank].

Results and Discussions

Morphological identification: The spiders in our study showed following morphological characteristics [Table 2, Table 3]. In case of specimen T1, T2, T4, T7 the anterior median eyes [AME] were bigger than anterior lateral eyes [ALE], which were bigger than posterior median eyes [PME]. The posterior median eyes were bigger than posterior lateral eyes [PLE]. The patella and tibia of leg I were shorter than the cephalothorax, which was longer than both the metatarsus and tarsus of leg IV. Each tarsal claw was with 3 denticles. The prolateral side of maxillae was with few thorn setae and a row of paddle setae. Chelicerae has prominent spinules on the retrolateral side. The palp bulb was oblong, with an embolus that was long, broad, and flat before abruptly narrowing near the tip. Its prolateral superior and inferior keels were visible, and it was twisting inward close to the base [figure 2-7; figure 11-13; figure 20-22]. Eye diameter of specimen T3 was AME>ALE>PME>PLE; the specimen no. T3's cephalothorax was longer than the patella and tibia of leg I and shorter than the patella and tarsus of leg IV. Tarsal claw 2 had three denticles on each side. The retrolateral side of the chelicerae of specimen T3 had prominent spinules, while the prolateral side of the maxillae had a row of paddle setae and a few thorn setae. The palp bulb had a pear-like shape, a long, flat, broad embolus, and was internally twisted close to the base. Prolateral superior and inferior keels were also visible [figure 8-10]. In case of specimen T5, T6 the eye diameter was ALE>AME=PLE>PME. The patella and tibia of leg I were shorter than the cephalothorax, which was also longer than the metatarsus and tarsus of leg IV. Each tarsal claw was with 2 denticles. The anterior 3/4th of metatarsus IV was without scapulae; a row of paddle setae and a few thorn setae could be seen on the prolateral side of the maxillae, and distinct spinules could be seen on the retrolateral side of the chelicerae. Male palp bulbs are globose, compressed at both ends, and have long, straight emboli that eventually narrow and point upward. The morphospecies in two genera and two families were used to represent our samples [Table 4]. The specimens were identified morphologically up to species level but they have unavailability in their morphological data for sub-adults and juveniles. Therefore, our study generated the DNA sequences to do DNA barcode of the morphospecies for further analysis.

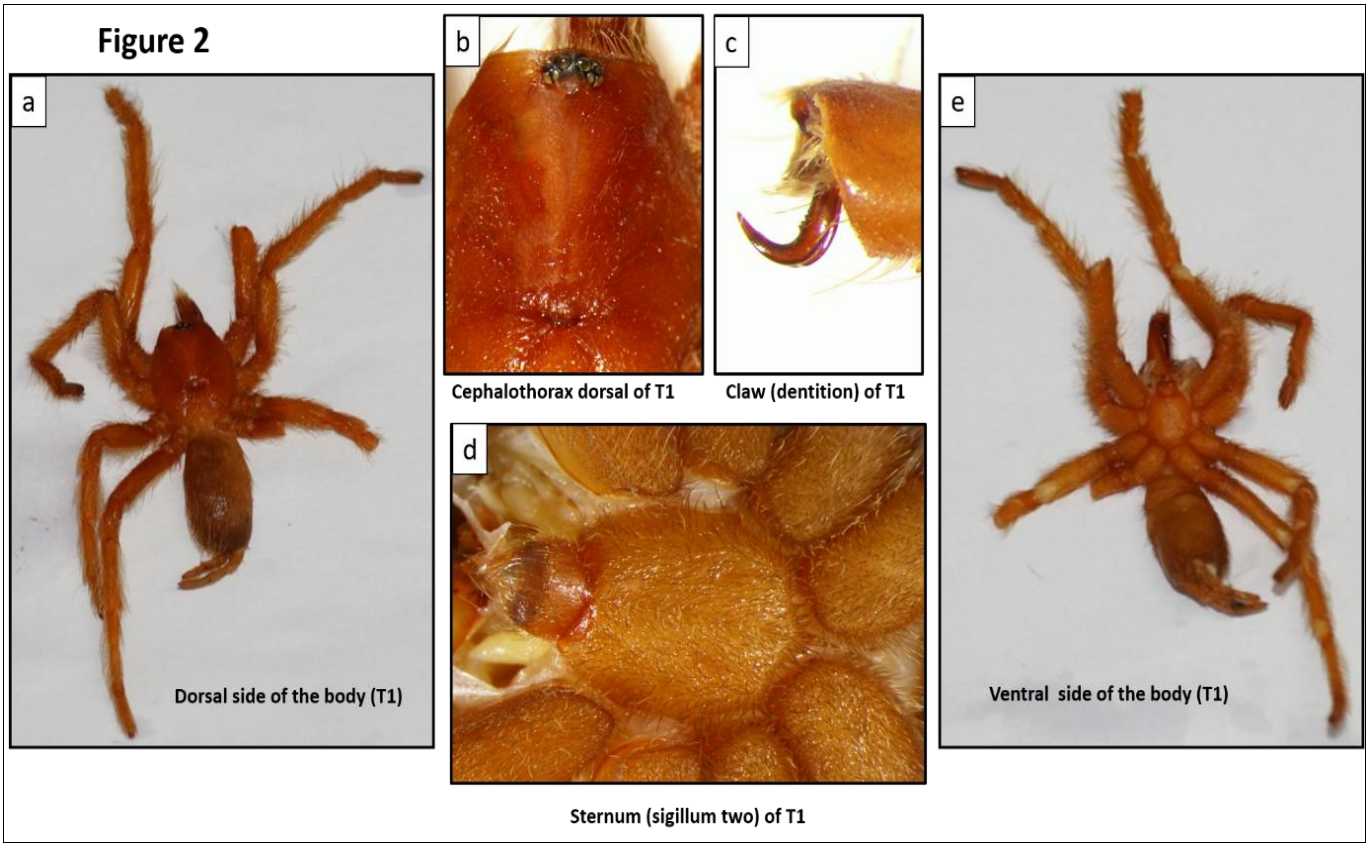

Fig 2: Different body parts of T1 specimen- a) Dorsal side of the body (T1), b) Cephalothorax dorsal of T1, c) Claw (dentition) of T1, d) Sternum (sigillum two) of T1, e) Ventral side of the body (T1) were observed under Olympus SZX7 stereomicroscope

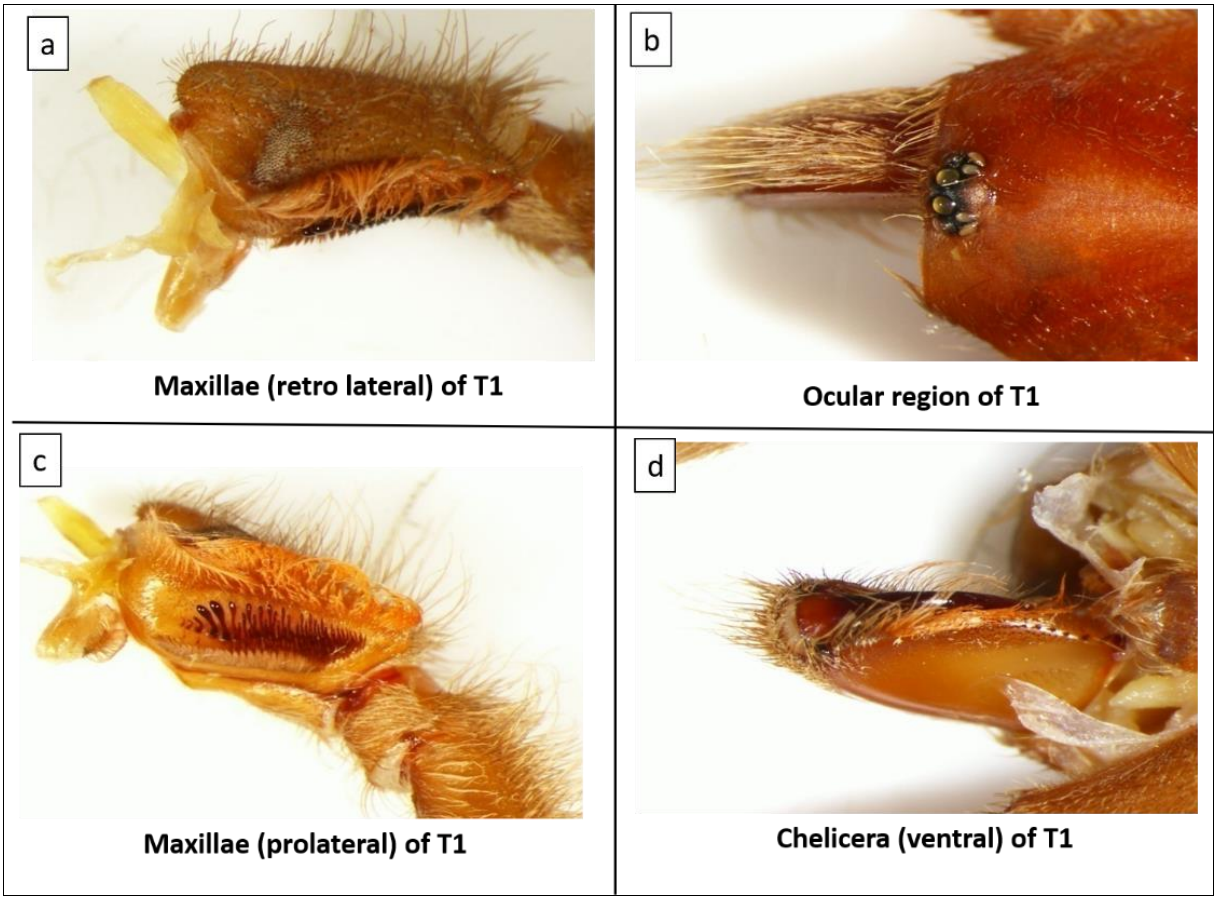

Fig 3: Different body parts of T1 specimen- a) Maxillae (retro lateral) of T1, b) Ocular region of T1, c) Maxillae (prolateral) of T1, d) Chelicera (ventral) of T1 were observed under Olympus SZX7 stereomicroscope.

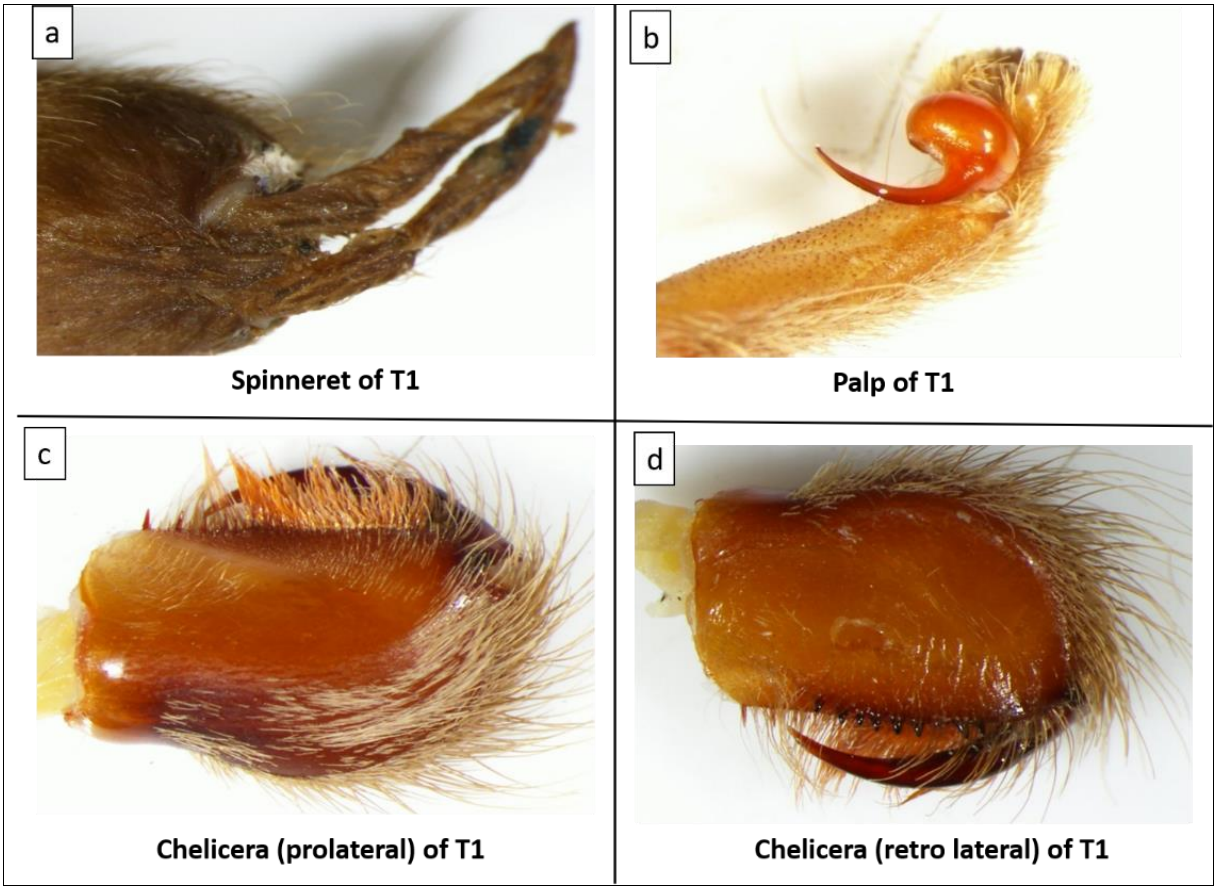

Figure 4: Different body parts of T1 specimen- a) Spinneret of T1, b) Palp of T1, c) Chelicera (prolateral) of T1, d) Chelicera (retro lateral) of T1 were observed under Olympus SZX7 stereomicroscope.

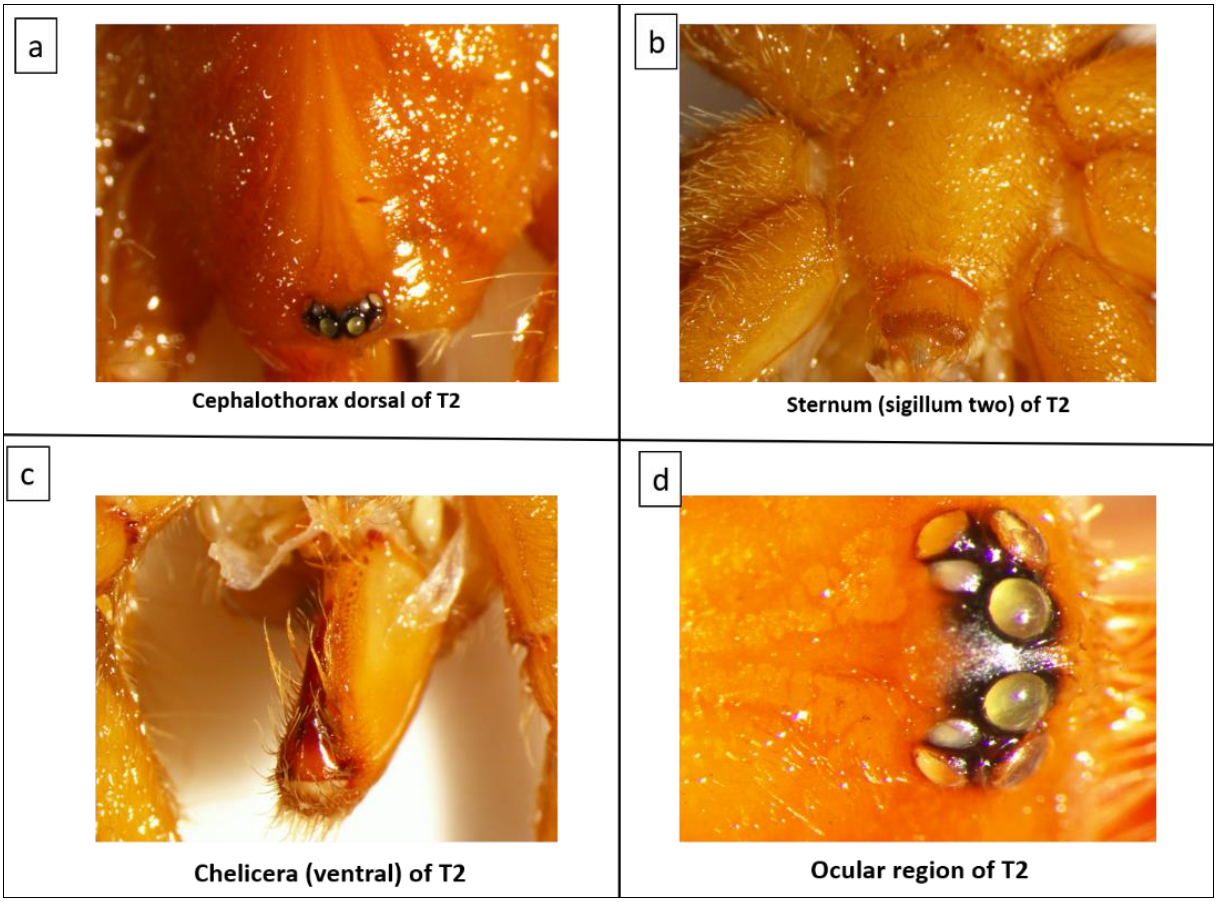

Fig 5: Different body parts of T2 specimen- a) Cephalothorax dorsal of T2, b) Sternum (sigillum two) of T2, c) Chelicera (ventral) of T2, d) Ocular region of T2 were observed under Olympus SZX7 stereomicroscope.

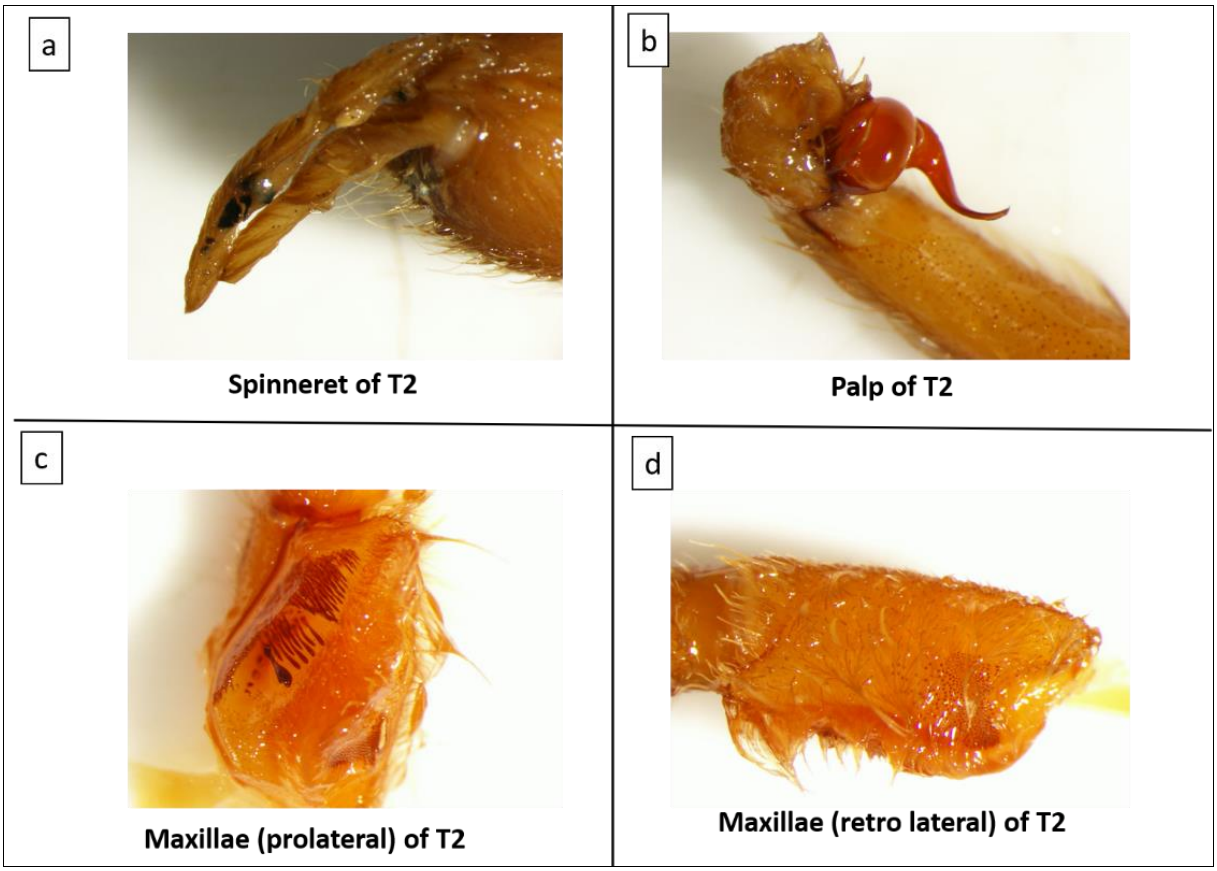

Fig 6: Different body parts of T2 specimen- a) Spinneret of T2, b) Palp of T2, c) Maxillae (prolateral) of T2, d) Maxillae (retro lateral) of T2 were observed under Olympus SZX7 stereomicroscope.

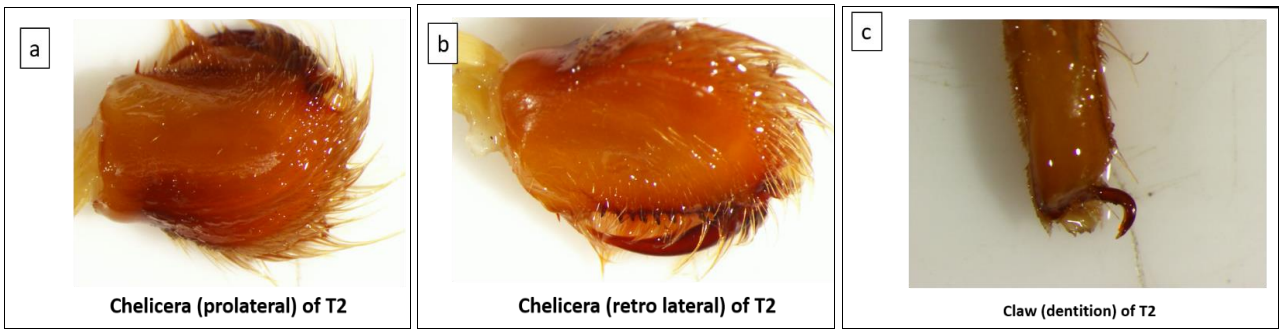

Fig 7: Different body parts of T2 specimen- a) Chelicera (prolateral) of T2, b) Chelicera (retro lateral) of T2, c) Claw (dentition) of T2 were observed under Olympus SZX7 stereomicroscope.

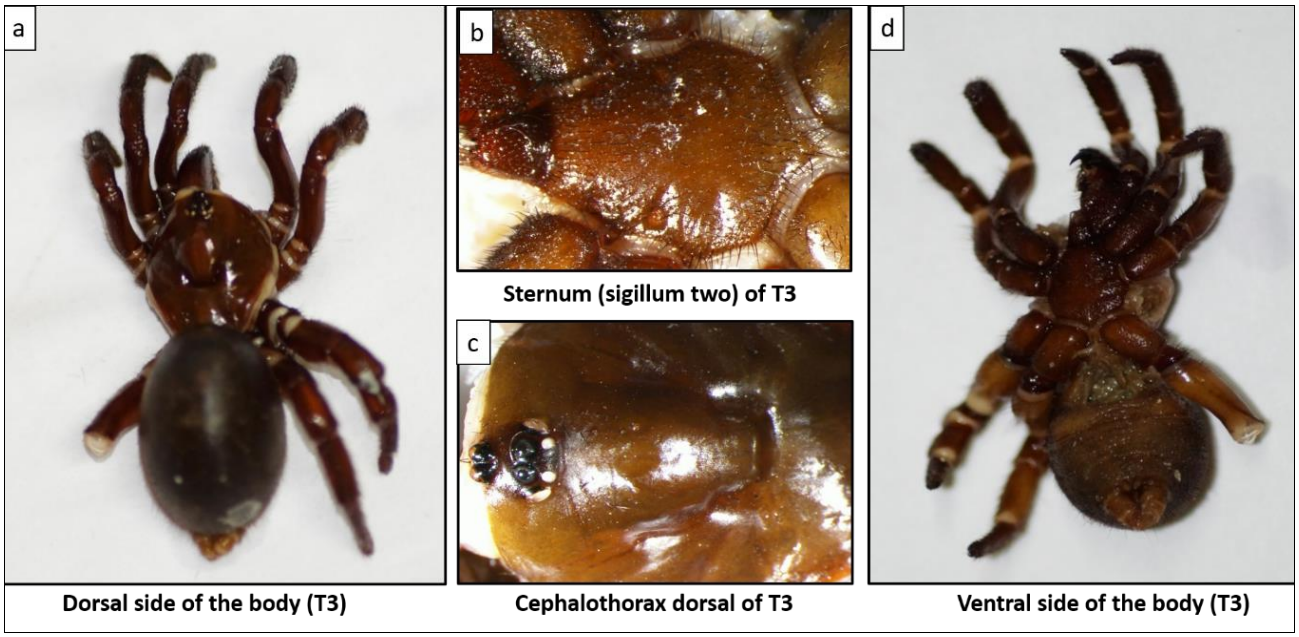

Fig 8: Different body parts of T3 specimen- a) Dorsal side of the body (T3), b) Sternum (sigillum two) of T3, c) Cephalothorax dorsal of T3, d) Ventral side of the body (T3) were observed under Olympus SZX7 stereomicroscope.

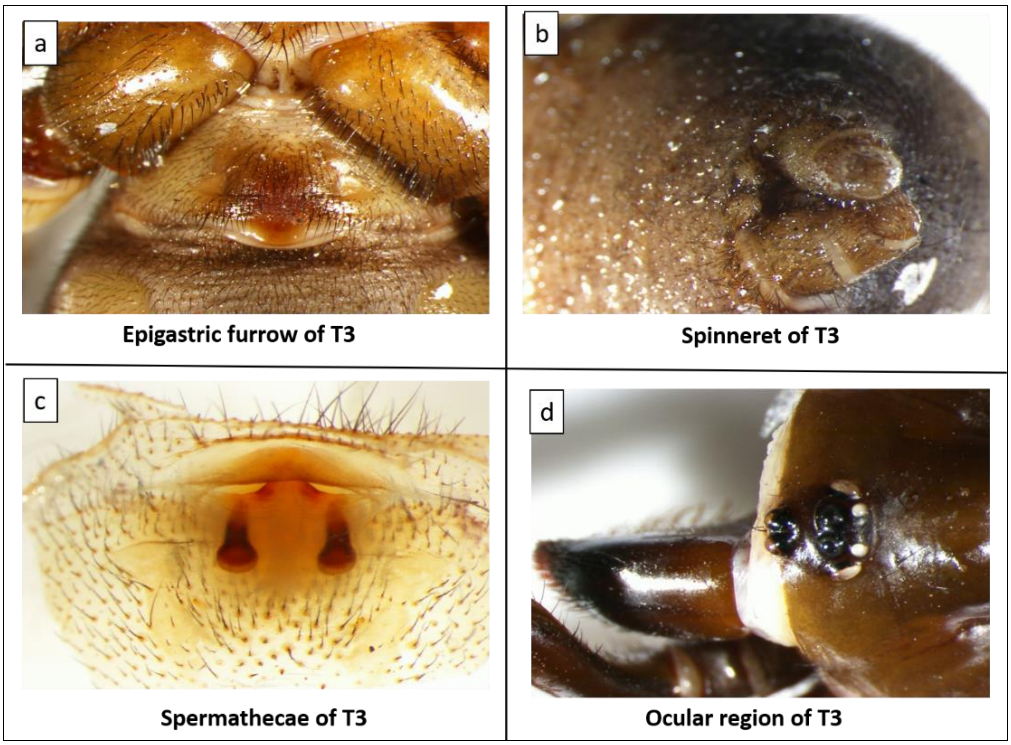

Fig 9: Different body parts of T3 specimen- a) Epigastric furrow of T3, b) Spinneret of T3, c) Spermathecae of T3, d) Ocular region of T3 were observed under Olympus SZX7 stereomicroscope.

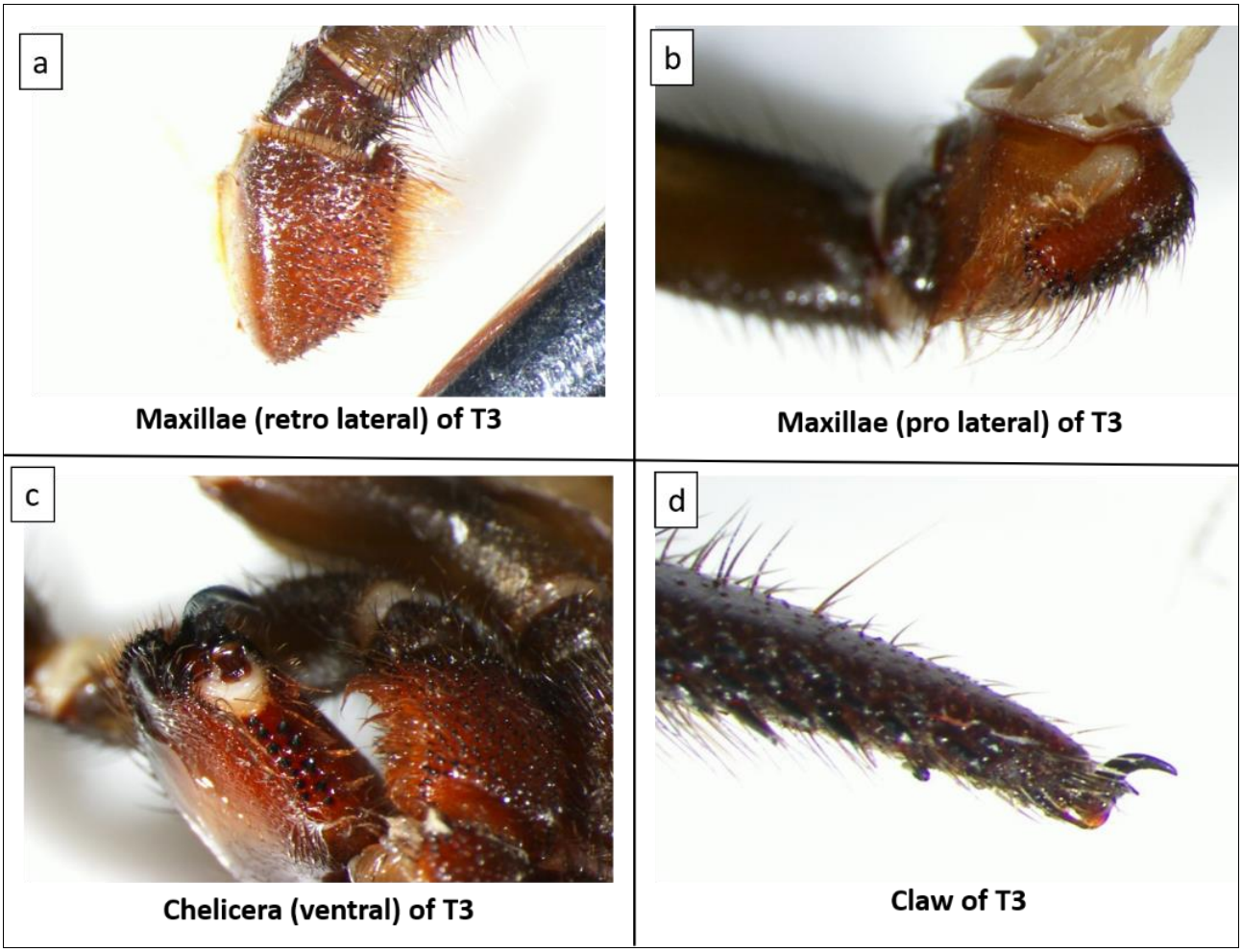

Fig 10: Different body parts of T3 specimen- a) Maxillae (retro lateral) of T3, b) Maxillae (pro lateral) of T3, c) Chelicera (ventral) of T3, d) Claw of T3 were observed under Olympus SZX7 stereomicroscope.

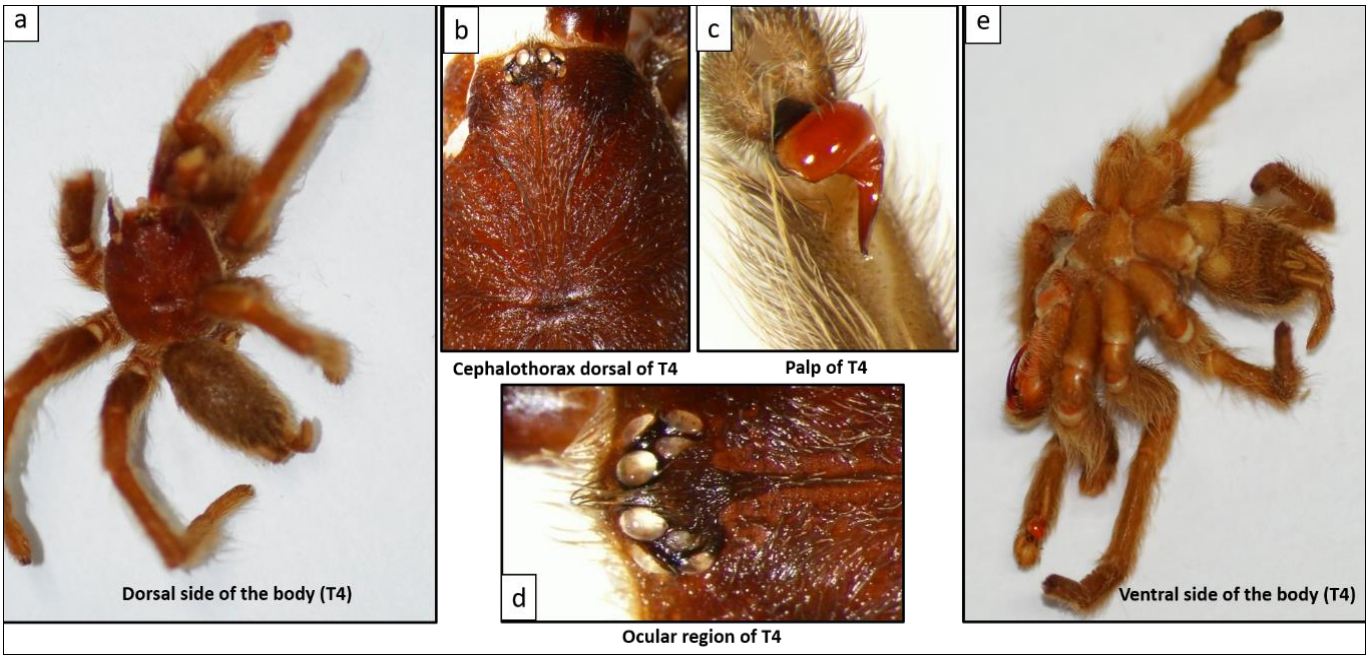

Fig 11: Different body parts of T4 specimen- a) Dorsal side of the body (T4), b) Cephalothorax dorsal of T4, c) Palp of T4, d) Ocular region of T4, e) Ventral side of the body (T4) were observed under Olympus SZX7 stereomicroscope.

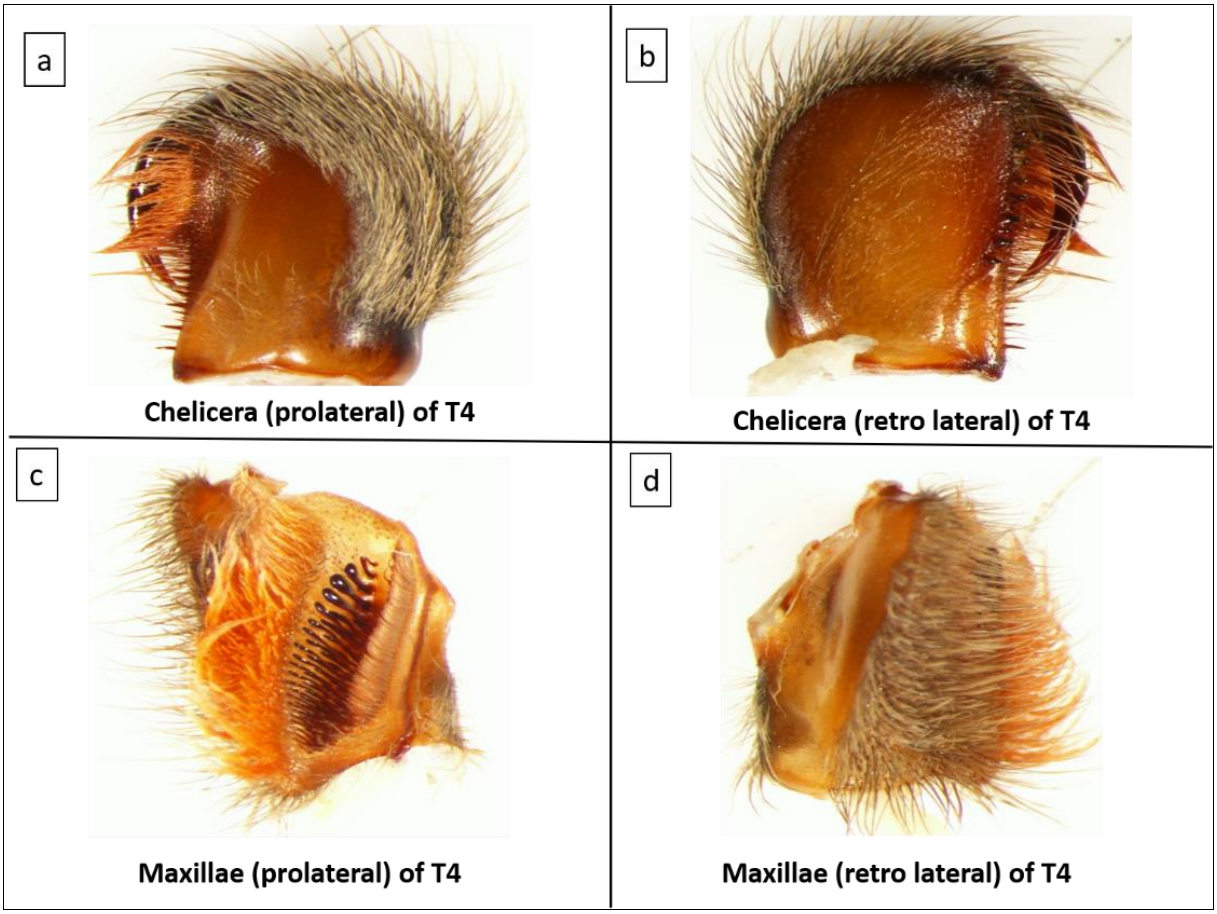

Fig 12: Different body parts of T4 specimen- a) Chelicera (prolateral) of T4, b) Chelicera (retro lateral) of T4, c) Maxillae (prolateral) of T4, d) Maxillae (retro lateral) of T4 were observed under Olympus SZX7 stereomicroscope.

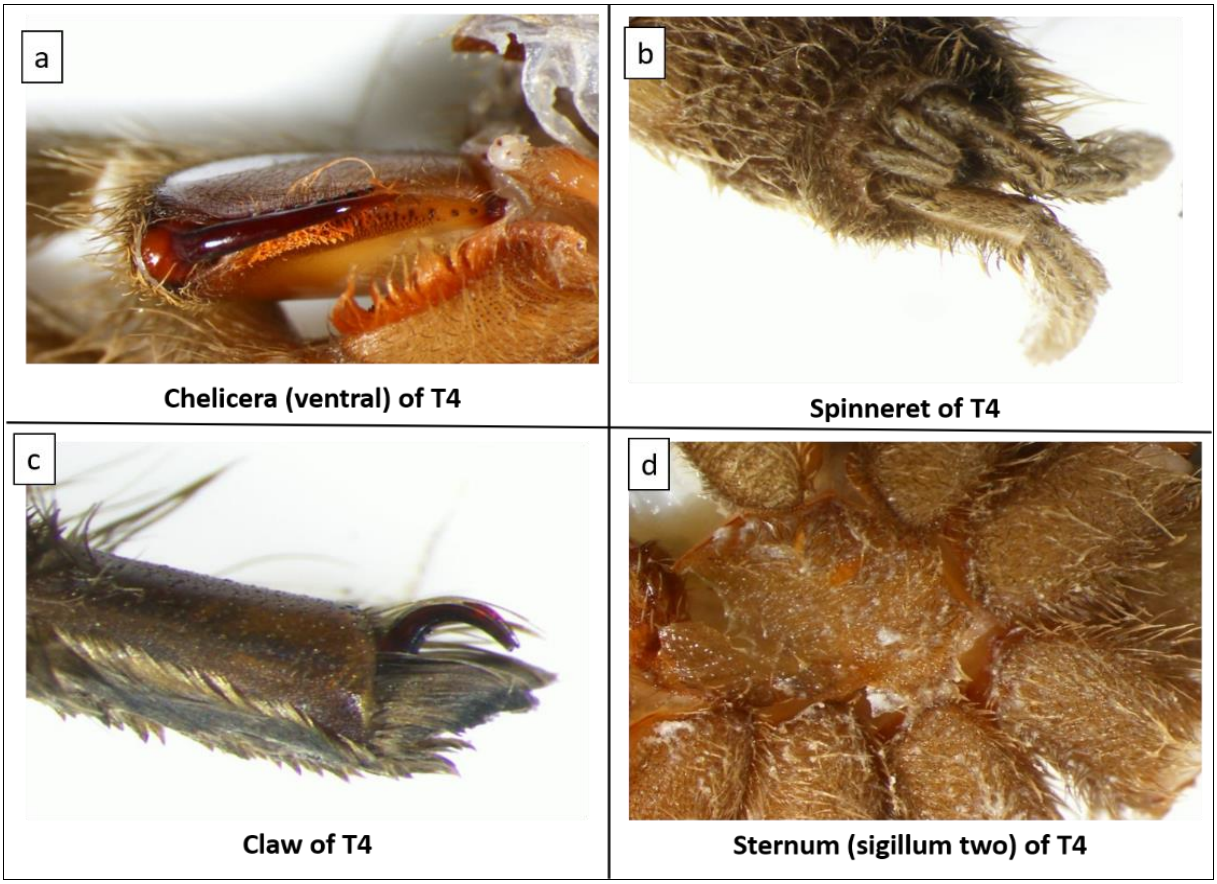

Fig 13: Different body parts of T4 specimen- a) Chelicera (ventral) of T4, b) Spinneret of T4, c) Claw of T4, d) Sternum (sigillum two) of T4 were observed under Olympus SZX7 stereomicroscope.

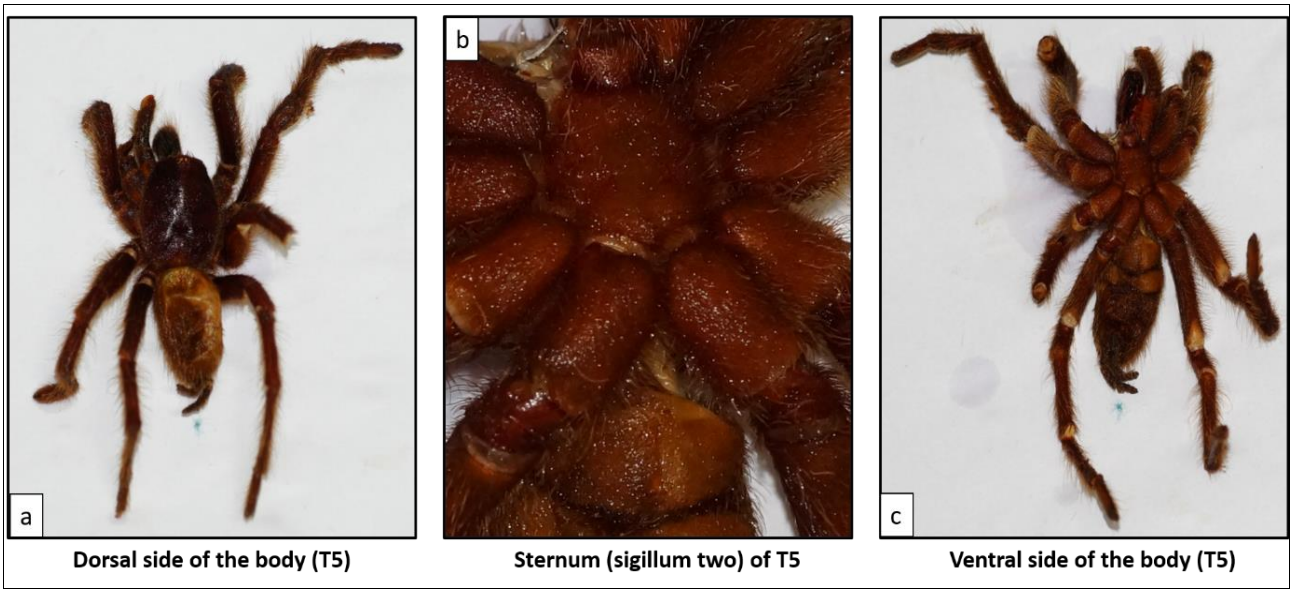

Fig 14: Different body parts of T5 specimen- a) Dorsal side of the body (T5), b) Sternum (sigillum two) of T5, c) Ventral side of the body (T5) were observed under Olympus SZX7 stereomicroscope.

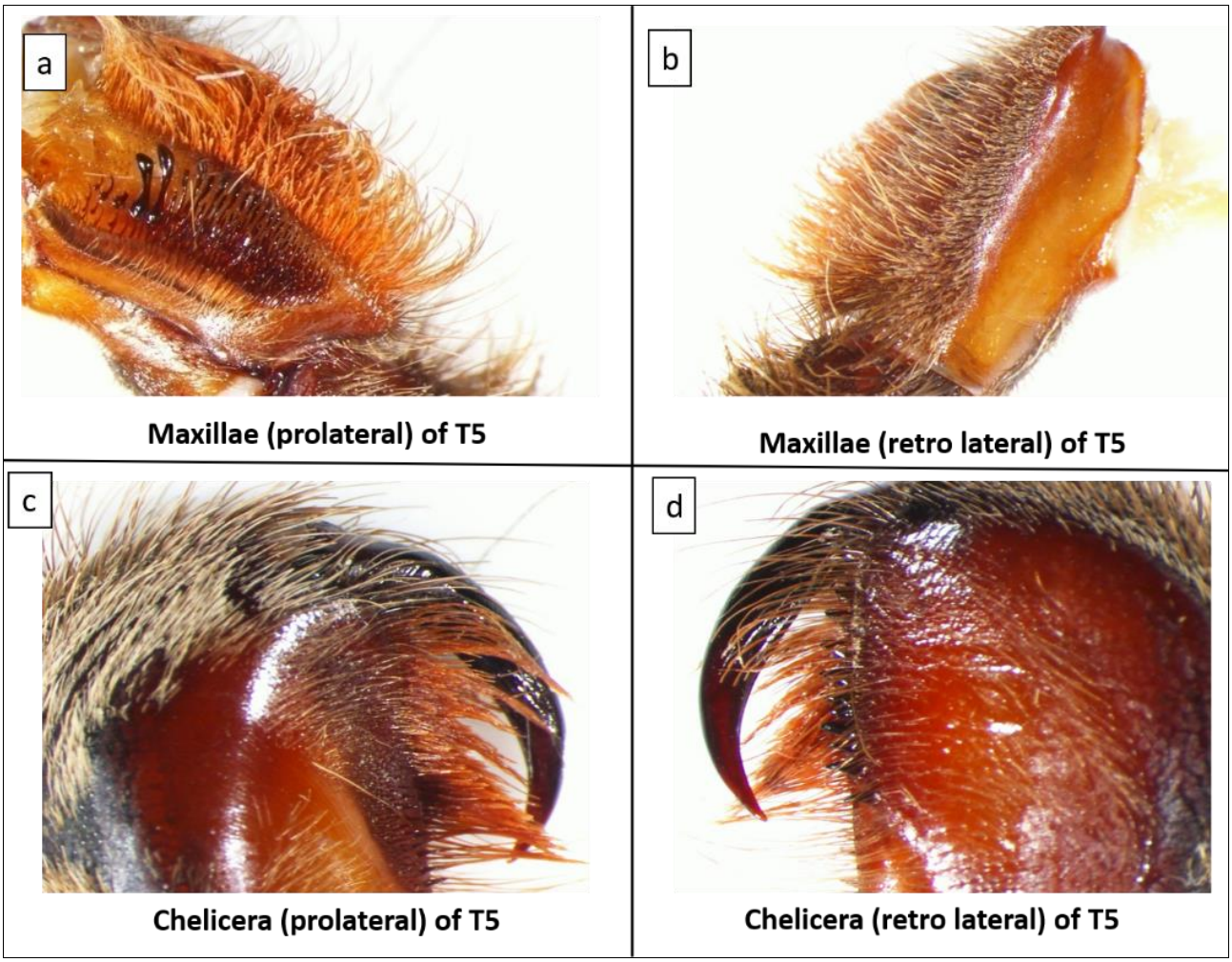

Fig 15: Different body parts of T5 specimen- a) Maxillae (prolateral) of T5, b) Maxillae (retro lateral) of T5, c) Chelicera (prolateral) of T5, d) Chelicera (retro lateral) of T5 were observed under Olympus SZX7 stereomicroscope

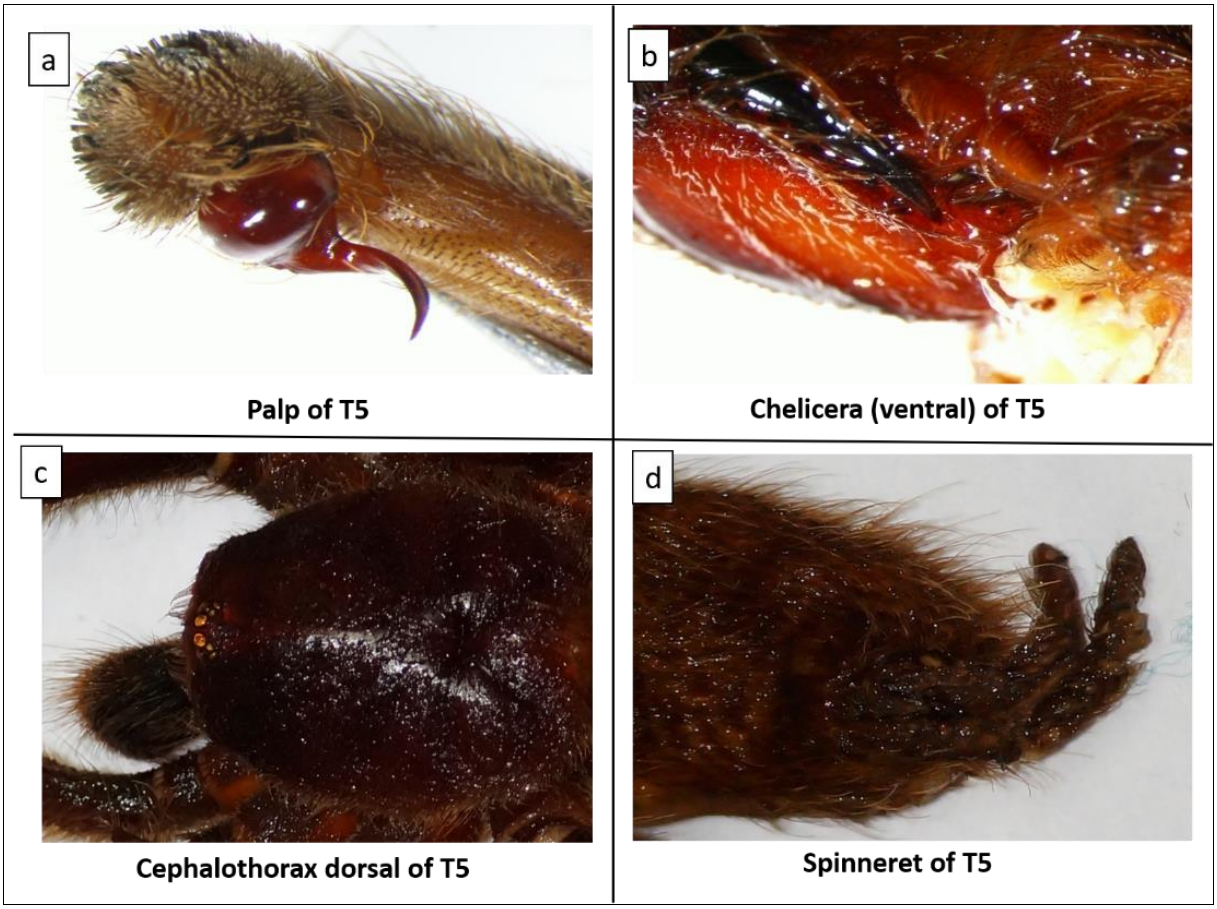

Fig 16: Different body parts of T5 specimen- a) Palp of T5, b) Chelicera (ventral) of T5, c) Cephalothorax dorsal of T5, d) Spinneret of T5 were observed under Olympus SZX7 stereomicroscope.

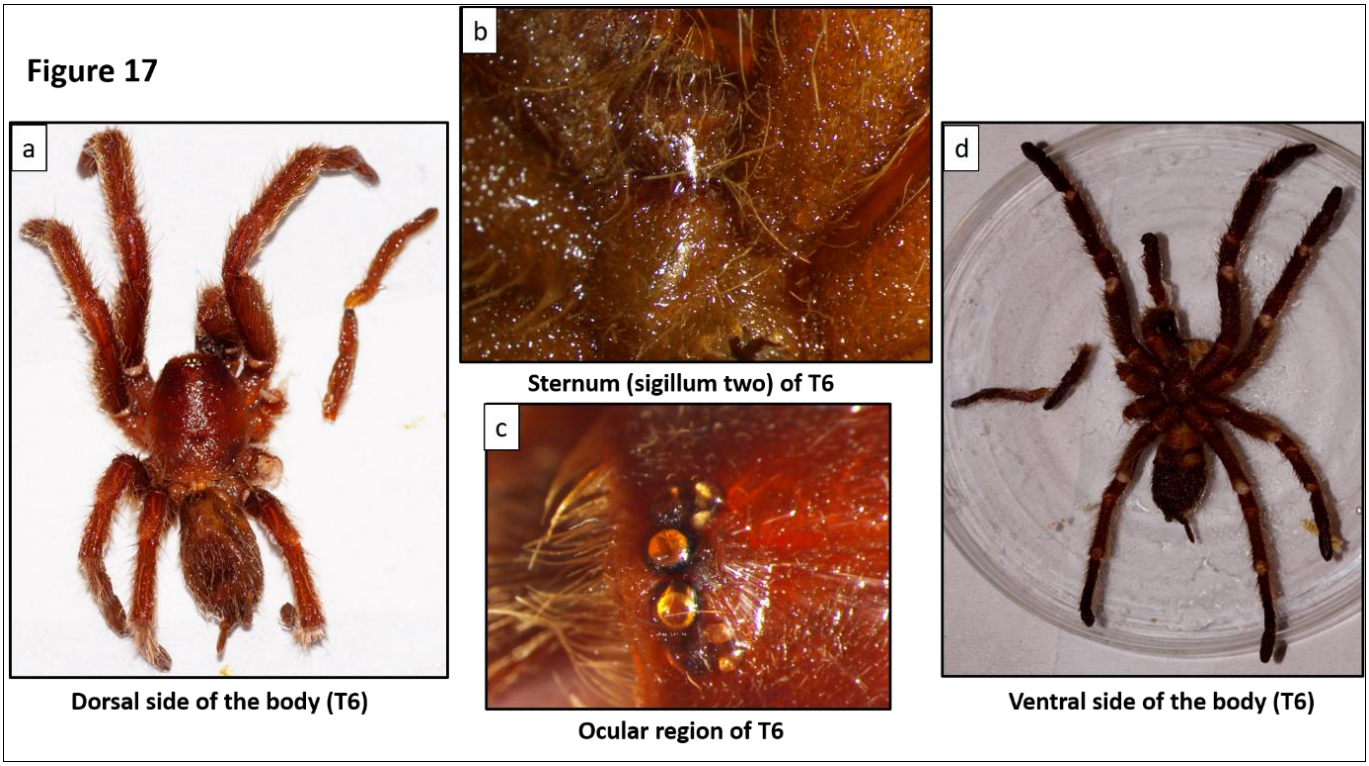

Fig 17: Different body parts of T6 specimen- a) Dorsal side of the body (T6), b) Sternum (sigillum two) of T6, c) Ocular region of T6, d) Ventral side of the body (T6) were observed under Olympus SZX7 stereomicroscope.

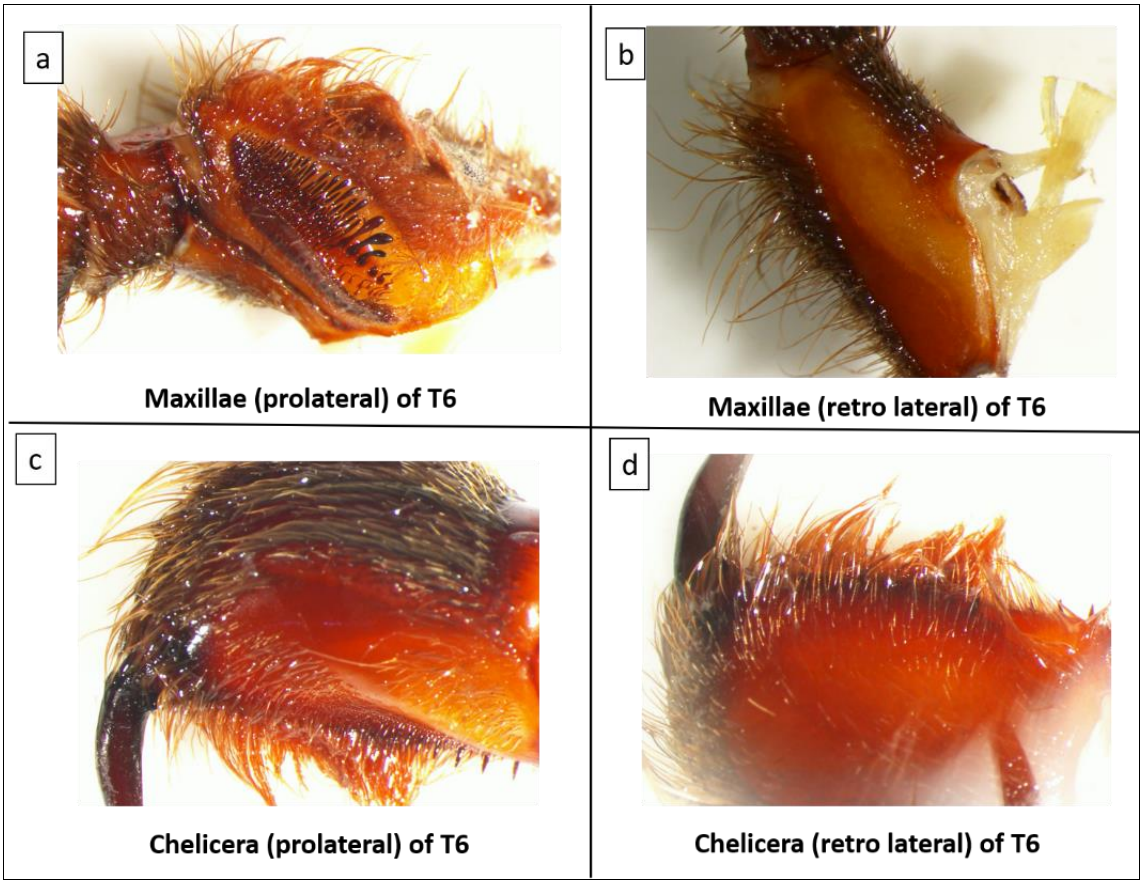

Fig 18: Different body parts of T6 specimen- a) Maxillae (prolateral) of T6, b) Maxillae (retro lateral) of T6, c) Chelicera (prolateral) of T6, d) Chelicera (retro lateral) of T6 were observed under Olympus SZX7 stereomicroscope.

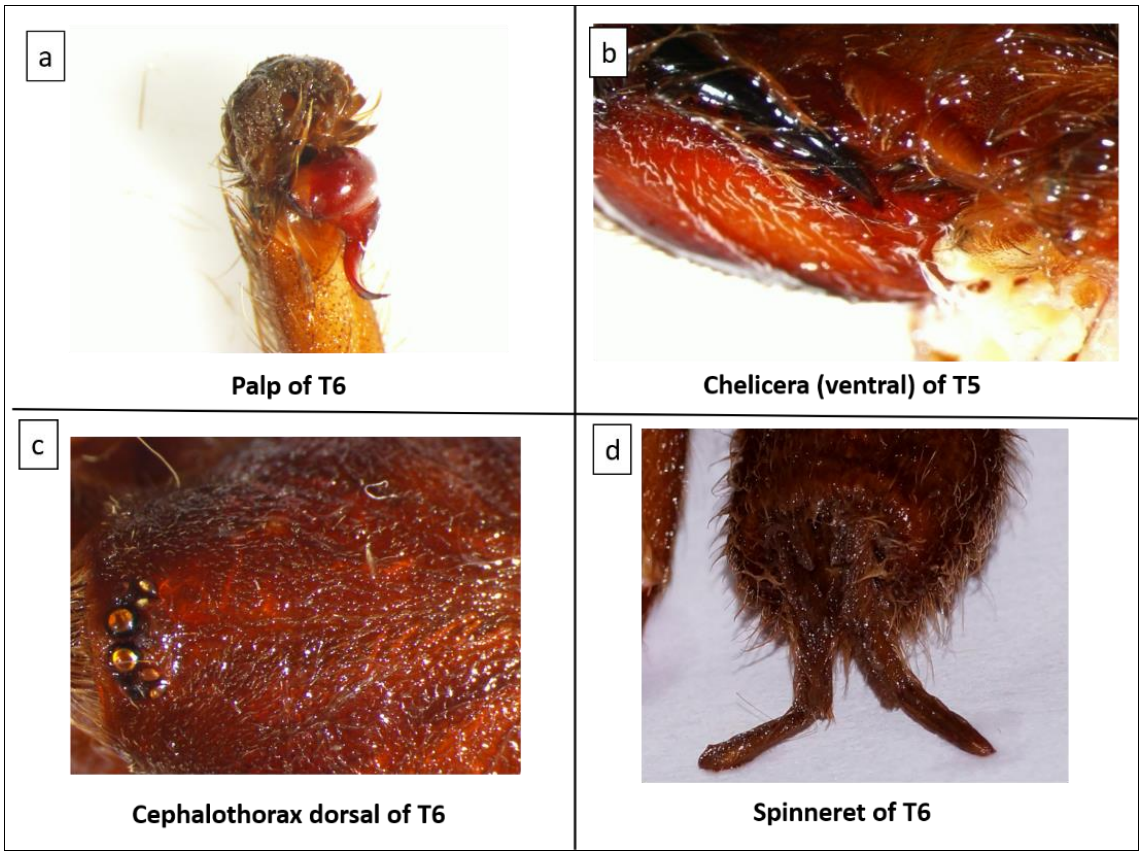

Fig 19: Different body parts of T6 specimen- a) Palp of T6, b) Chelicera (ventral) of T6, c) Cephalothorax dorsal of T6, d) Spinneret of T6 were observed under Olympus SZX7 stereomicroscope.

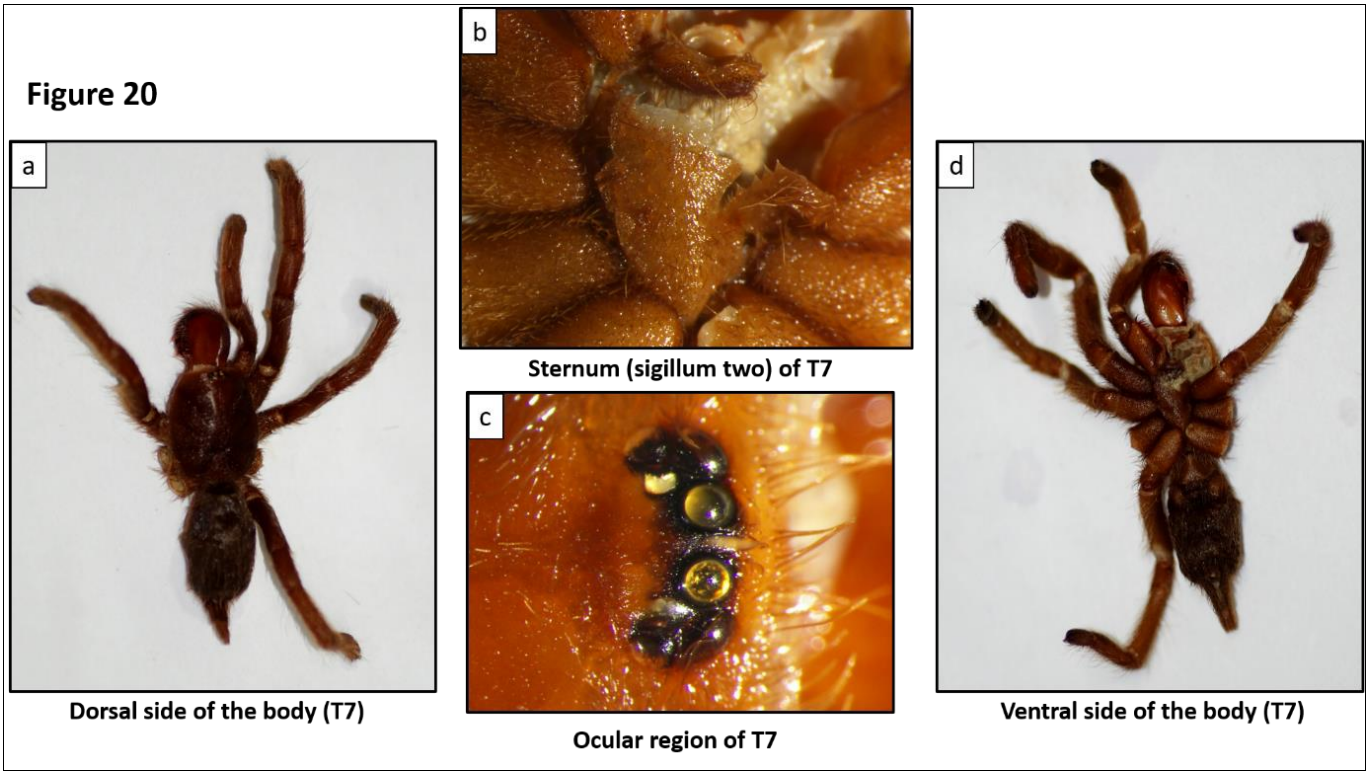

Fig 20: Different body parts of T7 specimen- a) Dorsal side of the body (T7), b) Sternum (sigillum two) of T7, c) Ocular region of T7, d) Ventral side of the body (T7) were observed under Olympus SZX7 stereomicroscope.

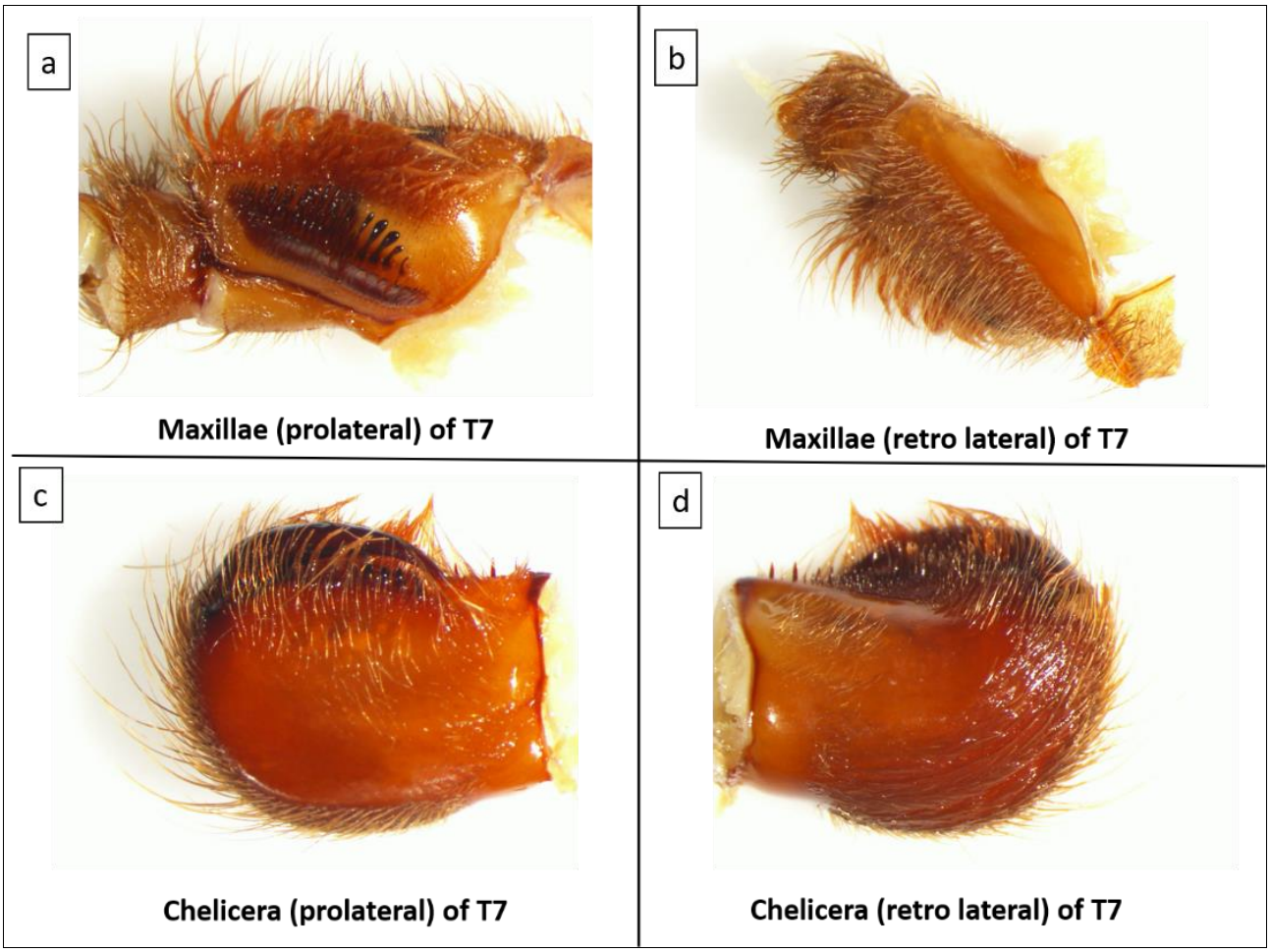

Fig 21: Different body parts of T7 specimen- a) Maxillae (prolateral) of T7, b) Maxillae (retro lateral) of T7, c) Chelicera (prolateral) of T7, d) Chelicera (retro lateral) of T7 were observed under Olympus SZX7 stereomicroscope.

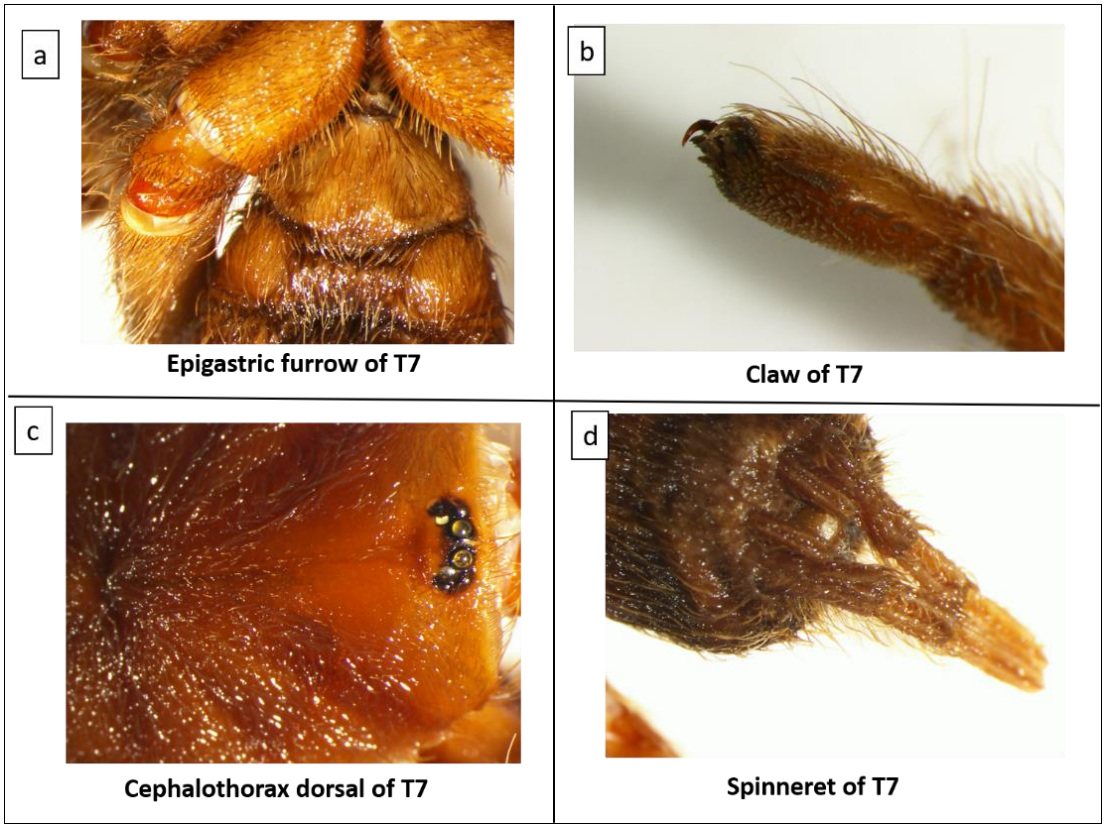

Fig 22: Different body parts of T7 specimen- a) Epigastric furrow of T7, b) Claw of T7, c) Cephalothorax dorsal of T7, d) Spinneret of T7 were observed under Olympus SZX7 stereomicroscope.

Table 2: Morphological characteristics of the spider samples: The measurements indicated in the text are in millimeters, made with an eye piece graticule. Abbreviations used: CL= Cephalothorax length, CW= Cephalothorax width, AL= Abdominal length, AW= Abdominal width, TL= Total length, AME= Anterior median eyes, ALE= Anterior lateral eyes, PME= Posterior median eyes, PLE= Posterior lateral eyes.

Spider specimens no.	CL (mm)	CW (mm)	AL (mm)	AW (mm)	TL (mm)	Eye group (mm)	Eye diameter			
							AME (mm)	ALE (mm)	PME (mm)	PLE (mm)
T1	10.5	8.3	11.2	5.68	21.7	L= 1, W= 1.7	0.39	0.36	0.32	0.29
T2	10.18	8	10.81	5.1	20.99	L= 1.02, W= 1.63	0.37	0.35	0.31	0.28
T3	9.97	9.4	12.4	10.5	22.44	L= 1.79, W= 2.2	0.24	0.5	0.2	0.4
T4	9.22	7.25	9.79	5.16	19.02	L= 0.93, W= 1.48	0.34	0.31	0.28	0.26
T5	17.4	14.68	19.51	10.35	36.9	L= 1.67, W= 3.2	0.46	0.7	0.23	0.46
T6	15.14	11.9	16.9	8.4	31.9	L= 1.45, W= 2.85	0.4	0.61	0.2	0.4
T7	10	9.5	10.6	5.7	20.6	L= 1, W= 1.61	0.37	0.34	0.3	0.28

Table 3: Morphological characteristics of the spider samples: The measurements indicated in the text are in millimeters, made with an eye piece graticule. Abbreviations used: PMS= Posterior median spinnerets, PLS= Posterior lateral spinnerets.

Spider specimens no.	Chelicerae		Spinneret length		Legs			
	Length (mm)	Width (mm)	PMS (mm)	PLS (mm)	I (mm)	II (mm)	III (mm)	IV (mm)
T1	6.36	4.41	2.58	9.9	28.43	24.95	21.99	27.39
T2	6.13	4.25	2.49	9.54	27.40	24.05	21.1	26.4
T3	4.93	2.99	0.61	3.95	16.12	15.17	13.57	22.19
T4	5.55	3.85	2.26	8.65	24.83	21.7	19.21	23.9
T5	10.49	7.14	2.96	10.04	52.77	40.3	35.67	45.01
T6	9.1	6.2	2.57	8.7	45.9	35	31	39.14
T7	6.02	4.17	2.45	9.38	26.9	23.63	20.8	25.94

Table 4: Our specimens were represented by the morphospecies in two genera under two families.

Spider specimens no.	Possible cryptic species or possible species complexes	Family
T1	*Chilobrachys hardwickei*	Theraphosidae
T2	*Chilobrachys hardwickei*	Theraphosidae
T3	*Heligmomerus barkudensis*	Idiopidae
T4	*Chilobrachys hardwickei*	Theraphosidae
T5	*Chilobrachys himalayensis*	Theraphosidae
T6	*Chilobrachys himalayensis*	Theraphosidae
T7	*Chilobrachys hardwickei*	Theraphosidae

Results of PCR products by gel electrophoresis with COIF/COIR primers: The discovery of molecular and genetic techniques has opened up new avenues for studying the species delimitation issue. These techniques are less reliant on the availability of particular materials and are become more affordable as sequencing prices come down. In this study, we have successfully isolated DNA from unknown spider samples using Invitrogen DNA extraction kit. These samples were tissue samples collected from the legs of the unknown spiders. PCR of these samples was successful in all the cases. Mitochondrial Cytochrome C Oxidase subunit I [COI] is the more widely used barcoding markers for animals (Vences *et al*., 2012) [30]. The agarose gel electrophoresis result showed PCR positive bands [figure 23] for all samples [around 650 bp] as compared to the DNA Marker [the range of the marker was 100bp-1200bp].

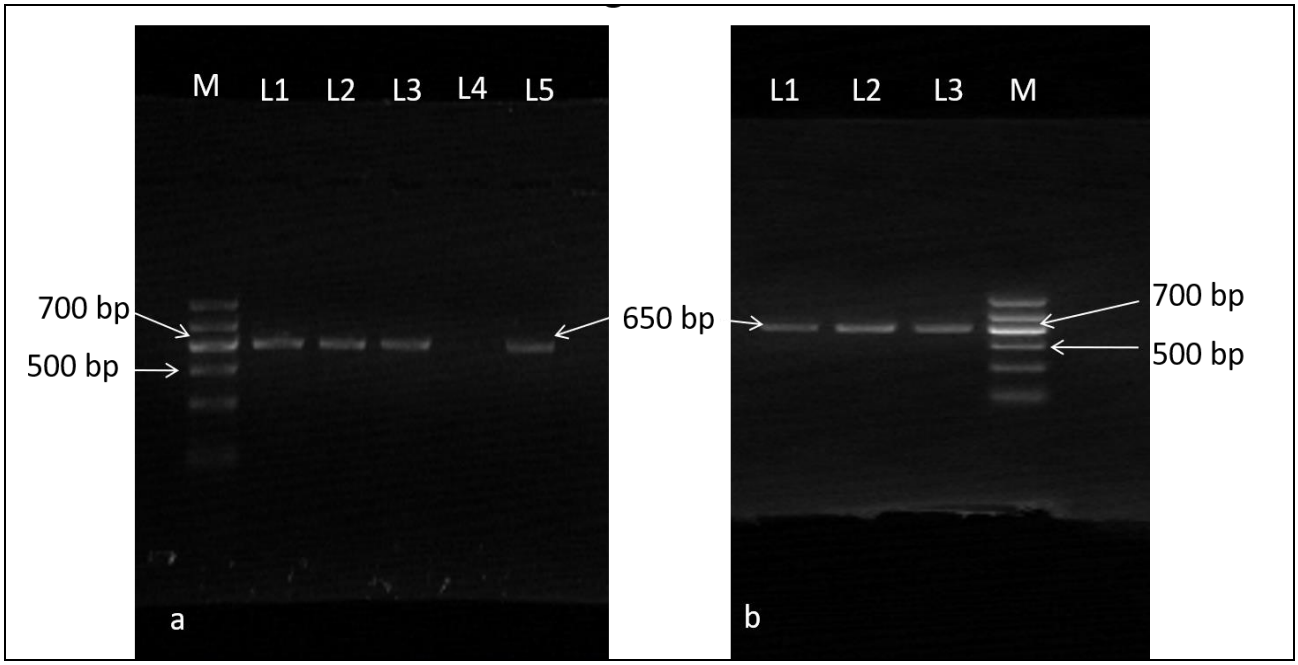

Fig 23: Results of PCR products by gel electrophoresis with COIF/COIR primers: PCR product was around 650 bp. (In figure a, M=marker, L1=T1, L2=T2, L3=T3, L4=empty, L5=T4 and in figure b, L1=T5, L2=T6, L3=T7).

Sequencing and data analysis: Obtained sequences were edited with BIOEDIT software. Sequences of both the samples were subjected to BLAST [Basic Local Sequence Alignment Tool] for species identification. The sequencing result of T1, T2 showed maximum similarity [85.99%] with *Aphonopelma anax and Selenotholus* sp. [85.38%]. The sequencing result of T3 showed maximum similarity [84.75%] with *Chaetopelma* sp. and *Grammostola anthracina* [84.34%]. The sequencing result of T4 showed maximum similarity [85.08%] with *Aphonopelma* sp. and *Chilobrachys* sp. [84.42%]. The sequencing result of T5 showed maximum similarity [83.93%] with *Lyrognathus crotalus* and *Brachypelma vagans* [83.91%]. The sequencing result of T6 showed maximum similarity [85.07%] with *Ischnocolus elongatus*. The sequencing result of T7 showed maximum similarity [85.31%] with *Selenotholus* sp. Sequence of 15 species which showed relatively higher similarity to the sequences were downloaded from NCBI database. Sequences of all the species were trimmed; and aligned along with the 15 other downloaded sequences using MEGA X software. MUSCLE program was used for this alignment. Maximum likelihood Tree [ML] was constructed for this data to conduct phylogenetic analysis. K2P model [Kimura 2 Parameter model] was used for constructing the tree. 1000 Bootstrap was conducted. iTOL Tree visualization software was used for visualizing the tree (Letunic & Borc 2016). The generated sequences were submitted to GenBank. Their accession numbers are "SUB10865759 T1 OM089662" of T1; "SUB10894529 T2 OM090167" of T2; "SUB10894533 T3 OM103380" of T3; "SUB10894541 T4 OM090168" of T4; "SUB10894544 T5 OM103379" of T5; "SUB10894549 T6 OM103381" of T6; "SUB10894554 T7 OM090169" of T7.

The tree showed that, our sequences were in the same clad with a bootstrap value of 2000, which indicates their similarity [Figure 24]. Neighbor joining method [NJ] of tree construction was also performed using the same data, to check whether the uniqueness of our samples under query remains constant. The result showed that the samples showed their individuality in the NJ tree [Figure 24]. By further sequence analysis we found that, both the specimens were not reported before. Thus, in this study, we are reporting new spiders' sequences found in West Bengal, India. The pairwise distances are also analyzed [Figure 25]. Our findings suggested that DNA barcoding is reliable for identifying spiders down to the species level. In the ongoing effort to characterise and comprehend the evolutionary patterns and processes defining the biodiversity landscape, the discovery of spider species offers a significant first step.

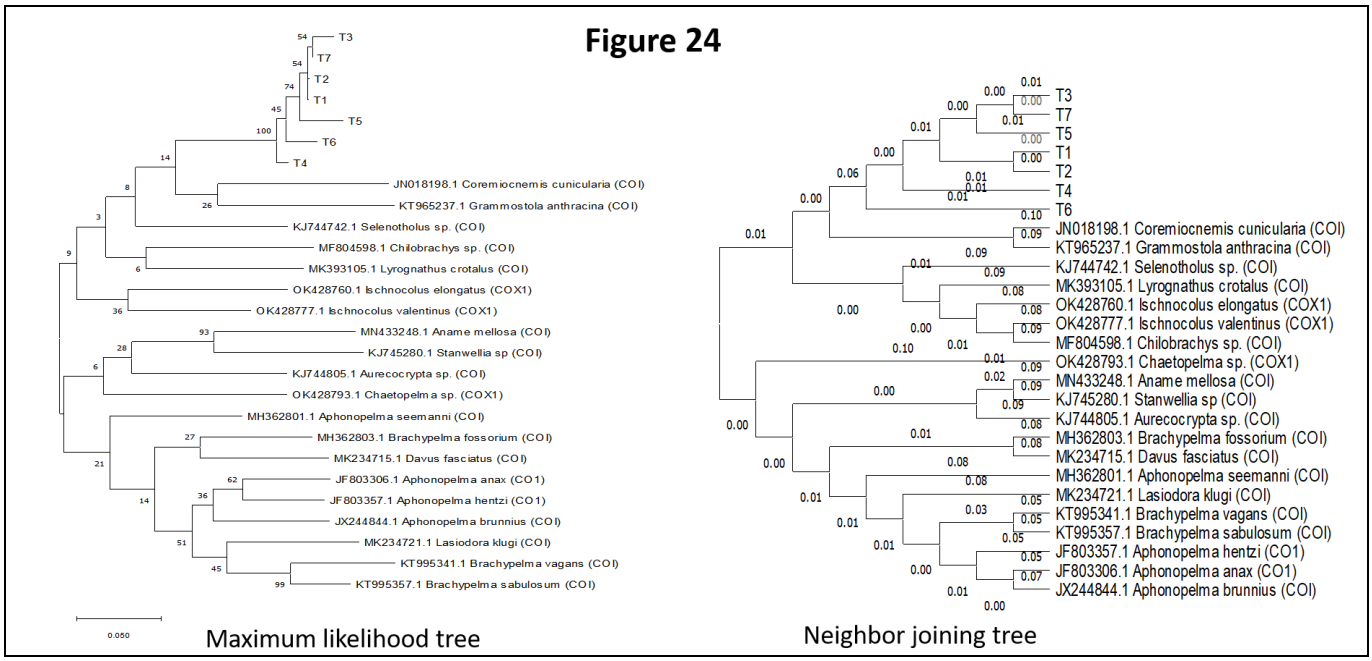

Fig 24: Components of a phylogenetic tree. External nodes, also called 'tips', represent actual organisms sampled and sequenced. They are the 'taxa' in the terminology of evolutionary biology. The internal nodes represent hypothetical ancestors for the tips. The root is the common ancestor of all species in the tree. The horizontal lines are branches and represent evolutionary changes measured in a unit of time or genetic divergence. The bar at the bottom provides the scale of these branch lengths.

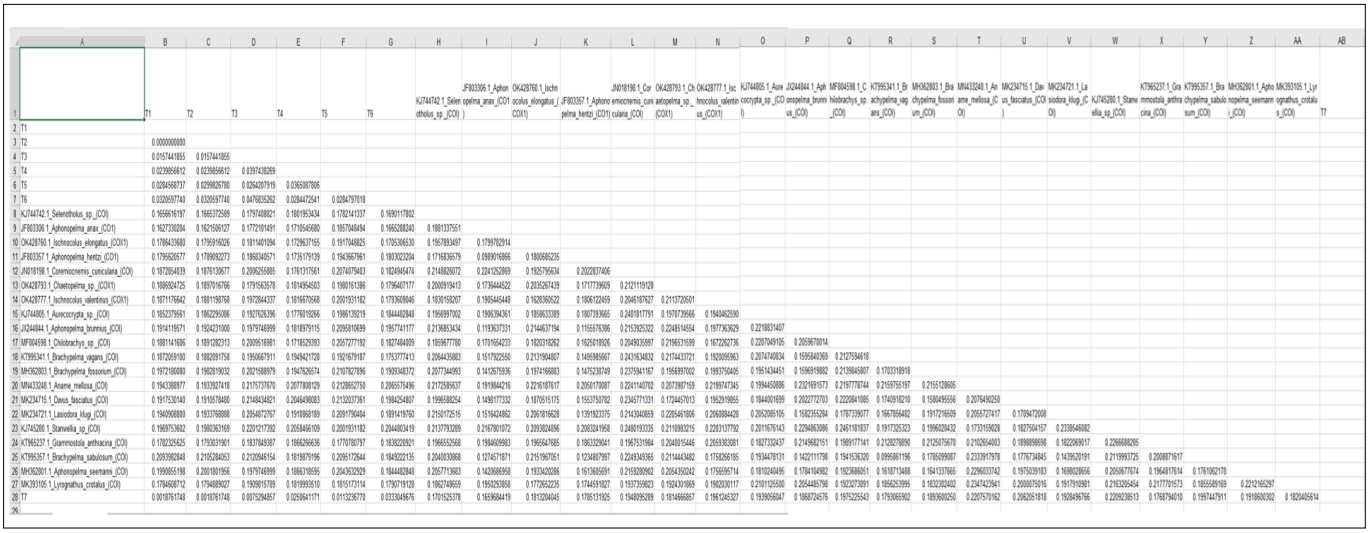

Fig 25: The pairwise distances.

Table 5: Comparison of percentage of similarity (NCBI Blast) between morphologically identified species and other species.

Animal species tested	% of nucleotide similarity with morphologically identified spider	% of highest nucleotide similarity with other spider species
T1	83% With Chilobrachys sp.	85.9% with Aphonopelma anax
T2	83% With Chilobrachys sp.	85.9% with Aphonopelma anax
T3	75% With Heligmomerus sp.	84.7% with Chaetopelma sp.
T4	84.4% With Chilobrachys sp.	85.08% with Aphonopelma sp.
T5	81% With Chilobrachys sp.	83.9% with Lyrognathus crotalus
T6	80% With Chilobrachys sp.	85% with Ischnocolus elongatus
T7	80% With Chilobrachys sp.	85.3% with Selenotholus sp.

Conclusion: One or more linked, highly variable, and easily amplified DNA segments are employed in DNA barcoding, one of the most used techniques for identifying species. Although it was first suggested as a means of identification, (Hebert *et al*., 2003) [12], it has since been applied to the discovery of new species (Fujita *et al*., 2012; Pons *et al*., 2006; Zhang *et al*., 2013) [18, 32]. In several taxonomic groups, barcoding techniques have been utilised to identify cryptic diversity. These techniques have also been suggested for large-scale discovery tactics, particularly in taxa that have not been well investigated (Čandek & Kuntner, 2015; Frézal & Leblois, 2008; Hebert *et al*., 2003; Riedel *et al*., 2013; Tautz *et al*., 2003) [4, 8 12, 23, 26].

The majority of morphological characteristics have been used

in the systematics and identification of tarantulas. They have been classified into different species primarily based on sexual characteristics. Males typically have traits that are more instructive than females, although juveniles are rarely useful. Theraphosids frequently display significant homoplasy and a combination of great intra- and low interspecific morphological variability, like the majority of mygalomorphs. The group's taxonomy has been impeded as a result (Prentice, 1997) [19]. DNA barcoding proponents have argued that, while acknowledging that there may be some variation around a single sequence, one should use DNA sequences of one [or a few] specific genes to identify species. This argument is based on the notion that each species has its own 'diagnostic' sequence, which is a distinct set of base pair mutations (Blaxter, 2004; Gaston & O'Neill, 2004; Godfray, 2002; Hebert *et al*., 2003; Hebert & Gregory, 2005; Tautz *et al*., 2003) [3, 12, 13, 26].

Despite molecular biology's advancements, the great majority of species are still classified according to their physical characteristics. One of the key factors affecting an organism's ecological function is its body size. When it comes to spiders, alpha taxonomy is often treated from a morphological standpoint that focuses mostly on genital structure variations. For spiders and other arthropod groups [such as Diplopoda], it is generally assumed that genital traits evolve quickly with speciation as a result of female sexual selection and/or sexual conflict. Our spider specimens were morphologically similar with the previously identified specimens but their molecular information showed a different result.

The problem of species delimitation, which has historically been controlled by morphology, is now better understood thanks to molecular knowledge. Theraphosidae appears to benefit from COI as a DNA barcoding marker. In many studies, mitochondrial COI is the only source of genetic data used for species delimitation and identification, making it the de facto standard animal barcoding marker. In this study we have reported 7 species of spiders, specifically tarantula, collected from West Bengal, India. These 7 spiders are morphologically very similar to some of the previously discovered species but genetically they are quite different [Table 5]. Through DNA barcoding, our results showed how the 7 spiders have genetically changed and given birth to new cryptic species. So, a quick and effective way for identifying species is DNA barcoding. The taxonomic diversity may therefore be overestimated if just mitochondrial markers are used. Our result also indicates that, new cryptic tarantula species has appeared in the field of Indian spider species and DNA barcoding is a very effective identification tool for the spider and an important advance for future biomonitoring programs. According to the results of the current study, DNA barcoding is a more accurate method, particularly for young spiders whose morphological characteristics are confusing and for species of cryptic spiders whose morphological characteristics are relatively similar.

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Statements and Declarations

Competing interests: There are no competing interests, according to the authors.

Author contributions: PP conceived the content, worked on all parts of this study, collated the results and wrote the manuscript. ERB helped in information extraction, revised the manuscript and approved the final version.

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