**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. All the individuals 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 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. A 658-base pair sequence of COI (Cytochrome c Oxidase Subunit I) of 5 specimens was retrieved successfully, which confirmed the presence of 3 families, 4 genera and 4 species. On the basis of molecular results, one misidentified specimen was then allotted the correct taxon. The overall accuracy of morphometry-based identification was 80%. It is concluded from the present study that morphometric analysis to identify a spider, is not satisfactory and hence to enhance the accuracy, pace and credibility of results, molecular technique like DNA barcoding is considerably recommendable. Furthermore, research is needed to magnify the authenticity of the evaluation of spiders 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 generalist predators and are known as natural enemies of pests [4]. Their role in forest and agricultural environment as a stabilizer of insect pest density is very critical [5]. These environments also support a wide range of prey types [6] for these specialist predators. Spiders are strong bio-control agents due to the features like mortality of non-consumed pests in webs, excessive killing and partial consumption of prey.

It is time-consuming and laborious job to identify the Spider species using morphometric analysis for different reasons [7]. Sexual dimorphism and the absence of diagnostic characteristics in juveniles are the main hurdles in the authentic evaluation of spiders [8]. DNA barcoding is a molecular identification tool which is being employed to overcome these kinds of problems [9]. It is a novel protocol which 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 diversity of standardized regions (658 base pairs) of the mitochondrial genome called biological barcode, which allows the species level identification [12]. DNA barcoding has been successfully used as a tool to evaluate organisms relating to a different group including the 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 not only used in the successful molecular descriptions of already identified species but also assists in discovering novel species [24]. The success and future of DNA Barcoding dependents upon the fact that genetic differences within a species are less than the 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 the molecular and morphometric approaches to identify and describe a species [26]. The objective of the study was to explore the accountability of identifying the spider’s fauna of the Mayiladuthurai District, Tamil Nadu, India by morphometric analysis and assessing its accuracy comparing with the Barcoding technique. Another motive of the study is to establish an add-on data to the genetic reference library for future study of spider species at molecular 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**

Different sampling methods including the jerking, sweep net and hand picking were employed to catch the spiders [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. The 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 assistance of multiple available keys. Frequently used keys 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 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 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 mix was added to each well in the sequencing plate containing sequencing PCR product.
3. Vortex by Mixmate vortex and Incubated at room temperature for 30 minutes
4. Spun at 3700 rpm for 30 minutes
5. Decanted the supernatant and added50 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 on morphological basis. The most abundant family on the ground under fallen leaves, detritus material and soil crevices in the fields was Lycosidae. However, family Oxyopidae was most common on foliage. 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 the Table 1 (mean value + standard deviation in mm). On the basis of the morphometric analysis and referring the literatures, the species identification was done and 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 greenalliea*** | 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**

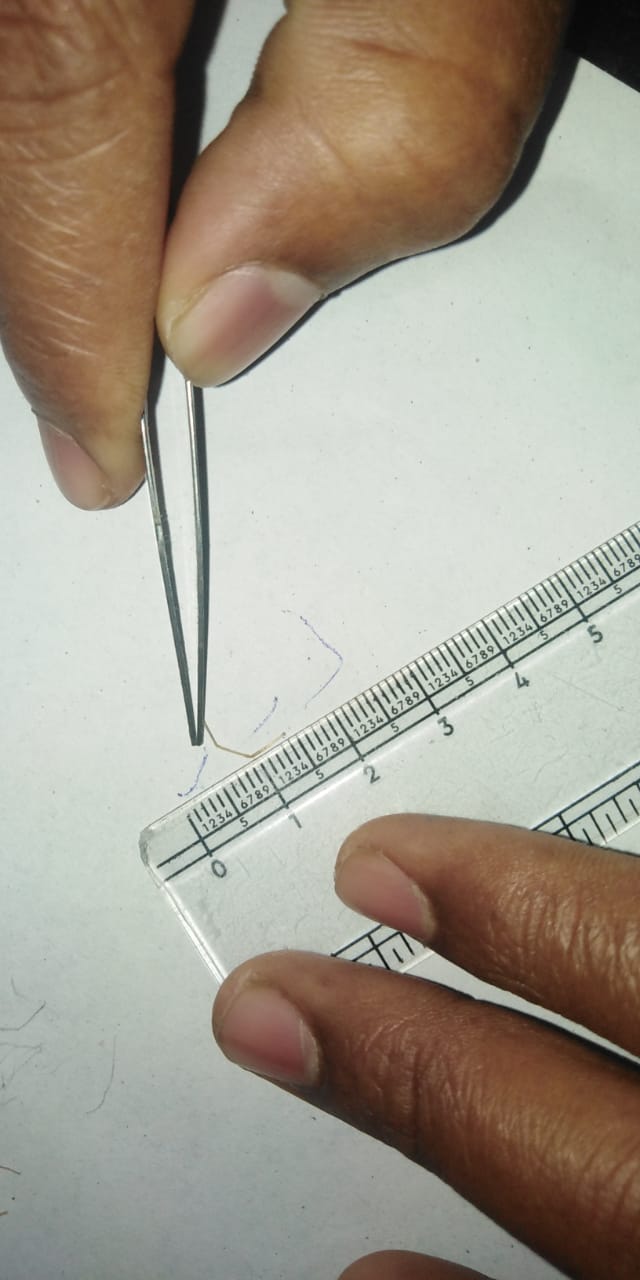
**CW – Cephalothorax Width AL – Abdomen Length**

**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 of the genus *Hippasa*. It is 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 the genus *Oxyopes*. The adult is 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***

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**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 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 precise evaluation of 5th specimens (Specimen code: AVCC05), which was then allotted the correct taxon on the premise of biological barcode sequence as depicted in the Table 4. Actually, the 5th sample was the subadult species of the 3rd sample, the species *Pardosa pseudoannulata.* DNA barcoding affirm the presence of 3 families, 4 genera and 4 species. Overall, accuracy of morphology-based identification was 80%.

**Table 4. Morphologically misidentified specimens along with their 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 diversity and to compare the efficacy of DNA barcoding with morphological-based evaluation for species identification. Comparing to molecular-based evaluation, morphological-based identification’s success rate was 80%. Absence of diagnostic characters and availability of identification keys for juvenile and subadult spiders could be the possible factors for low success rate. DNA sequence retrieval for all the 5 specimens belonging to different genera was successful.

In the present study, a total of 5 spiders species were identified morphologically belonging to the family Lycosidae, Oxyopidae and Tetragnathidae. We could report only a proportion of spider’s species due to insufficient time and sampling effort and also due to the high-cost DNA sequencing technology. Although, many researchers across the world have reported large number of species. Overall, in 5 specimens, Family Lycosidae caught was the highest on ground which was reported by many scientists. Tahir *et al.* (2015) [33] reported Lycosidae as a dominant family on ground. During the present study, hand picking and jerking method were used for spider’s collection. Robinson *et al.* (2009) [8] also used the hand picking and jerking methods for sampling.

The 5′ end of Biological barcode (COI) was selected for species discrimination through DNA barcoding because of availability of the primers for recovery of required DNA from wide range of taxa [34]. During the morphometric analysis, we misidentified 1 specimen and then according to the molecular results, we allotted them their correct taxa. Thus, the evaluation of specimens by identifying the DNA barcode for specimens is apt with fewer diagnostic characters itself. Goldstein and DeSalle (2003) [35] reported the recovery of DNA from century old specimens thorough molecular techniques. We concluded from these results that molecular methods like DNA barcoding are necessary for complete and accurate species identification. Hebert *et al.* (2003, 2004) [34,24] reported the DNA barcoding, a technique with 100% accuracy.

**Phylogenetic analysis of the sequences:**

Neighbor joining tree separated the specimens into different species with genetic difference of 2% or more in the present study. For family Araneidae, a significant 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. Slowik and Blagoev (2012) [36] reported the same results for family Araneidae as we did in our study. There was no overlap between the intra and inter-specific values for family Araneidae in present study but Čandek and Kunter (2015) [37] found the overlapping in the divergence’s values for family Araneidae.

However, in the present study, the 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] has 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 confusions regarding the identification of this species are prevailing among the researchers around the world. Many researchers like Naseem and Tahir (2018) [38] across the 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 misidentified specimens were allotted their exact taxon. Robinson *et al.* (2009) [8] described hybridization, introgression and quick morphological divergences as the possible causes for these kinds of variations. 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**

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

For the total of 5 specimens of present study, a significant barcode gap was observed in the intra and inter-specific divergences indicating the reliability of the results [38]. Moreover, values of maximum intra-specific divergences were lower than the distance to NN for every species. These results showed the 100% accuracy in identifying the juvenile and adult spiders using the molecular method in this study. 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 tool of DNA barcoding. Čandek and Kunter [37] also suggested the use of DNA barcoding for evaluation of spider’s 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 lack of knowledge on Sexual dimorphism and absence of diagnostic characters for juveniles and subadults. Because of these hurdles, the fifth sample’s taxonomic evaluation was done upto family level (Lycosidae sp.).

## To evaluate the authenticity of morphological identification, tissue samples of 5 specimens were sent to RGCB, Trivandrum. A 658-base pair sequence of COI (Cytochrome c Oxidase Subunit I) of 5 specimens was retrieved successfully, which confirmed the presence of 3 families, 4 genera and 4 species. 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. 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 appeared to be a standard species discriminatory technique due to its cheap, fast 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, combination of DNA barcoding is advantageous. Furthermore, to magnify authenticity of evaluation of spiders taxonomically, studies with large sample size is needed.

**REFERENCES**

[1] Wise, D.H. (1993): Spiders in Ecological Webs. Cambridge. Univ. Press, Cambridge, UK

[2] Coddington, J.A. and Levi, H.W. (1991): Systematics an d evolution of spiders Araneae. Ann. Rev. Eco. Syst., 22: 565-592.

[3] Platnick, N. I. (2014). The world spider catalog, version 15.0. American Museum of Natural History. <http://research.amnh.org/iz/spiders/catalog/INTRO3.html>.)

[4] Maloney D. 2003. Spider predation in agroecosystems: can spiders effectively control pest populations? Orono (ME): Maine Agricultural and Forest Experiment Station, The University of Maine.

[5] Ghavami S. 2008. The potential of predatory spiders as biological control agents of cotton pests in Tehran provinces of Iran. Asian J Exp Sci. 22: 303–306.

[6] Sunderland K, Samu F. 2000. Effects of agricultural diversification on the abundance, distribution, and pest control potential of spiders: a review. Entomol Exp Appl. 95(1): 1–13. 

[7] Barrett RDH, Hebert PDN. 2005. Identifying spiders through DNA barcodes. Can J Zool. 83(3): 481–491.

[8] Robinson EA, Blagoev GA, Hebert PDN, Adamowicz SJ. 2009. Prospects for using DNA barcoding to identify spiders in species-rich genera. ZooKeys. 16: 27–46.

[9] Bond JE, Hedin MC, Ramirez MG, Opell BD. 2001. Deep molecular divergence in the absence of morphological and ecological change in the Californian coastal dune endemic trapdoor spider *Aptostichus simus*. Mol Ecol. 10(4): 899–910.

[10] Hebert PDN, Gregory TR. 2005. The promise of DNA barcoding for taxonomy. Syst Biol. 54(5): 852–859.

[11] Miller SE. 2007. DNA barcoding and the renaissance of taxonomy. Proc Nat Acad Sci. 104(12): 4775–4776.

[12] Hajibabaei M, Singer GA, Hebert PDN, Hickey DA. 2007. DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. Trends Genet. 23(4): 167–172. 

[13] Clare EL, Lim BK, Engstrom MD, Eger JL, Hebert PD. 2007. DNA barcoding of Neotropical bats: species identification and discovery within Guyana. Mol Ecol Resour. 7(2): 184–190.

[20] Vences M, Thomas M, Bonett RM, Vieites DR. 2005. Deciphering amphibian diversity through DNA barcoding: chances and challenges. Phil Trans R Soc B. 360 (1462): 1859–1868. 

[14] Lukhtanov VA, Sourakov A, Zakharov EV, Hebert PDN. 2009. DNA barcoding Central Asian butterflies: increasing geographical dimension does not significantly reduce the success of species identification. Mol Ecol Resour. 9(5): 1302–1310.

[15] Kerr KC, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PDN. 2007. Comprehensive DNA barcode coverage of North American birds. Mol. Ecol. Resour. 7(4): 535–543.

[16] Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. 2005. DNA barcoding Australia's fish species. Phil Trans R Soc B. 360(1462):1847–1857. 

[17] Meier R, Shiyang K, Vaidya G, Ng PK. 2006. DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Syst Biol. 55(5): 715–728.

[18] Saunders GW. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Phil Trans R Soc B. 360 (1462): 1879–1888.

[19] Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, *et al.* 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Nat Acad Sci. 109(16): 6241–6246.

[20] Vences M, Thomas M, Bonett RM, Vieites DR. 2005. Deciphering amphibian diversity through DNA barcoding: chances and challenges. Phil Trans R Soc B. 360 (1462): 1859–1868. 

[21] Smith MA, Fisher BL, Hebert PDN. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. Phil Trans R Soc B. 360(1462): 1825–1834.

[22] Witt JD, Threloff DL, Hebert PDN. 2006. DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. Mol Ecol. 15 (10): 3073–3082.

[23] Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Janzen DH, Hallwachs W, Hebert PDN. 2008. Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. Proc Nat Acad Sci. 105(34): 12359–12364.

[24] Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proc Nat Acad Sci USA. 101(41): 14812–14817. 

[25] Rubinoff D. 2006. Utility of mitochondrial DNA barcodes in species conservation. Conserv Biol. 20(4): 1026–1033.

[26] Gibbs J. 2009. Integrative taxonomy identifies new (and old) species in the Lasioglossum (Dialictus) tegulare (Robertson) species group (Hymenoptera, Halictidae). Zootaxa. 2032(1): 1–38. 

[27] Tahir HM, Naseem S, Akhtar S, Ashfaq M, Butt A, Mukhtar MK. 2016. DNA barcode record of some common spiders from Punjab, Pakistan. Pakistan J Zool. 48(1): 159–164. 

[28] Sabestin, P. A. and Peter, K. V. 2009. Spiders of India, Universities Press, Hyderabad, India.

[29] Barrion AT, Litsenger JA. 1995. Riceland spiders of South and Southeast Asia. CAB International, Wallingford, UK.

[30] Tikadar BK, Molhotra MS. 1980. Lycosidae (Wolf Spiders). Fauna India (Araneae), 1: 248–447.

### [31] Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Heled J, Kearse M, Moir R, Stones-Havas S, Sturrock S, Thierer T and Wilson A (2010) Geneious v5.1, Available from <http://www.geneious.com>

[32] Zhang, F., Chen, X., Zeng, C., Wen, L., Zhao, Y. and Peng Y. (2021). Modest sexual size dimorphism and allometric growth: a study based on growth and gonad development in the wolf spider *Pardosa* *pseudoannulata* (Araneae: Lycosidae). The Company of Biologists, 10: 1-11.

[33] Tahir HM, Yaqoob, Naseem S, Sherawat SM, Zahra K. 2015. Effect of insecticides on predatory performance of spiders. Biologia (Pakistan): 61: 127–131.

[34] Hebert PDN, Cywinska A, Ball SL, DeWaard JR. 2003. Biological identifications through DNA barcodes. Proc R Soc Lond B. 270(1512): 313–321.

[35] Goldstein PZ, Desalle R. 2003. Calibrating phylogenetic species formation in a threatened insect using DNA from historical specimens. Mol Ecol. 12(7): 1993–1998. 

[36] Slowik J, Blagoev GA. 2012. A survey of spiders (Arachnida: Araneae) of Prince of Wales Island, Alaska; combining morphological and DNA barcode identification techniques. Insecta Mundi. 251: 1–12. 

[37] Čandek K, Kuntner M. 2015. DNA barcoding gap: reliable species identification over morphological and geographical scales. Mol Ecol Resour. 15(2): 268–277.

[38] Naseem S, Tahir HM. 2018. Use of mitochondrial COI gene for the identification of family Salticidae and Lycosidae of spiders. Mitochondrial DNA. 29(1): 96–101.