**DNA Barcoding, an assessment tool for assessing the accuracy of morphometric identification of selected spider species of Mayiladuthurai District, Tamil Nadu, India**

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**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 5 species were captured from May 1st week to 3rd week of 2022. Every individual collected 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 base pair of COI (Cytochrome c Oxidase Subunit I) sequences of 5 specimens were deciphered successfully, through which the presence of 3 families, 4 genera and 4 species. Based on the sequenced results, one mistaken 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 analysis for identifying the spider taxon, is unsatisfactory. Hence to enhance the pace, accuracy and credibility of results, molecular-based taxon identification like DNA barcoding is considerably recommendable. Also, research is needed to confirm the genuineness of the identification of spider species with a large sample size is necessary.

**Keywords -** Spiders, Morphometry, Barcoding, [agroecosystems](https://www.tandfonline.com/keyword/Agroecosystems), [COI](https://www.tandfonline.com/keyword/COI)

1. **INTRODUCTION**

## Spiders are found everywhere on the entire landscapes on earth where life is supported. Spiders are important predators in most terrestrial habitats [1]. Arachnids constitute 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 common hunters and are well-known as common opponents of pests [4]. They are protagonists in forest and agricultural environments and 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 analytical features for young ones are the key steeplechases 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 novel protocol that is used to deliver fast and cost-efficient species identification by which standard taxonomic information [10,11] can be obtained. 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 new discovery of species [24]. The sustainability of DNA Barcoding relays on the fact that the differences in sequences within a species are less than the sequence differences between the species [7, 16, 21, 24,12]. Though there are limitations in Barcoding, many scientists over the world have given possible solutions by introducing the idea of “*integrated barcodes*” [25]. Integrated barcoding combines both DNA and morphometric methods to classify and label a species [26]. The objective of the study was to explore the accountability of identifying the spider wildlife of 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 future study of spider species at the DNA level.

1. **MATERIALS AND METHODS**

### **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.

### **Sampling methods**

To catch the spiders, different sampling methods like, sweep net, hand picking and jerking were employed [8,27]. Foliage spiders were sampled through handpicking and sweep net while spiders from shrubs were captured by jerking [8].

### **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.

### **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 often used for identification were Sebastian, P. A (2009) [28], Barrian and Listinger (1995) [29], Tikader and Malhotra (1980) [30] and other available catalogues 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.

### **DNA Barcoding**

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

* **DNA Barcoding using universal primers of CO1**

**I Protocols**

**Genomic DNA Isolation:**

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. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56oC in a water bath until the tissue was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 minutes. 200 µl of B3 buffer was added and incubated at 70oC for 10 minutes. 210 µl of 100% ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

**Figure 1: Measurement of *Hippasa greenalliae***

* **Agarose Gel Electrophoresis for DNA Quality check**

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel documentation system (Bio-Rad) (Figure 1).

* **PCR Analysis**

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

**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).

* **PCR amplification profile**

**COX1**

98oC - 30 sec

98oC - 5 sec

45oC - 10 sec 10 cycles

72oC - 15 sec

98oC - 5 sec

50oC - 10 sec 30 cycles

72oC - 15 sec

72oC - 60 sec

4oC - ∞

* **Agarose Gel electrophoresis of PCR products**

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 4 µl of PCR products and was loaded and 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. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using the Gel documentation system (Bio-Rad) (Figure 2).

* **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 37oC for 15 minutes followed by enzyme inactivation at 85oC for 5 minutes (as per the User Guide, GE Healthcare).

* **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 |

* **Sequencing PCR amplification profile**

96oC - 2min

96oC - 30sec

50oC - 40sec 30 cycles

60oC - 4min

4oC - ∞

* **Post Sequencing PCR Clean up**

|  |  |
| --- | --- |
| D/W | 5 µl |
| 3M Sodium Acetate | 1 µl |
| EDTA | 0.1 µl |
| 100% Ethanol | 44 µl |

1. Mix D/W, 125mM EDTA, 3M sodium acetate pH 4.6 and ethanol were prepared and were properly mixed.
2. 50 µl of the mix was added to each well in the sequencing plate containing sequencing the PCR product.
3. Vortex by Mixmate vortex and Incubated at room temperature for 30 minutes
4. Spun at 3700 rpm for 30 minutes
5. Decant the supernatant and add 50 µl of 70% ethanol to each well
6. Spun at 3700 rpm for 20 minutes.
7. Decanted the supernatant and repeated 70% ethanol wash
8. Decanted the supernatant and air dried the pellet.

The cleaned-up air-dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

1. **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].

1. **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.

1. **RESULTS AND DISCUSSION**

### **Morphology-based identification:**

A total of 5 specimens representing 3 families, 4 genera and, 4 species were identified morphometrically. Lycosidae was the most abundant family on the ground under detritus material, fallen leaves, and soil crevices during the collection in the fields. However, the family Oxyopidae was the most common 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.

**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 CL – Cephalothorax Length**

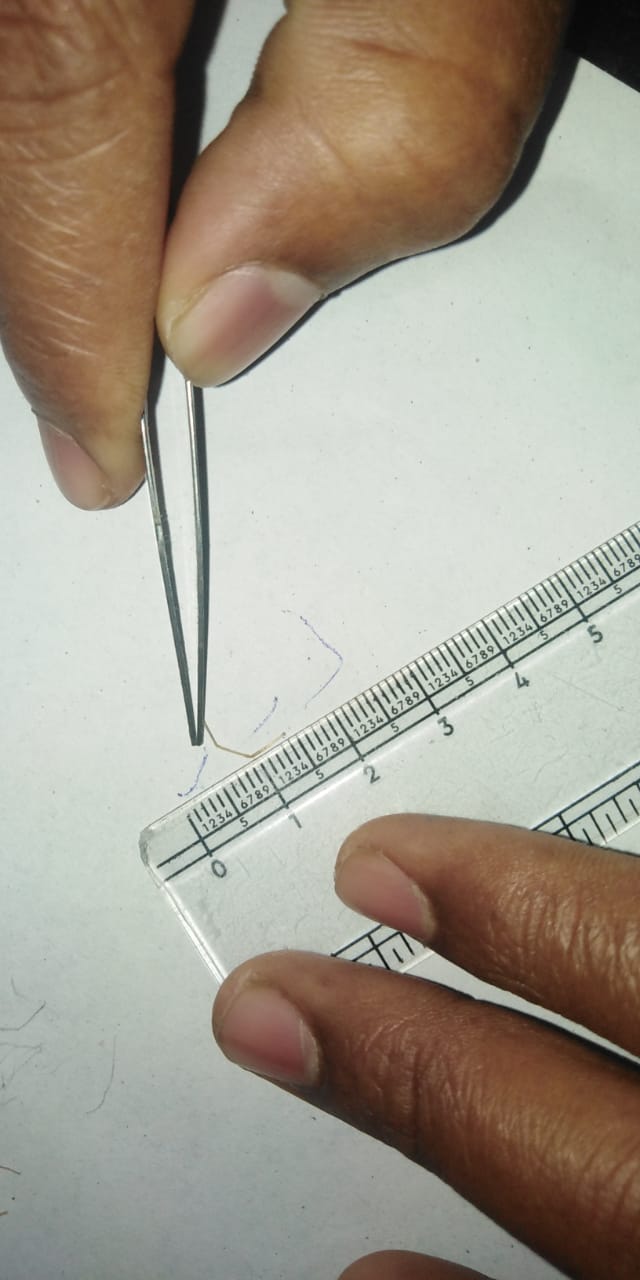
**CW – Cephalothorax Width AL – Abdomen Length**

**AW – Abdomen Width PL – Pedipalp Length**

**Table 2. Details of morphological based 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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**Photos taken during Morphometric analysis**

***Biology of the spider species collected:***

The taxonomic position of the spider species collected is represented in the Table 3. ***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) commonly inhabits arable farm fields and similar open habitats. It is one of the most common surface-dwelling spider species Cosmo politically and plays an important role as a control agent against pests and insects within the crop fields. 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 specie**s

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Sample 1 | Sample 2 | Ample 3 | Sample 4 |
| Kingdom: | Animalia | [Animalia](https://en.wikipedia.org/wiki/Animal) | [Animalia](https://en.wikipedia.org/wiki/Animal) | [Animalia](https://en.wikipedia.org/wiki/Animal) |
| Phylum: | Arthropoda | [Arthropoda](https://en.wikipedia.org/wiki/Arthropod) | [Arthropoda](https://en.wikipedia.org/wiki/Arthropod) | [Arthropoda](https://en.wikipedia.org/wiki/Arthropod) |
| Subphylum: | Chelicerata | [Chelicerata](https://en.wikipedia.org/wiki/Chelicerata) | [Chelicerata](https://en.wikipedia.org/wiki/Chelicerata) | [Chelicerata](https://en.wikipedia.org/wiki/Chelicerata) |
| Class: | Arachnida | [Arachnida](https://en.wikipedia.org/wiki/Arachnid) | [Arachnida](https://en.wikipedia.org/wiki/Arachnid) | [Arachnida](https://en.wikipedia.org/wiki/Arachnid) |
| Order: | Araneae | [Araneae](https://en.wikipedia.org/wiki/Spider) | [Araneae](https://en.wikipedia.org/wiki/Spider) | [Araneae](https://en.wikipedia.org/wiki/Spider) |
| Infraorder: | Araneomorphae | [Araneomorphae](https://en.wikipedia.org/wiki/Araneomorphae) | [Araneomorphae](https://en.wikipedia.org/wiki/Araneomorphae) | [Araneomorphae](https://en.wikipedia.org/wiki/Araneomorphae) |
| Family: | Lycosidae | [Oxyopidae](https://en.wikipedia.org/wiki/Lynx_spider) | [Lycosidae](https://en.wikipedia.org/wiki/Wolf_spider) | Tetragnathidae |
| Genus: | *Hippasa* | [*Oxyopes*](https://en.wikipedia.org/wiki/Oxyopes) | [*Pardosa*](https://en.wikipedia.org/wiki/Pardosa) | *Tetragnatha* |
| **Species:** | ***H. greenalliae*** | ***O. hindostanicus*** | ***P. pseudoannulata*** | ***T. javana*** |



**Figure 2: *Oxyopes hindostanicus***

**Figure 1: *Hippasa greenalliae0***

### 

**Figure 3: *Pardosa pseudoannulata (Female)***

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**Figure 4: *Tetragnatha javana***

**Figure 5: *Pardosa pseudoannulata (Male)***

### **Genetic-based identification:**

To verify the authenticity of the morphology-based identification of spiders, 5 specimens were subjected to DNA barcoding. Genomic sequence of COI up to 600 base pairs was successfully retrieved from those individuals. Morphological identification failed in the precise evaluation of the 5th specimen (Specimen code: AVCC05), which was then allotted the correct taxon on the premise of biological 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 were not 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 growth and developmental differences between both sexes appeared at early life stages, and there was allometric growth in the carapace, abdomen, and gonads between males and females.

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.



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

## 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**

TTGTTACTGCTCATGCTTTTGTAATAATTTTTTTTATAGTTATACCGATTTTAATTGGTGGTTTTGGAAATTGATTAGTACCATTAATATTAGGTGCTCCTGATATATCATTTCCTCGAATGAATAATCTTTCATTTTGATTATTACCTCCTTCTTTATTTTTATTATTTATATCTTCTATAGTATAAATAGGAGTTGGAGCTGGATGAGCTGTTTATCCACCTTTAACTTCTAG

**>SR2856-AV01-COR\_H04.ab1**

CAGGTAAAGAAAGTAATAATAAAATAGCAGTAATTAAAACTGACCAAACAAATAAAGGAACTTTTTCCATTCTTATTCCTATTAATCGTATATTAATAATAGTTGAAATAAAATTTACTGCTCCTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATCTATTGATCTCCCTATATGTCCTATTCTAGAAGCTAAAGGTGGATAAACAGTTCATCCAGCTCCAACTCCTATTTCTACTATAGAAGATATAAATAATAAAAATAAAGAAGGAGGTAATAATCAAAATGAAAGATTATTCATTCGAGGAAATGATATATCAGGAGCACCTAATATTAATGGTACTAATCAATTTCCAAAACCACCAATTAAAATCGGTATAACTATAAAAAAAATTATTACA

**Sample 02:**

**>SR2856-AV02-COF\_D05.ab1**

CAATAAGAGTATTGATTCGAATGGAATTAGGACATTCTGGAAGAATATTAGGAGATGATCATTTGTATAATGTAATTGTTACTGCTCATGCTTTTGTAATGATTTTTTTTATAGTTATACCAATTTTAATTGGTGGATTTGGGAATTGATTAATTCCTTTAATATTAGGAGCTCCTGATATATCTTTTCCTCGTATAAATAATTTATCATTTTGGTTACTTCCTCCTTCTTTATTTTTGTTATTTATATCTTCTATAGTTGAAACTGGGGTTGGGGCAGGATGGACAGTATATCCTCCATTAGCTTCGACTACTGGTCATATAGGAAGATCAATGGATTTTGCTATTTTTTCTTTACATTTAG

**>SR2856-AV02-COR\_D06.ab1**

GCAGGATCAAAAAATGAAGTATTAAAATTTCGATCAGTTAACAATATAGTAATAGCCCCTGCTAATACTGGTAAAGATAACAATAATAAAATAGCAGTAATAAAAACAGATCACACAAATAAAGGAACCTTCTCTATTCTTATACTACTTAATCGTATATTAATAATAGTAGAAATAAAATTTACAGCTCCTATAATAGAAGAAGCACCAGCTAAATGTAAAGAAAAAATAGCAAAATCCATTGATCTTCCTATATGACCAGTAGTCGAAGCTAATGGAGGATATACTGTCCATCCTGCCCCAACCCCAGTTTCAACTATAGAAGATATAAATAACAAAAATAAAGAAGGAGGAAGTAACCAAAATGATAAATTATTTATACGAGGAAAAGATATATCAGGAGCTCCTAATATTAAAGGAACTAATCAATTCCCAAATCCACCAATTAAAATTGGTATAACTATAAAAAAAATCATTACAAAAGCATGAGCAGTAACAATTACATTATACAAATGATCATCTCCTAATATTCTTCCAGAATGTCCTAATTCCATTCGAATCAATACTCTTATTGCTGTTCCAACTATAGCCGATCAAACTCCAAATATTAAATATAAAGTCCCCATTATCTTTTATGA

**Sample 03:**

**>SR2856-AV03-COF\_B05.ab1**

AGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGAATGGAATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAATGTGATGGTTACTGCACATGCTTTTGTGATAATTTTTTTTATAGTAATACCAATTCTTATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCTGATATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACCTTCTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCTGGTTGAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAGTTCGATAGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATTATAGGAGCTGTAAATTTTATTTCTACTATTATTAATATACGAGTGACTGGAATATCAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCAGTTTTATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGTTAACGGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTG

**>SR2856-AV03-COR\_B06.ab1**

CCCCTCCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTTAACAATATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAAAACTGCAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTGATATTCCAGTCACTCGTATATTAATAATAGTAGAAATAAAATTTACAGCTCCTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATCTATCGAACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAGTTCAACCAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAAAATAAAGAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAAAAGATATATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAACCACCAATAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATGGGCAGTAACCATCACATTATATAAATGATCATCACCTAATAATCTACCAGGATTTCCTAATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCAAAGCCGATCAAACTCCAAACATTAAATATAAG

**Sample 04:**

**>SR2856-AV04-COF\_E05.ab1**

GTTTTAATCCGTATTGAATTAGGACAGTCTGGGAGATTTCTTGGGGACGACCAGCTTTATAATGTTATTGTTACTGCTCATGCTTTTGTAATAATTTTTTTTATAGTGATACCTATTTTGATTGGGGGATTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCCCCAGATATAGCTTTTCCTCGTATAAATAATTTAAGTTTTTGGCTTTTACCTCCCTCTCTTTTTATATTATTTGTATCTTCTATGGTGGATATCGGAGTAGGGGCTGGATGAACGGTGTATCCCCCTCTAGCTTCTTTGGAGGGTCATTCGGGAAGATCTGTGGATTTTGCTATTTTTTCGCTTCATTTAGCGGGAGCCTCGTCTATTATAGGAGCAATTAATTTTATTTCTACAATTATTAATATGCGAATGAAAGGAGTCTCGATAGAAAAGGTTCCTCTTTTCGTTTGATCTGTTTTGATTACAGCGGTTTTATTGCTTTTATCCCTTCCCGTTCTACTGGGGCTATTACT

**>SR2856-AV04-COR\_E06.ab1**

GAAAATGAGGTATTAAAATTTCGATCTGTTAATAATATAGTAATAGCCCCAGCTAGAACGGGAAGGGATAAAAGCAATAAAACCGCTGTAATCAAAACAGATCAAACGAAAAGAGGAACCTTTTCTATCGAGACTCCTCTCATTCGCATATTAATAATTGTAGAAATAAAATTAATTGCTCCTATAATAGACGAGGCTCCCGCTAAATGAAGCGAAAAAATAGCAAAATCCACAGATCTTCCCGAATGACCCTCCAAAGAAGCTAGAGGGGGATACACCGTTCATCCAGCCCCTACTCCGATATCCACCATAGAAGATACAAATAATATAAAAAGAGAGGGAGGTAAAAGCCAAAAACTTAAATTATTTATACGAGGAAAAGCTATATCTGGGGCCCCTAATATTAAAGGAACTAATCAATTTCCAAATCCCCCAATCAAAATAGGTATCACTATAAAAAAAATTATTACAAAAGCATGAGCAGTAACAATAACATTATAAAGCTGGTCGTCCCCAAGAAATCTCCCAGACTGTCCTAATTCAATACGGATTAAAACTCTTATTGCAGTCCCCACTATAGCTGATCATACACCAAATAAAAAATATAAACTTCCAATATCTTTATG

## Sample 05:

**>SR2856-AV05-1-COF\_C05.ab1**

TGGAGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGAATGGAATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAATGTGATGGTTACTGCACATGCTTTTGTGATAATTTTTTTTATAGTAATACCAATTCTTATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCTGATATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACCTTCTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCTGGTTGAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAGTTCGATGGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATTATAGGAGCTGTAAACTTTATTTCTACTATTATTAATATACGAGTGACTGGAATATCAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCAGTTTTATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGTTAACGGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTGGTGGAGGGGATCCAATTTTATTTCAACATTTGTTT

**>SR2856-AV05-1-COR\_C06.ab1**

CCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTTAACAATATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAAAACTGCAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTGATATTCCAGTCACTCGTATATTAATAATAGTAGAAATAAAGTTTACAGCTCCTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATCCATCGAACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAGTTCAACCAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAAAATAAAGAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAAAAGATATATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAACCACCAATAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATGTGCAGTAACCATCACATTATATAAATGATCATCACCTAATAATCTACCAGGATTTCCTAATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCATAGCCGATCAAACTCCAAACATTAAATATAA

**Sample 6:**

**>SR2856-AV05-2-COF\_G05.ab1**

TTGGAGTTTGATCGGCTATGATAGGAACTGCTATAAGAGTATTGATTCGAATGGAATTAGGAAATCCTGGTAGATTATTAGGTGATGATCATTTATATAATGTGATGGTTACTGCACATGCTTTTGTGATAATTTTTTTTATAGTAATACCAATTCTTATTGGTGGTTTTGGAAATTGATTAGTTCCTTTAATATTAGGGGCTCCTGATATATCTTTTCCTCGAATAAATAATCTTTCTTTTTGGTTATTACCACCTTCTTTATTTTTATTATCTATATCTTCTATAGTAGAAATAGGGGTTGGTGCTGGTTGAACTGTTTATCCACCGTTAGCGTCTACGGTGGGGCACATGGGAAGTTCGATGGATTTTGCTATTTTTTCTCTTCATTTGGCTGGGGCTTCTTCTATTATAGGAGCTGTAAACTTTATTTCTACTATTATTAATATACGAGTGACTGGAATATCAATAGAAAAGGTTCCTCTATTTGTTTGATCAGTATTAATTACTGCAGTTTTATTATTACTTTCTTTACCTGTTTTAGCAGGAGCTATTACTATATTGTTAACGGATCGAAATTTTAATACTTCTTTTTTTGATCCTGCTGGTGGAGGGGATCCAATTTT

**>SR2856-AV05-2-COR\_G06.ab1**

CCCCTCCACCAGCAGGATCAAAAAAAGAAGTATTAAAATTTCGATCCGTTAACAATATAGTAATAGCTCCTGCTAAAACAGGTAAAGAAAGTAATAATAAAACTGCAGTAATTAATACTGATCAAACAAATAGAGGAACCTTTTCTATTGATATTCCAGTCACTCGTATATTAATAATAGTAGAAATAAAGTTTACAGCTCCTATAATAGAAGAAGCCCCAGCCAAATGAAGAGAAAAAATAGCAAAATCCATCGAACTTCCCATGTGCCCCACCGTAGACGCTAACGGTGGATAAACAGTTCAACCAGCACCAACCCCTATTTCTACTATAGAAGATATAGATAATAAAAATAAAGAAGGTGGTAATAACCAAAAAGAAAGATTATTTATTCGAGGAAAAGATATATCAGGAGCCCCTAATATTAAAGGAACTAATCAATTTCCAAAACCACCAATAAGAATTGGTATTACTATAAAAAAAATTATCACAAAAGCATGTGCAGTAACCATCACATTATATAAATGATCATCACCTAATAATCTACCAGGATTTCCTAATTCCATTCGAATCAATACTCTTATAGCAGTTCCTATCATAGCCGATCAAACTCCAAACATTAAATATAAAGTTCCAATATCTTTATGT

**Table 5. Specimens and GENBANK ACCESSION Nos with their taxonomic identification and coordinates of the collection sites.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **S.No** | **Sample Code** | **GenBank Accession No** | **Taxonomic identification** | **COLLECTION PLACE** | **Coordinates of collection sites** |
| **1** | AVCC01 | ON817272 | *Hippasa greenalliae* | Mannampandal | 11.1064, 79.6750 |
| **2** | AVCC02 | ON834461 | *Oxyopes hindostanicus* | Karraimedu | 11.8137, 79.7316 |
| **3** | AVCC03 | ON817273 | *Pardosa pseudoannulata* | Mannampandal | 11.1064, 79.6750 |
| **4** | AVCC04 | ON817271 | *Tetragnatha javana* | Korkai | 8.62777, 78.0443 |
| **5** | AVCC05-1 | ON908677 | *Pardosa pseudoannulata* | Korkai | 8.62777, 78.0443 |
| **6** | AVCC05-2 | ON892065 | *Pardosa pseudoannulata* | Korkai | 8.62777, 78.0443 |

## Discussion

The main goal of our study was to evaluate the best identification tool for exploring the spider’s identity so 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 sample number is very low due 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. Although, many researchers across the world have reported a large number of species. Overall, in 5 specimens, Family Lycosidae caught was the highest on the ground which was reported by many scientists. Tahir *et al.* (2015) [33] reported Lycosidae as a highly persisting family on the ground. The collection methods that we followed were also recommended by Robinson *et al.* (2009) [8] for sampling.

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 according to the COI sequencing results, it was allotted the correct taxa. Thus, the evaluation of specimens by identifying the COI marker is aptly comparing morphogenetic identification. Goldstein and DeSalle (2003) [35] also reported 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 complete and accurate identification of species. Hebert *et al.* (2003, 2004) [34,24] also published their results in compliance with our results that the DNA barcoding technique is 100% accurate.

**Phylogenetic analysis of the sequences:**

In the present study, the phylogenetic analysis of the sequences obtained was studied by the Neighbor-joining tree. 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. Though all four species belong to the family Araneidae, a noteworthy barcode gap was also observed between the intra and inter-specific divergences. Furthermore, the distance to NN is lower than the maximum intra-specific values. Similar results were reported by Slowik and Blagoev (2012) [36] for the family Araneidae. Overlapping in the divergence’s values for the family Araneidae was observed by Čandek and Kunter (2015) [37] but it was not found in our study which was the only difference observed.

However, in the present study, sample 3 (*Pardosa pseudoannulata*) showed 100% resemblance with sequences of Sample 5*,* when matched with the available sequences at the GenBank. 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 potential growth allometry among the carapace, abdomen, and gonads of spiders in both sexes. Even confusion regarding the identification of this species is prevailing among researchers around the world. Many researchers like Naseem and Tahir (2018) [38] across Pakistan reported this species as *Pardosa birmanica.* Thus, there is still confusion existing in confirming the exact taxon of this particular species. After the results of DNA barcoding, those mistaken specimens were allotted their exact taxon. Robinson *et al.* (2009) [8] described interbreeding, introgression and quick morphological divergences as the possible causes of these kinds of variations. The approach of “integrated barcoding” was also used by Slowik and Blagoev (2012) [36] to overcome these types of issues. There was no overlap between the intra and inter-specific divergences. However, Čandek and Kunter (2015) [37] reported an overlap of the intra and inter-specific values.



**Figure 6: Phylogenetic tree created using the MEGA 11 software**

During the morphological identification of family Oxyopidae specimens due to variations of colour and body patterns, great indistinctness was recorded. Finally, it was identified correctly. Bond *et al.* (2001) [9] also reported the problem of morphological differences in the same species and suggested molecular approaches like DNA barcoding to overcome these hurdles.

For the total of 5 specimens of the present study, the reliability of the results depends on a significant barcode gap which was observed in the intra and inter-specific divergences [38]. Moreover, values of maximum intra-specific divergences were lower than the distance to NN for every species. When the results were blasted, they showed 100% similarity to the species identified. These 100% successful results are in accordance with the results of Barrett and Hebert ([2005](https://www.tandfonline.com/doi/full/10.1080/23802359.2019.1693283)) [7], who correctly evaluated the 168 species of spiders using molecular tools of DNA barcoding. Čandek and Kunter [37] also suggested the use of DNA barcoding for the evaluation of spider species. Tahir *et al.* (2016) [27] also identified 5 spider species with 100% success using DNA barcoding. Robinson *et al.* (2009) [8] also successfully described the 19 species-rich genera using DNA barcoding. All these results validate the point of relying on DNA barcoding for highly accurate and authentic results for species evaluation.

**Conclusion:**

## A total of 30 spiders were captured and morphometrically analyzed for species identification. Morphological identification revealed the presence 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 absence of diagnostic characteristics for juveniles 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 molecular results, one species was misidentified. 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%.

## DNA barcoding has proved to be a standard species discriminatory technique due to its fast, cheap and authentic results [27]. In conclusion, it can be said that morphometric-based approaches to describe any spider species are satisfactory but to improvise the pace and credibility of the results, a combination of DNA barcoding is advantageous. Furthermore, to magnify the authenticity of the evaluation of spiders taxonomically, studies with large sample size is needed.

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