**ABSTRACT**

Spiders are important insect predators and being used as model organisms in various investigatory studies however, work on this group is difficult due to the constraints in their species level identification. DNA barcoding is a promising approach to minimize the difficulties of taxonomists to delimit species. Current study was designed to provide first barcode record of some common spiders from Punjab, Pakistan. The standard barcode region (658 base pairs of Cytochrome C oxidase subunit I), in multiple specimens of all species, was sequenced. DNA based data corresponded the morphological study, confirming the presence of five species i.e., Clubiona analis Thorell, 1895, Cyrtophora citricola (Forskål, 1775), Leucauge decorata (Blackwall, 1864), Neoscona theisi (Walckenaer, 1841) and Oxyopes javanus Thorell, 1887. There was clear gap in intraspecific and interspecific values of genetic distances. For every species, the maximum interspecific divergence was less than the minimum distance to its nearest-neighbour. The divergence values within and between species ranged from 0–1.6% and 17.6–23.7%, respectively. Five species were also clearly separated based on parsimony analysis. It is concluded that DNA barcoding is a reliable tool to delimit studied spider species.

**INTRODUCTION**

Species level identification is the basis for understanding species diversity, phylogenetic patterns and evolutionary processes. Up till now about 45,000 species of spiders have been described (Platnick, 2014) and a large number of species awaiting discovery (Agnarsson et al., 2013). Routine taxonomy, based on morphology alone, is often time consuming and complex (Barret and Hebert, 2005). There are many reasons for this complexity i.e., most life stages are unidentifiable as most identification keys rely on examination of adults and many species show sexual dimorphism so different criteria is required to identify male and female. Furthermore, we cannot differentiate cryptic species (Bickford et al., 2007), or species complexes with morphological characters (Hajibabaei et al., 2006). Due to all these complications spider identification at species level is a challenging task.

Different molecular methods are increasingly being applied to overcome complexities of taxonomists (Navajas and Fenton, 2000). Among these DNA barcoding is a popular and most commonly used molecular method for species identification (Nagoshi et al., 2011; Van der Bank et al., 2012). In this method shortstandardized (658 base pairs) gene region, usually the 5' end part of the mitochondrial Cytochrome C oxidase subunit I (COI), is used (Hebert et al., 2003a). This specific sequence is known as DNA barcode as it is like a barcode tag for each taxon (Jinbo et al., 2011). The COI is a good target for animal barcoding as it is present in all animals, and insertions and deletions in this region are rare. Furthermore, it possesses enough sequence divergence required for species discrimination (Hebert et al., 2003 b).

DNA barcoding is an innovative tool in molecular systematics. Among different molecular approaches, it has received increase acceptance for identification and delimitation of new species from various groups of animals (Hebert et al., 2004; Kerr et al., 2009; Smith et al., 2007), as it is simple, fast and affordable method (Padial and De La Riva, 2007). DNA barcoding also help to identify individuals at any stage of development. Using DNA barcoding even a non-taxonomist can easily identify and discriminate a matrix containing a mixture of biological species (Casiraghi et al., 2010).

Present study was designed to evaluate the effectiveness of COI barcode region in identifying common spiders from agro-ecosystems of Punjab, Pakistan and to assess and compare the magnitude of divergence in COI sequences within and between species. This study would provide a first barcode record of some common spiders from Pakistan. Present study is also a
first step to develop a DNA barcode reference library for the spiders of Pakistan.

**MATERIALS AND METHODS**

**Collection of spiders**

Spiders were collected from different localities of Punjab, Pakistan by visual searching and using a portable battery operated suction device (SIEMENS VK). GPS coordinates were recorded with the help of a portable Garmin™ GPS. The species distribution map, based on all locality points within Pakistan available from the literature (Mukhtar and Mushtaq, 2005; Butt and Siraj, 2006; Butt and Tahir, 2010; Ursani and Soomroo, 2010; Tahir et al., 2011, 2012; Mukhtar, 2012; Mukhtar et al., 2012; Perveen and Jamal, 2012; Perveen et al., 2012; Sial et al., 2012; Rafay et al., 2014) was produced using ArcMap 10.1. Collected spiders were washed with 75% alcohol and preserved in absolute alcohol with the proper labelling of collection site, date of collection, collector name and other notes of importance.

**Morphological identification**

Each spider was identified to species level on the basis of morphological characters with the help of available literature (Dyal, 1935; Tikader and Malhotra, 1980; Tikader, 1982; Tikader and Biswas, 1981; Barrion and Litsinger, 1995; Changmin et al., 1997; Patnick, 2014), photographed and assigned a number. Multiple specimens of each species were stored at -40°C for subsequent DNA isolation and sequence.

**Extraction of DNA and mtCOI PCR amplification**

Genomic DNA was extracted from spiders using the Thermo Scientific Gene JET Genomic DNA Purification kit. Left leg of first pair of each spider was used as DNA source. Amplification of the barcode region was performed with primer pair LCO1490 (GGTCAACAAATCATAAAGATATTGG) HCO2198 (TAAAATATCGTGACCAAAA) following the PCR conditions; an initial denaturing step at 94°C for 5 min, 35 amplification cycles (94°C for 15 s, 49°C for 15 s, 72°C for 15 s), and a final step at 72°C for 7 min in a thermocycler. Specific conditions were optimized for spiders of different taxa. PCR products were verified on 1% agarose/TBE electrophoresis gel. PCR products, which yielded good quality bands, were selected for sequencing. Amplicons were sequenced bi-directionally using the BigDye Terminator Cycle Sequencing Kit (v3.1) (Applied Biosystems) on an Applied Biosystems 3730XL DNA Analyzer.

**Genetic distance and phylogenetic tree**

Sequences (both forward and reverse) were cleaned and edited using DNA baser. MUSCLE, a multiple sequence alignment programs in TNT software, was used for sequence alignment. Aligned sequences were converted into FASTA format. MEGA5.2 was consulted to verify that the sequences are free of stop codons and gaps. MEGA5.2 was also used for pairwise distance analysis. Distance histograms and distance ranks were generated using online version of Automatic Barcode Gap Discovery (ABGD). A species was considered distinct from its nearest neighbour (NN) if its maximum intraspecific distance was less than the distance to its NN sequence (Ashfaq et al., 2014). The sequences generated during this study were also compared to barcode sequences on GenBank using "BLAST" function. A phylogenetic tree, using the maximum parsimony (MP) method, was also constructed with the help of MEGA5.2. Finally all sequences generated during this study were submitted to BOLD in the dataset DS-MTSPD.

**RESULTS**

Morphologically five species of spiders, each belonging to different genera, were identified i.e., *Clabonia analis* Thorell 1895, *Cyrtophora citricola* (Forskål, 1775), *Leucauge decorata* (Blackwall, 1864), *Neoscona theisi* (Walckenaer, 1841) and *Oxyopes javanus* Thorell, 1887. Barcode sequences (658bp for all specimens) generated during the study also confirmed the presence of five species. Figure 2 shows the number of specimens of each species with a barcode sequence. Locality distribution points of all species based on previous known records have been plotted in Figure 1.

The histograms of distances and ranked distances computed using ABGD analysis is shown in Figure 3. The intraspecific and interspecific distances revealed a clear gap (Fig. 3). The maximum interspecific value for every species is less than the minimum distance to the nearest-neighbour (NN) (Fig. 4). The divergence of barcode sequences within and among different species ranged from 0–1.6% and 17.6–23.7%, respectively (Table I). Phylogenetic analysis clearly separated the spiders of five different genera (Fig. 5).

BLAST results showed that sequences of *N. theisi* and *C. citricola* are already available in GeneBank data. There are only two barcode records for *C. citricola* in GeneBank. Our barcode sequences were 98% similar with sequence of same species from India (Accession No. KJ206564). However, there was 94% resemblance with the spider of same species reported from Slovenia (Accession No. K849071). Similarly, for *N. theisi*, only two barcode sequences are available in GeneBank. One sequence is from Japan (Accession No. AB969823) and
Fig. 1. Maps plotting the known records of five spiders in Pakistan. In the above figure symbol ● is for *Clubiona analis* Thorell 1895; ○ for *Cyrtophora citricola* (Forskål, 1775); ▲ for *Leucauge decorata* (Blackwall, 1864); ► for *Oxyopes javanus* Thorell, 1887 and ■ for *Neoscona theisi* (Walckenaer, 1841). 
*Note:* ● nbol is indicating the common locality points of *Clubiona analis* Thorell 1895 and *Cyrtophora citricola* (Forskål, 1775).

![Map of Pakistan with spider records](image1)

Fig. 2. Number of specimens of each species with barcode sequence.

![Bar graph of spider species](image2)

other from South Korea (Accession No. JN817155). Both of these sequences showed 99% resemblance to our

<table>
<thead>
<tr>
<th>Species</th>
<th>Intraspecific Divergence (Mean±SEM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clubiona analis</em></td>
<td>0.48±0.026</td>
<td>0.00-0.90</td>
</tr>
<tr>
<td><em>Cyrtophora citricola</em></td>
<td>0.00±0.00</td>
<td>0.00-0.00</td>
</tr>
<tr>
<td><em>Leucauge decorata</em></td>
<td>0.13±0.06</td>
<td>0.00-0.20</td>
</tr>
<tr>
<td><em>Neoscona theisi</em></td>
<td>0.61±0.07</td>
<td>0.30-0.80</td>
</tr>
<tr>
<td><em>Oxyopes javanus</em></td>
<td>0.95±0.69</td>
<td>0.50-1.60</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Interspecific Divergence (Mean±SEM)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clubiona analis</em></td>
<td>22.76±0.54</td>
<td>21.70-23.70</td>
</tr>
<tr>
<td><em>Cyrtophora citricola</em></td>
<td>18.57±0.01</td>
<td>18.40-18.70</td>
</tr>
<tr>
<td><em>Leucauge decorata</em></td>
<td>18.26±0.12</td>
<td>17.60-18.90</td>
</tr>
<tr>
<td><em>Neoscona theisi</em></td>
<td>18.26±0.12</td>
<td>17.60-18.90</td>
</tr>
<tr>
<td><em>Oxyopes javanus</em></td>
<td>21.81±0.10</td>
<td>21.00-22.60</td>
</tr>
</tbody>
</table>
evaluate its potential to delimit spider species. We got 100% success to discriminate five spider species using this method. There was strong correspondence between morphological identifications and the identification based on barcode sequences. Blagoev et al. (2013) and Raso et al. (2014) also observed strong correspondence in morphological and molecular data while identifying spiders. These results suggest that although DNA barcoding is an effective tool for spider identification, morphological studies have also been effective in recognizing spider species. Slowik and Blagoev (2012), Franzini et al. (2013) and Candek and Kuntner (2014) combined morphological and DNA barcode data for identification of spiders and got more success than using morphological data alone.

In this study we used DNA barcoding technique to identify five spider species. We did not find any close match for *C. analis*, *L. decorata* and *O. javanus* in GeneBank.

**DISCUSSION**

In this study we used DNA barcoding technique to

specimens. We did not find any close match for *C. analis*, *L. decorata* and *O. javanus* in GeneBank.

**DISCUSSION**

In this study we used DNA barcoding technique to
gap between intra and interspecific genetic distances for all five species, suggesting the reliability of results (Table I).

Our result, that there is no overlap between mean nucleotide divergences at the intraspecific and interspecific levels, is in accordance with the findings of Barret and Hebert (2005). Robinson et al. (2009) used DNA bar-coding to discriminate species of species rich genera. The average maximum intraspecific divergence and the mean divergence between nearest interspecific neighbours recorded by them were 3.16% and 6.77%, respectively. Similarly, Blagoev et al. (2013) reported that in most cases of spiders, the maximum intraspecific sequence variation was <1%. Barret and Hebert (2005) recorded at least 3% sequence divergence in congeneric species pairs. Furthermore, they did not record any overlap between mean nucleotide divergences at the intra- and inter-specific levels. The effectiveness of barcode region is greatly reduced for the families of spiders with high overlap between mean nucleotide divergences at the intraspecific and interspecific levels.

Ashfaq et al. (2013, 2014) have successfully identified the butterflies and mosquitoes of Pakistan using DNA barcoding. In butterflies they recorded 1.6% divergence in conspecifics while 1.7–14.3% divergence from the nearest neighbour species. Similarly, the barcode sequence divergence in conspecific mosquitoes ranged from 0–2.4% compared with 2.3–17.8% in congeneric species. In our study, the interspecific divergence ranged from 17.6–23.7%. The interspecific divergence for Cyrtophora citricola recorded during our study was between 18.4% and 18.7% as depicted in Table I. These results are in correspondence with Keith (2010) and Franzini et al. (2013). They also recorded high interspecific divergence in clusters of Cyrtophora species.

Based on the literature it could be concluded that divergence values differ within different taxa and defining a universal threshold in the bar-coding gap is difficult (Yassin et al., 2010). Furthermore, the success of DNA barcodes based identification is largely dependent on the establishment of DNA barcode reference library for the specimens with confirmed taxonomy. Achievement of this goal requires cooperation of scientists and institutions internationally (Barrett and Hebert, 2005).

REFERENCES


Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T. S. and Francis, C.


