DNA BARCODING, AN ASSESSMENT TOOL FOR ASSESSING THE ACCURACY OF MORPHOMETRIC IDENTIFICATION OFSELECTED SPIDER SPECIESOF MAYILADUTHURAI DISTRICT, TAMIL NADU, INDIA

Abstract

In the present study, DNA barcoding was used to assess the percentage accuracy of morphological base identification of spiders from the agriculture fields of Mayiladuthurai district, Tamil Nadu, India. A total of 30 spiders, 6 individuals from 5species were captured fromMay 1st week to 3rd week of 2022. Everyindividualcollected were brought to the College laboratory and sedated with Chloroform for performing morphometric analysis. One individual from each species was preserved in 70% ethanol and stored at −20 °C until the DNA extraction. Spiders were taxonomically evaluated morphologically on the basis of different identification Keys and Catalogues. Morphological identification revealed the presence of 3 families, 4 genera and 4 species. To evaluate the authenticity of morphological identification, tissue samples of 5 specimens were sent to Rajiv Gandhi Centre for Biotechnology (RGCB), Trivandrum, Kerala. About 650 bp of Cytochrome c Oxidase Subunit I (COI) sequencesfor5 samples weredecipheredeffectively, which concludedthe presence of 3 families, 4 genera and 4 species. Based on the sequenced outcomes, onemistaken specimen was then corrected and placed in the appropriate taxon. The all-over accuracy of identification based on morphometry was 80%. Thus, the present study concluded that morphometric analysisfor identifying thespider taxon, is unsatisfactory. Hence to

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improve the credibility, accuracy and pace of results, molecular-based taxon identification like barcoding of DNA is considerably recommendable. Also, research is needed to confirmthe genuineness of the identification of spider species with a large sample size is necessary.

Keywords: Agroecosystems,Spiders, Morphometry, DNA Barcoding, Taxonomic classification, Accuaracy

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I. INTRODUCTION

Spiders are found everywhere on the entire landscapes on earth where life is supported. In most of the terrestrial habitats,Spiders are the important hunters[1].They belong to the class, Arachnids which is the second largest class representing 7% of total documented arthropods [2]. About 44,906 species of spiders belonging to the 114 families and 3935 genera have been described so far [3].

Spiders are commonhunters and are well-known as commonopponents of pests [4]. They areprotagonists in forest and agricultural environmentsand are very critical as insect pest density stabilizers[5].These environments also support a wide range of prey types for these specialist predators. Spiders are strong bio-control agents due to the features like partial consumption of prey,mortality of pests due to enallenes in webs, and extreme killing [6].

It is a time-consuming and laborious job to identify Spider species using morphometric analysis for different reasons [7]. Sexual dimorphism and the lack of analyticalfeaturesfor young ones are the keysteeplechases in the determination of the taxon of spiders [8]. DNA barcoding is a molecular identification tool that is being employed to crush such problems[9]. It is a newprotocol which is used to deliver quick and cost-effective species identification by which standard taxonomic classification[10,11] can be done. This protocol is based on the differences in the standard COI region (658 base pairs) of the DNA of mitochondria called genetic barcode, from which the identification of species taxon can be carried out[12]. DNA barcoding is successfully used as a tool to evaluate species relating to various groups including bats [13], butterflies [14], birds [15], fishes[16], Diptera[17], Algae [18], Fungi [19], amphibians [20], ants [21], crustaceans [22],wasps and aphids [23].

DNA barcoding is now-a-days commonly used for the successful identification of species. Beyond the identification, it can also be used for assisting newdiscovery of species [24]. The sustainability of DNA Barcoding relays on the fact that the differences in sequences are less in intra species than the inter species [7, 16, 21, 24,12]. Though there are limitations in Barcoding, many scientists over the world have given possible solutions by introducing the awareness of "*integrated barcodes*" [25]. Integrated barcoding combinesbothDNA and morphometricmethods to classify and label a species [26]. The objective of the study was to explore the accountability of identifying the spider wildlifeof the Mayiladuthurai District, Tamil Nadu, India by morphometric analysis and assessing its accuracy compared with the Barcoding technique. Another motive of the study is to establish add-on data to the genetic reference library for forthcoming study of spider species at the DNA level.

II. MATERIALS AND METHODS

1. **Sampling of Spiders:** Spiders were collected from agricultural fields of Mayiladuthurai District, India (Please refer Table 5 for the collection site and their Coordinates). They were collected from the leaf litter, cotton field, black gram field, Banana field, sesame field and among the grasses.

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2. **Sampling Methods:** To catch the spiders,different sampling methods like,hand picking,sweep net and jerking were hired[8,27]. The spiders that were in between the leaves were sampled through sweep net and handpicking and the spiders present in theshrubs were captured by jerking [8].

3. **Preservation Technique:** Spiders were collected in plastic bottles and were brought to the laboratory of Zoology department, A.V.C. College, Mannampandal, Tamil Nadu, India. One individual from each species was then preserved in 70% ethanol as per the instructions given by RGCB. Preserved samples were sent to RGCB immediately for sequencing.

4. **Morphological Identification:** Before applying the molecular technique for evaluation, spiders were identified on the basis of specific diagnostic morphological characters like total body length, length and width of Cephalothorax, Length and width of Abdomen, 1st, 2nd, 3rd and 4th pair of legs and Pedipalp. Identification was possible with the help of various available keys. The keys that were oftenused for identification were Sebastian, P. A (2009)[28], Tikader and Malhotra (1980)[29], Barrian and Listinger (1995)[30] and other obtainablecollections and literature. The morphological examination of all the specimens was done by placing the specimen on a graph sheet and observing it under a stereomicroscope (CXM4 Model). Each and every part was dissected carefully and measured. Figure 1 shows the measurement of the sample 1 placed on a graph sheet.

5. **DNA Barcoding:** DNA sequencing was outsourced from Rajiv Gandhi Centre for Biotechnology, The protocol for the sequencing is given below.

6. **DNA Barcoding Using Universal Primers of CO1 IProtocols Genomic DNA Isolation:**

Figure 1: Measurement of *Hippasa Greenalliae*

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Genomic DNA was isolated from the tissues using NucleoSpin® Tissue Kit (Macherey-Nagel) following the manufacturer's instructions.

Tissues were placed in a 1.5 ml microcentrifuge tube. Incubate at 56°C in a water bath until the tissue was completely lysedafter adding 180 µl of T1 buffer and 25 µl of proteinase K. After lysis, incubated at room temperature for 5 minutes after adding5 µl of RNase A (100 mg/ml). incubated at 70°C for 10 minutesafter adding 200 µl of B3 buffer. Vortex it thoroughly after adding210 µl of 100% ethanol. Centrifuge at 11000 x g for 1 minutethe mixture after pipetting into NucleoSpin® Tissue column placed in a 2 ml collection tube. Then transfer it to a new 2 ml tube and wash with 500 µl of BW buffer. Repeat the wash step using 600 µl of B5 buffer. Placed it in a clean 1.5 ml tube after washing the NucleoSpin® Tissue column and elute the DNA out using 50 µl of BE buffer.

7. **Agarose Gel Electrophoresis for DNA Quality check:** Agarose gel electrophoresiswas used to check the quality of the DNA isolated. 5µl of DNAwas taken and 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to it. 0.5 µg/ml ethidium bromidewas added and the samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer. Electrophoresis buffer at 75 Vused for Electrophoresis with 0.5X TBE until bromophenol dye front has migrated to the bottom of the gel. Using the Gel documentation system (Bio-Rad), the gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light

8. **PCR Analysis:**

2X Phire Master Mix	5µL
D/W	4µL
Forward Primer	0.25µL
Reverse Primer	0.25µL
DNA	1µL

9. **Primers Used:**

Target	**Primer Name**	**Direction**	**Sequence (5' → 3')**
COX1	LCO	Forward	GGTCAACAAATCATAAAGATATTGG
	HCO	Reverse	TAAACTTCAGGGTGACCAAAAAATCA

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

10. **PCR Amplification Profile:**

COX1

98°C - 30 sec
98°C - 5 sec
45°C - 10 sec 10 cycles
72°C - 15 sec

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98°C - 5 sec
50°C - 10 sec 30 cycles
72°C - 15 sec
72°C - 60 sec
4°C - ∞

11. **Agarose Gel electrophoresis of PCR products:** 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromideto check the PCR products. Electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel by adding1 µl of 6X loading dye with 4 µl of PCR products before loading. The molecular standard used was a 2-log DNA ladder (NEB). The Gel documentation system (Bio-Rad) was used to visualize the gel in a UV transilluminator (Genei) and the image was captured under UV light.

12. **ExoSAP-IT Treatment:** ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five microlitres of PCR product is mixed with 0.5µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 85°C for 5 minutes (as per the User Guide, GE Healthcare).

13. **Sequencing using BigDye Terminator v3.1:** Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Referring the User manual, Applied Biosystems, USA) following manufactures protocol.The Sequencing PCR mix consisted of the following components:

D/W	6.6µL
5X Sequencing Buffer	1.9µL
Forward Primer	0.3µL
Reverse Primer	0.3µL
Sequencing Mix	0.2µL
Exosap treated PCR product	1µL

14. **Sequencing PCR Amplification Profile:**

96°C - 2min
96°C - 30sec
50°C - 40sec 30 cycles
60°C - 4min
4°C - ∞

15. **Post Sequencing PCR Clean Up:**

D/W	5 µl
3M Sodium Acetate	1 µl

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EDTA	0.1 µl
100% Ethanol	44 µl

- Mix D/W, 125mM EDTA, 3M sodium acetate pH 4.6 and ethanol were prepared and were properly mixed.
- 50 µl of the mix was added to each well in the sequencing plate containing sequencing the PCR product.
- Vortex by Mixmate vortex and Incubated at room temperature for 30 minutes
- Spun at 3700 rpm for 30 minutes
- Decant the supernatant and add50 µl of 70% ethanol to each well
- Spun at 3700 rpm for 20 minutes.
- Decanted the supernatant and repeated 70% ethanol wash
- Decanted the supernatant and air dried the pellet.
- The cleaned-upair-dried product was sequenced in ABI 3500 DNA Analyzer(Applied Biosystems).

16. **Sequence Analysis:** The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 [31].

17. **Sequence Submission:** Generated sequences were submitted to BankIt. Then MEGA 11 software was utilised to align the present study sequences. To compute the barcode gap which arises when the interspecific genetic divergences exceed the intra-specific divergences, we used the BOLD online system v3. By applying the Kimura 2 parameter as a distance model, COI-5P- Cytochrome Oxidase Subunit 1, 5′ Region as a marker, BOLD Aligner as a sequence aligner and sequence length of ≥600 base pairs as a filter in BOLD software, we generated the barcode gaps of all the under-study specimens.

III. RESULTS AND DISCUSSION

1. **Morphology-Based Identification:** A total of 5 specimens representing 3 families, 4 genera and, 4 species were identified morphometrically.Figure 2 shows a collection of photographs while performing the morphometric analysis. Lycosidaefamily was ample on the ground under detritus material, fallen leaves, and soil crevices during the collection in the fields. However, the Oxyopidae family was the most communal on vegetation. Six individuals from each species were collected, sedated with chloroform and the morphometry studies were carried out. Details of morphometric analysis and the data got for each species is given in Table 1 (mean value + standard deviation in mm). On the basis of the morphometric analysis and referring to the literature, species identification was done. The list of species identified is given the Table 2.

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Table 1: Morphometric Analysis of Spider Individuals (*Mean of 6 INDIVIDUALS + SD in mm)

Species name	TL	CL	CW	AL	AW	1st Pair of Leg	2nd Pair of Leg	3rd Pair of Leg	4th Pair of Leg	PL
Hippasa greenalliae	14.1±0.6	6.6±0.4	3.6±0.4	6.6±0.4	4.1±0.8	16.1±0.6	15.8±0.3	14.3±0.4	21.6±0.7	3.3±0.4
Oxyopes hindostanicus	7.3±0.7	3.1±0.6	2.3±0,4	4.6±0.4	1.3±0.4	13.6±0.7	13.1±0.3	11.3±0.4	13.1±0.3	3±0
Pardosa pseudoannulata	10.1±0.3	3.8±0.3	3.1±0.1	5.8±0.3	3.1±0.3	14.1±0.3	16.8±0.3	13.6±0.7	20.3±0.4	4±0
Tetragnatha javana	15.8±0.4	3.5±0.5	1±0	11.8±0.6	1±0	23.8±0.6	12.3±0.4	4.8±0.6	12.3±0.4	1±0
Lycosidae sp.	6.6±0.4	3.1±0.6	2.1±0.3	3.8±0.6	2.8±0.3	6.6±0.4	8.3±0.3	7.2±0.2	10.3±0.3	3±0

***TL – Total Length** **AL – Abdomen Length**
CL – Cephalothorax Length **AW – Abdomen Width**
CW – Cephalothorax Width **PL – Pedipalp Length**

Table 2: Details of Morphologicalbased Identified Spider Species

Sample Code	Morphological Identification	Family Belonging to
AVCC01	*Hippasa greenalliae*(Blackwall, 1867)	Lycosidae (Sundevall, 1833)
AVCC02	*Oxyopes hindostanicus* (Pocock, 1901)	Oxyopidae (Thorell, 1870)
AVCC03	*Pardosa pseudoannulata*(Bösenberg & Strand, 1906)	Lycosidae (Sundevall, 1833)
AVCC04	*Tetragnatha javana* (Thorell, 1890)	Tetragnathidae (Menge, 1866)
AVCC05	Lycosidae sp.	Lycosidae (Sundevall, 1833)

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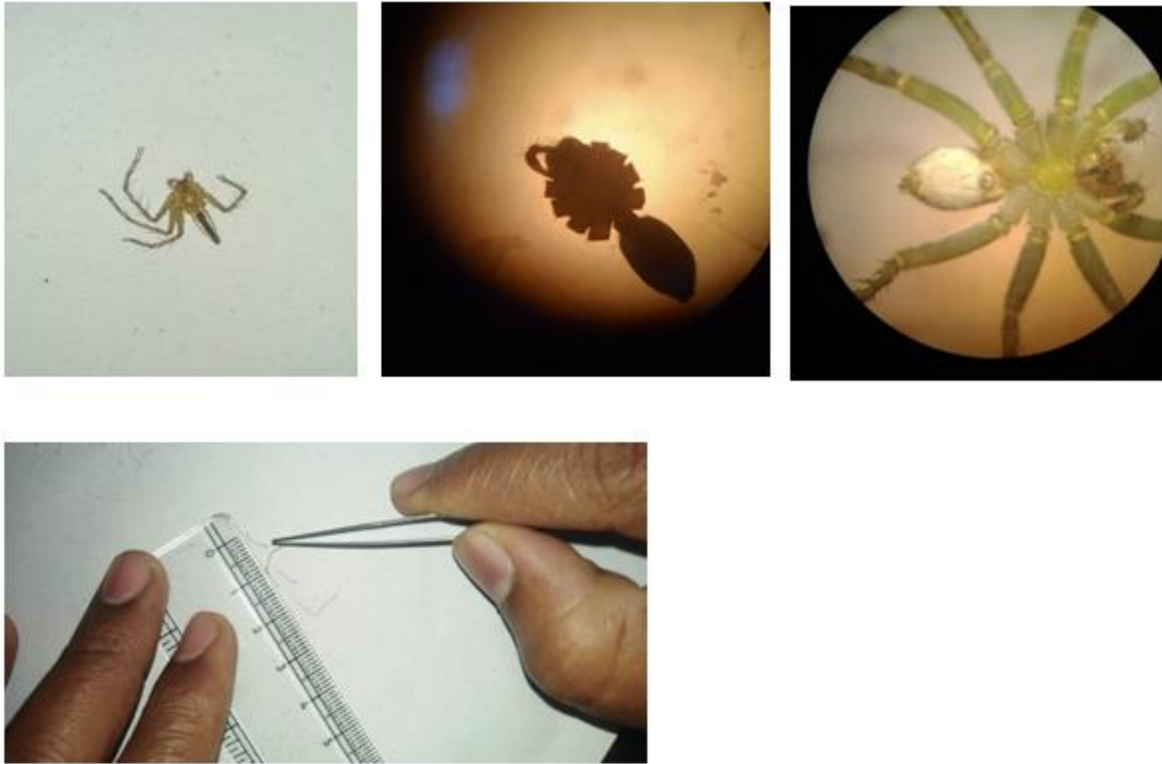

Figure 2: Photos taken during Morphometric Analysis

2. **Biology of the Spider Species Collected:** The taxonomic position of the spider species collected is represented in the Table 3. Figures 3 to 7 are the sample photographs of the 5 species collected. ***Hippasa greenalliae***(Blackwall, 1867) is a species of spider native to India, Bangladesh and Sri Lanka. The adult is about 14.10 mm in length. ***Oxyopes hindostanicus*** (Pocock, 1901) is a species of spider of about 7.3 mm in length. It is found in India, Pakistan and Sri Lanka. *Pardosa pseudoannulata* (Bösenberg & Strand, 1906) normally inhabits in open habitats and similararable farm fields. Also, it is cosmopolitical surface-dwelling spider species which plays an important role in controlling the pests and insects inside the agricultural lands. The adult is about 10.10 mm in length *Tetragnatha javana* (Thorell, 1890) is a common agricultural spider inhabiting the wetlands. The adult is about 7.0 mm in length.

Table 3: The Taxonomic position of the selected spider species

	Sample 1	Sample 2	Ample 3	Sample 4
Kingdom:	Animalia	Animalia	Animalia	Animalia
Phylum:	Arthropoda	Arthropoda	Arthropoda	Arthropoda
Subphylum:	Chelicerata	Chelicerata	Chelicerata	Chelicerata
Class:	Arachnida	Arachnida	Arachnida	Arachnida
Order:	Araneae	Araneae	Araneae	Araneae

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Infraorder:	Araneomorphae	Araneomorphae	Araneomorphae	Araneomorphae
Family:	Lycosidae	Oxyopidae	Lycosidae	Tetragnathidae
Genus:	*Hippasa*	*Oxyopes*	*Pardosa*	*Tetragnatha*
Species:	***H. greenalliae***	***O. hindostanicus***	***P. pseudoannulata***	***T. javana***

Figure 3: *Hippasa greenalliae0*

Figure 4: *Oxyopes hindostanicus*

Figure 5: *Pardosa pseudoannulata (Female)*

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Figure 6: *Tetragnatha javana*

Figure 7: *Pardosa pseudoannulata (Male)*

3. **Genetic-Based Identification:** 5 specimens were subjected to DNA barcoding in order to verify the authenticity of the morphology-based identification of spiders. DNA sequence of the mitochondrial COI upto 650 base pairs was successfully retrieved from those specimens. The precise morphological evaluation of the 5th specimen (Specimen code: AVCC05), was identified wronglywhich was then fixed the correct taxon on the evidence of biological DNA barcode sequence as depicted in Table 4. Actually, the 5th sample was the subadult species of the 3rd sample, the species *Pardosa pseudoannulata.* DNA barcoding affirms the presence of 3 families, 4 genera, and 4 species.Totally, the accurateness of DNA-based identification was 80%.

Table 4: Morphologically Misidentified Specimen along with its Correct Taxon.

Sample Code	Morphological Identification	Molecular Identification	Family belonging to
AVCC05	Lycosidae sp.	*Pardosa pseudoannulata*(Bösenberg & Strand, 1906)	Lycosidae (Sundevall, 1833)

Though the fifth sample's family was identified as Lycosidae, the genus and species identification werenot possible. About 3 species was guessed using the morphometry values as *Plexipus paykuli, Pirata subpiraticus and Rabidosa rabita* but there were vast differences among the actual values. So, we thought that, after the COI sequencing results, we could know it by Blasting and can confirm. But the blasting results confirmed it as *Pardosa pseudoannulata.* We couldn't accept it, as the patterns and the morphometric values were entirely different. Hence, we requested the outsourcing agent, the RGCB to do the COI sequencing again for the 5th sample alone.

They also accepted our request and did the sequencing and mailed the results. We blast the sequence to know the species which was shocking. It showed 100% similarity to the third sample, *Pardosa pseudoannulata.* Then we searched for literature for the sexual size dimorphism (SSD) among the species *Pardosa pseudoannulata.* We found the answer that SSD exist among this species. Research on it was done by Zhang *et*

al. (2021)[32] which indicated that the developmental and growth differences between both sexes look as if at early life stages, and there was allometric growth observed between males and females in the carapace, abdomen, and gonads.From this literature, we could understand that the subadult male are entirely different from the adult females. These differences are depicted in the images shown below (Figure 8 and 9).

Figure 8: Picture courtesy from Ecology and Evolution Journal, Wiley Online Library
Figure 9: Pictures of Female (Top) and Male (Bottom) *Pardosa pseudoannulata* taken in this study

4. **Sequences Obtained:** The sequences obtained for the given five samples and the repeated 5th sample sequences with sample codes, AVCC01, AVCC02, AVCC03, AVCC04, AVCC05-1 and AVCC05-2 are given below. All together 6 sequences that were obtained were submitted in NCBI through online submission to BankIt for deposition to GenBank and the accession numbers were got, which were given in the Table 5.

- **Sample 01:**
  >SR2856-AV01-COF_E11.ab1
  TTGTTACTGCTCATGCTTTTGTAATAATTTTTTTATAGTTATACCGATTTT
  AATTGGTGGTTTTGGAAATTGATTAGTACCATTAATATTAGGTGCTCCTGA
  TATATCATTTCCTCGAATGAATAATCTTTCATTTTGATTATTACCTCCTTCT
  TTATTTTTATTATTTATATCTTCTATAGTATAAATAGGAGTTGGAGCTGGAT
  GAGCTGTTTATCCACCTTTAACTTCTAG

  >SR2856-AV01-COR_H04.ab1
  CAGGTAAAGAAAGTAATAATAAAATAGCAGTAATTAAAACTGACCAAAC
  AAATAAAGGAACTTTTTCCATTCTTATTCCTATTAATCGTATATTAATAAT
  AGTTGAAATAAAATTTACTGCTCCTATAATAGAAGAAGCCCCAGCCAAAT
  GAAGAGAAAAAATAGCAAAATCTATTGATCTCCCTATATGTCCTATTCTA
  GAAGCTAAAGGTGGATAAACAGTTCATCCAGCTCCAACTCCTATTTCTACT

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ATAGAAGATATAAATAATAAAAATAAAGAAGGAGGTAATAATCAAAATG
AAAGATTATTCATTCGAGGAAATGATATATCAGGAGCACCTAATATTAAT
GGTACTAATCAATTTCCAAAACCACCAATTAAAATCGGTATAACTATAAA
AAAAATTATTACA

- **Sample 02:**

>SR2856-AV02-COF_D05.ab1
CAATAAGAGTATTGATTCGAATGGAATTAGGACATTCTGGAAGAATATTA
GGAGATGATCATTTGTATAATGTAATTGTTACTGCTCATGCTTTTGTAATG
ATTTTTTTTATAGTTATACCAATTTTAATTGGTGGATTTGGGAATTGATTAA
TTCCTTTAATATTAGGAGCTCCTGATATATCTTTTCCTCGTATAAATAATTT
ATCATTTTGGTTACTTCCTCCTTCTTTATTTTTGTTATTTATATCTTCTATAG
TTGAAACTGGGGTTGGGGCAGGATGGACAGTATATCCTCCATTAGCTTCG
ACTACTGGTCATATAGGAAGATCAATGGATTTTGCTATTTTTTCTTTACATT
TAG
>SR2856-AV02-COR_D06.ab1
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AATAGCCCCTGCTAATACTGGTAAAGATAACAATAATAAAATAGCAGTAA
TAAAAACAGATCACACAAATAAAGGAACCTTCTCTATTCTTATACTACTTA
ATCGTATATTAATAATAGTAGAAATAAAATTTACAGCTCCTATAATAGAA
GAAGCACCAGCTAAATGTAAAGAAAAAATAGCAAAATCCATTGATCTTCC
TATATGACCAGTAGTCGAAGCTAATGGAGGATATACTGTCCATCCTGCCC
CAACCCCAGTTTCAACTATAGAAGATATAAATAACAAAAATAAAGAAGG
AGGAAGTAACCAAAATGATAAATTATTTATACGAGGAAAAGATATATCAG
GAGCTCCTAATATTAAAGGAACTAATCAATTCCCAAATCCACCAATTAAA
ATTGGTATAACTATAAAAAAAATCATTACAAAAGCATGAGCAGTAACAAT
TACATTATACAAATGATCATCTCCTAATATTCTTCCAGAATGTCCTAATTC
CATTCGAATCAATACTCTTATTGCTGTTCCAACTATAGCCGATCAAACTCC
AAATATTAAATATAAAGTCCCCATTATCTTTTATGA

- **Sample 03:**

>SR2856-AV03-COF_B05.ab1
AGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGAATGG
AATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAATGTGA
TGGTTACTGCACATGCTTTTGTGATAATTTTTTTATAGTAATACCAATTCT
TATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCTGA
TATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACCTTCT
TTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCTGGTT
GAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAGTTCG
ATAGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATTATAG
GAGCTGTAAATTTTATTTCTACTATTATTAATATACGAGTGACTGGAATAT
CAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCAGTTT
TATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGTTAAC
GGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTG
>SR2856-AV03-COR_B06.ab1

CCCCTCCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTT
AACAATATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAA
AACTGCAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTG
ATATTCCAGTCACTCGTATATTAATAATAGTAGAAATAAAATTTACAGCTC
CTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATC
TATCGAACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAG
TTCAACCAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAA
AATAAAGAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAA
AAGATATATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAA
CCACCAATAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATG
GGCAGTAACCATCACATTATATAAATGATCATCACCTAATAATCTACCAG
GATTTCCTAATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCAAAG
CCGATCAAACTCCAAACATTAAATATAAG

- **Sample 04:**

>SR2856-AV04-COF_E05.ab1
GTTTTAATCCGTATTGAATTAGGACAGTCTGGGAGATTTCTTGGGGACGAC
CAGCTTTATAATGTTATTGTTACTGCTCATGCTTTTGTAATAATTTTTTTA
TAGTGATACCTATTTTGATTGGGGGATTTGGAAATTGATTAGTTCCTTTAA
TATTAGGGGCCCCAGATATAGCTTTTCCTCGTATAAATAATTTAAGTTTTT
GGCTTTTACCTCCCTCTCTTTTTATATTATTTGTATCTTCTATGGTGGATAT
CGGAGTAGGGGCTGGATGAACGGTGTATCCCCCTCTAGCTTCTTTGGAGG
GTCATTCGGGAAGATCTGTGGATTTTGCTATTTTTTCGCTTCATTTAGCGG
GAGCCTCGTCTATTATAGGAGCAATTAATTTTATTTCTACAATTATTAATA
TGCGAATGAAAGGAGTCTCGATAGAAAAGGTTCCTCTTTTCGTTTGATCTG
TTTTGATTACAGCGGTTTTATTGCTTTTATCCCTTCCCGTTCTACTGGGGCT
ATTACT
>SR2856-AV04-COR_E06.ab1
GAAAATGAGGTATTAAAATTTCGATCTGTTAATAATATAGTAATAGCCCC
AGCTAGAACGGGAAGGGATAAAAGCAATAAAACCGCTGTAATCAAAACA
GATCAAACGAAAAGAGGAACCTTTTCTATCGAGACTCCTCTCATTCGCAT
ATTAATAATTGTAGAAATAAAATTAATTGCTCCTATAATAGACGAGGCTC
CCGCTAAATGAAGCGAAAAAATAGCAAAATCCACAGATCTTCCCGAATGA
CCCTCCAAAGAAGCTAGAGGGGGATACACCGTTCATCCAGCCCCTACTCC
GATATCCACCATAGAAGATACAAATAATATAAAAAGAGAGGGAGGTAAA
AGCCAAAAACTTAAATTATTTATACGAGGAAAAGCTATATCTGGGGCCCC
TAATATTAAAGGAACTAATCAATTTCCAAATCCCCCAATCAAAATAGGTA
TCACTATAAAAAAAATTATTACAAAAGCATGAGCAGTAACAATAACATTA
TAAAGCTGGTCGTCCCCAAGAAATCTCCCAGACTGTCCTAATTCAATACG
GATTAAAACTCTTATTGCAGTCCCCACTATAGCTGATCATACACCAAATAA
AAAATATAAACTTCCAATATCTTTATG

- **Sample 05:**

>SR2856-AV05-1-COF_C05.ab1

TGGAGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGAA
TGGAATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAATG
TGATGGTTACTGCACATGCTTTTGTGATAATTTTTTTATAGTAATACCAAT
TCTTATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCT
GATATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACCTT
CTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCTGG
TTGAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAGTTC
GATGGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATTATA
GGAGCTGTAAACTTTATTTCTACTATTATTAATATACGAGTGACTGGAATA
TCAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCAGTT
TTATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGTTAA
CGGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTGGTGGAGGGGATC
CAATTTTATTTCAACATTTGTTT

>SR2856-AV05-1-COR_C06.ab1
CCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTTAACAA
TATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAAAACTG
CAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTGATATTC
CAGTCACTCGTATATTAATAATAGTAGAAATAAAGTTTACAGCTCCTATAA
TAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATCCATCGA
ACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAGTTCAAC
CAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAAAATAAA
GAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAAAAGATAT
ATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAACCACCAA
TAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATGTGCAGTA
ACCATCACATTATATAAATGATCATCACCTAATAATCTACCAGGATTTCCT
AATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCATAGCCGATCAA
ACTCCAAACATTAAATATAA

- **Sample 6:**

>SR2856-AV05-2-COF_G05.ab1
TTGGAGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGA
ATGGAATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAAT
GTGATGGTTACTGCACATGCTTTTGTGATAATTTTTTTATAGTAATACCA
ATTCTTATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTC
CTGATATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACC
TTCTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCT
GGTTGAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAG
TTCGATGGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATT
ATAGGAGCTGTAAACTTTATTTCTACTATTATTAATATACGAGTGACTGGA
ATATCAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCA
GTTTTATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGT
TAACGGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTGGTGGAGGGG
ATCCAATTTT

>SR2856-AV05-2-COR_G06.ab1
CCCCTCCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTT
AACAATATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAA

AACTGCAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTG
ATATTCCAGTCACTCGTATATTAATAATAGTAGAAATAAAGTTTACAGCTC
CTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATC
CATCGAACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAG
TTCAACCAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAA
AATAAAGAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAA
AAGATATATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAA
CCACCAATAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATG
TGCAGTAACCATCACATTATATAAATGATCATCACCTAATAATCTACCAG
GATTTCCTAATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCATAG
CCGATCAAACTCCAAACATTAAATATAAAGTTCCAATATCTTTATGT

Table 5: Specimens and GENBANK ACCESSION Nos with their Coordinates of the Collection Sites and Taxonomic Identification.

S.No	Sample Code	GenBank Accession No	Taxonomic identification	Collection place	Latitude and Longitudes of collectionsites
1	AVCC01	ON817272	*Hippasa greenalliae*	Mannampandal, Tamilnadu, India	11.1064, 79.6750
2	AVCC02	ON834461	*Oxyopes hindostanicus*	Karraimedu, Tamilnadu, India	11.8137, 79.7316
3	AVCC03	ON817273	*Pardosa pseudoannulata*	Mannampandal, Tamilnadu, India	11.1064, 79.6750
4	AVCC04	ON817271	*Tetragnatha javana*	Korkai, Tamilnadu, India	8.62777, 78.0443
5	AVCC05-1	ON908677	*Pardosa pseudoannulata*	Korkai, Tamilnadu, India	8.62777, 78.0443
6	AVCC05-2	ON892065	*Pardosa pseudoannulata*	Korkai, Tamilnadu, India	8.62777, 78.0443

IV. DISCUSSION

The foremostgoal line of the study was to assess the best identification tool for exploring the spider's identityso that the taxonomy of the species could be identified flawlessly. When comparing the COI marker, morphological-based identification with keys that are designed already, the success rate of identification is 80%. The keys for the identification of juveniles and subadult is very rare for spider species especially the distinguishing features about the sexual dimorphism is not well explained by any researchers in this field. This is the main reason for the lowering in success rate.COI sequence was obtained for all the 5 specimens collected was successful.

We collected 5 different spider species and made a morphological identification using the keys (mainly the books) and classified the species under the family Lycosidae, Oxyopidae, and Tetragnathidae. The collection methods that we followed were also recommended by Robinson *et al.* (2009) [8]for sampling.The sample number is very low due

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to insufficient time and sampling effort, also barcoding of DNA is a bit costlier technology for a postgraduate student to carry out his research work, thoughmost of the scientist across the world have reported a large number of species. Out of the5 specimens collected, Lycosidae Family was the maximumon the ground which wasalso reported by most of thescientists in their literatures (Tahir *et al.*2015)[33].

The COI of mitochondria is represented as a Biological barcode for the identification of species.Through DNA barcoding,a wide range of taxa can be identified with the help of universal primers from the required DNA [34]. During the morphometric analysis, we misidentified 1 specimen and then rendering the help of DNA Barcoding,the appropriate taxawas assigned. Thus,the assessment of species by identifying theCOI markeris aptlycomparing morphogenetic identification. Goldstein and DeSalle (2003)[35]also described the same for the identification of century-old specimens through DNA barding. Thus, it was concluded that these results are in accordance with many researchers and it also emphasizes the necessity for ample and correct identification of species. Hebert *et al.* (2004)[24]also published their results in compliance with our results that the DNA barcoding technique is 100% accurate.

V. PHYLOGENETIC ANALYSIS OF THE SEQUENCES

In the current investigation, Neighbor-joining treewas utilized to study the phylogenetic relationship between the sequences obtained. The sequences of the 4 different species showed more than 2% genetic difference. Thus, it was confirmed that all four samples belonged to different species. A noteworthy barcode gap was also observed between the intra and inter-specific divergences though all four species belong to the Araneidaefamily. Additionally,the distance to NNis lower than the maximum intra-specific values. Similar results were reported by Slowik and Blagoev (2012)[36]for the family Araneidae. Lapping in the divergence's standards for the Araneidae familywas observed by Čandek and Kunter (2015)[37]but it was not found in our study which was the only difference observed.

Nevertheless, in the current investigation, sample 3 (*Pardosa pseudoannulata*) exhibited 100% similarity with sequences of Sample 5, during the blasting with the sequences ofGenBank. This was because of the misidentification of the subadult species of *Pardosa pseudoannulata* as a different species. Zhang *et al.* (2021) [32] have explained the Sexual dimorphism existing in *Pardosa pseudoannulata.* He investigated the allometric and potential growth differences among the abdomen, carapace and gonads of spiders among the two sexes. Even confusion regarding the identification of this species is prevailing among researchers around the world. Researchers like Naseem and Tahir (2018)[38]during their investigations in Pakistan have reported this species as *Pardosa birmanica.* Thus, there is silentmisperceptionexisting in confirming the correct taxon of this particular species. Though, their exact taxon were allotted after the performance of DNA barcoding. The possible causes of these kinds of variations may be due to introgression, quick morphological divergences and interbreeding as described by Robinson *et al.* (2009)[8]. Thus a novel approach of "integrated barcoding" was followed by Slowik and Blagoev (2012)[36] to overwhelm these kinds of problems. Between the intra and inter-specific divergences, there was no overlap. However, an overlap of the intra and inter-specific valueswas reportedby Čandek and Kunter (2015)[37].

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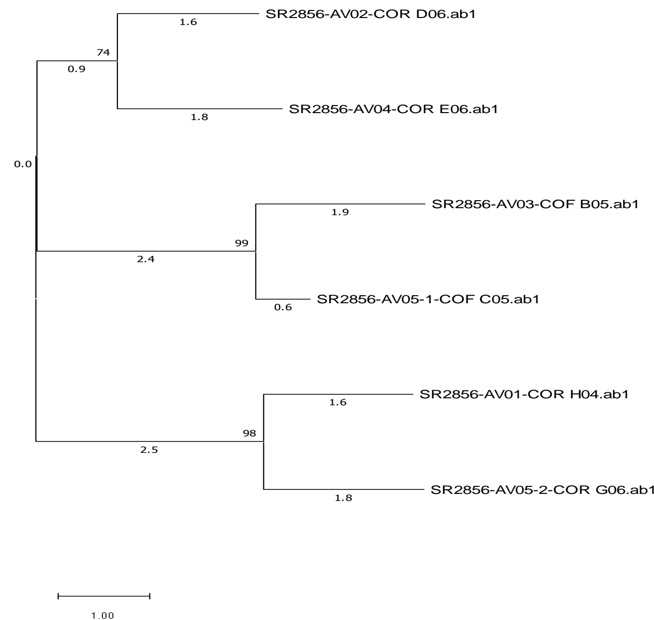

Figure 6: Phylogenetic Tree Created Using the MEGA 11 Software

While identifyingthe Lycosidae family,the specimens showed great variations of colour and body patterns morphologically. Finally, it was identified correctly.Such problems were also addressed by Bond *et al.* (2001)[9] and he too suggested the molecular-based approaches to overcome these kinds of issues.

For the total of 5 specimens of the present study, the reliability of the results depends on a barcode gap which was observed significantlyin the intra and inter-specific divergences [38]. Furthermore, values of the distance to NN for every species were higher than the maximum intra-specific divergences.When the outcomes were blasted, they displayed 100% similarity to the species identified. Such 100% positiveoutcomeswere in harmony with the outcomes of Barrett and Hebert (2005)[7], who acceptablyassessed the 168 species of spiders using molecular-based DNA barcoding. The application of DNA barcoding was also suggested by Čandek and Kunter [37]for the assessment of taxon of spider species. This technique was also followed by Tahir *et al.* (2016)[27]for identifying 5 spider species with 100% successful rate. 19 species-rich genera was described successfully by Robinson *et al.* (2009) [8]by means of DNA barcoding as a tool. Thus these literature act as a proof for validating the point of relying on DNA barcoding for highly authentic and accurate outcomes for species evaluation.

VI. CONCLUSION

30 spiders in total were caught and morphometrically analyzed for species identification. Taxonomic identification based on morphometry exposed the occurrence of 3 families, 4 genera and 4 species. The fifth species could not be identified because of a lack of knowledge of Sexual dimorphism and the nonappearance of analytical characteristics for youngsters and subadults. Because of these hurdles, the fifth sample's taxonomic evaluation was done upto the family level (Lycosidae sp.). The fifth sample was very much similar to

the 3rd and was then identified as males of *Pardosa pseudoannulata*while the 3rd sample was the females of *Pardosa pseudoannulata*.

On the basis of DNA Barcoding, one species that was misidentified was placed in the appropriate taxon. The overall accuracy of morphological-based identification was thus 80% only. Similar results were got by Tahir *et al.* (2016)[27]. He studies 872 spiders morphologically and while confirming with their Barcoding, he got an overall accuracy of 88%.

Molecular based identification has proved to be a standard technique for species discrimination due to its authentic,cheap and fast outcomes[27]. In conclusion, it can be said that morphometric-based identification of taxon of any spider species can be satisfactory, still it must be enhanced to improvise the credibility and pace of the outcomes, a blending of molecular and morphometric analysis would be more advantageous.Also, to validate this conclusion, studies with large sample size is in need tomagnify the genuineness of the assessment of spiders' taxon.

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