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DNA Barcode Record of Some Common Spiders from Punjab, Pakistan

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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 constrains 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 interaspecific 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.

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Authors' Contributions
HMT and AB analyzed the data and wrote the manuscript. SN and SA collected the animals, and preserved the tissues and prepared the map. MA extracted DNA for nucleotide sequencing.

Key words
Spiders, DNA barcode, genetic distance, divergence.

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

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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 researching 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; Platnick, 2014), photographed and assigned a number. Multiple specimens of each species were stored at -40°C for subsequent DNA isolation and sequences.

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
LCOI490 (GGTCAACAAATCATAAAGATATTGG)
HCO2198 (TAAACTTCAGGGTGACCAAAAAA)
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.*, *Clubiona 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 interaspecific 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. KC849071). Similarly, for *N. theisi,* only two barcode sequences are available in GeneBank. One sequence is from Japan (Accession No. AB969823) and

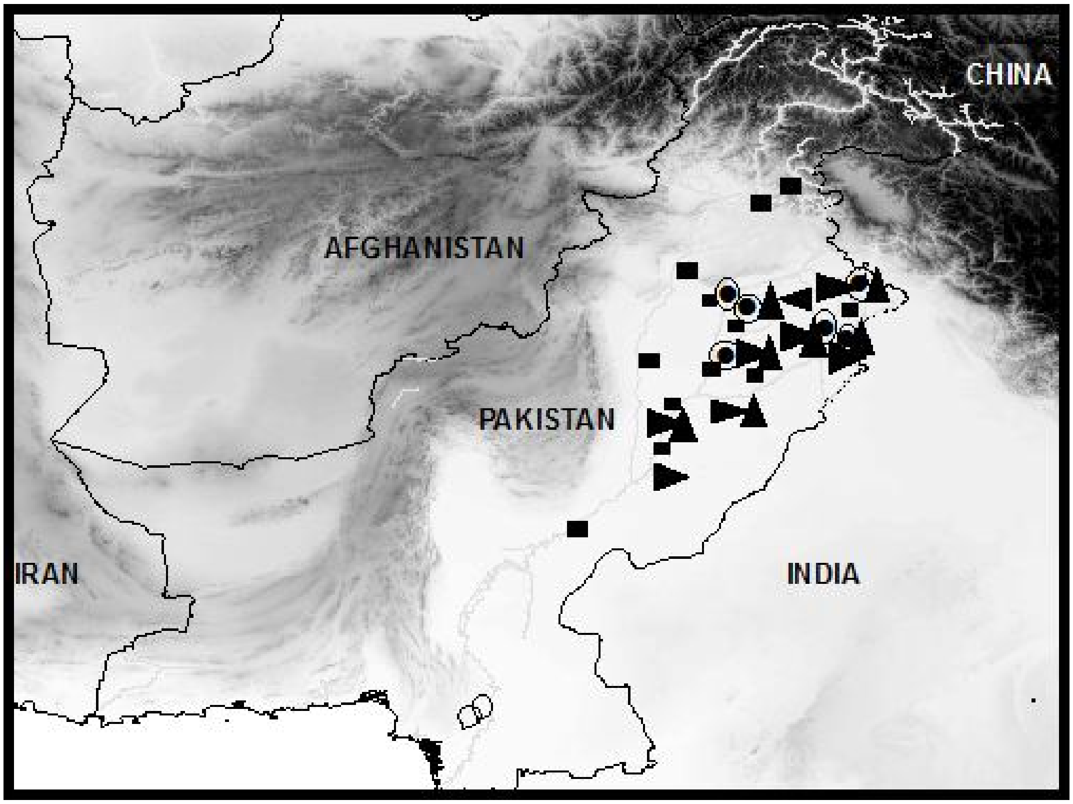

Fig. 1. Maps plotting the known records of five spiders in Pakistan. In the above figure symbol● is for *Clubiona analis* Thorell 1895; ○ for *Cyrtophora citricola* (Forskål, 1775); ▲ for *Leucauge decorata* (Blackwall, 1864); ► for *Oxyopes javanus* Thorell, 1887 and ■ for *Neoscona theisi* (Walckenaer, 1841).

Note: ◉ nbol is indicating the common locality points of *Clubiona analis* Thorell 1895 and *Cyrtophora citricola* (Forskål, 1775).

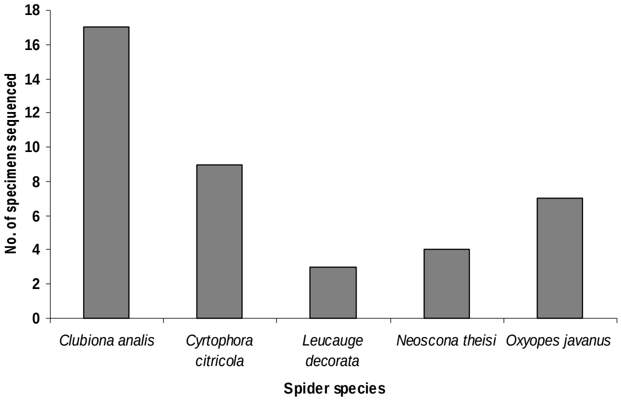

Fig. 2. Number of specimens of each species with barcode sequence.

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

Table I.- Intraspecific and iternspecific divergence for five spider species groups generated for COI gene.

Species	Divergence (Mean±SEM)	Range
Intraspecific divergence		
Clubiona analis	0.48±0.026	0.00-0.90
Cyrtophora citricola	0.00±0.00	0.00-0.00
Leucauge decorata	0.13±0.06	0.00-0.20
Neoscona theisi	0.61±0.07	0.30-0.80
Oxyopes javanus	0.95±0.69	0.50-1.60
Interspecific divergence		
Clubiona analis	22.76±0.54	21.70-23.70
Cyrtophora citricola	18.57±0.01	18.40-18.70
Leucauge decorata	18.26±0.12	17.60-18.90
Neoscona theisi	18.26±0.12	17.60-18.90
Oxyopes javanus	21.81±0.10	21.00-22.60

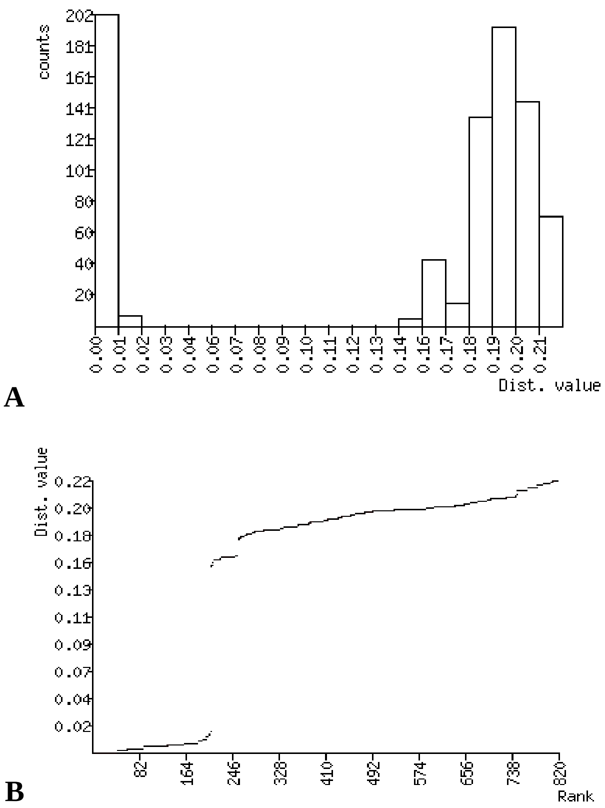

Fig. 3. Histograms of distances (A) and rank distances (B) generated by ABGD.

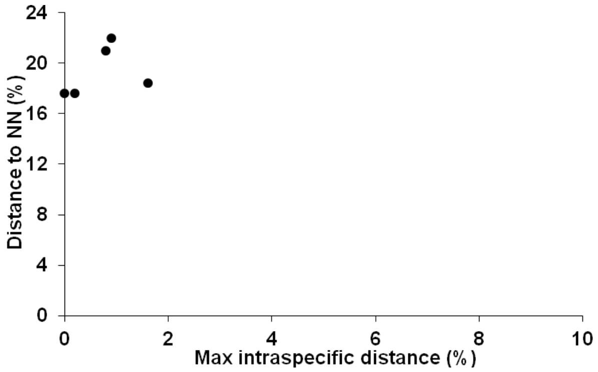

Fig. 4. Barcode gap distances at COI for five common species of spiders. NN= Nearest neighbour.

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 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.

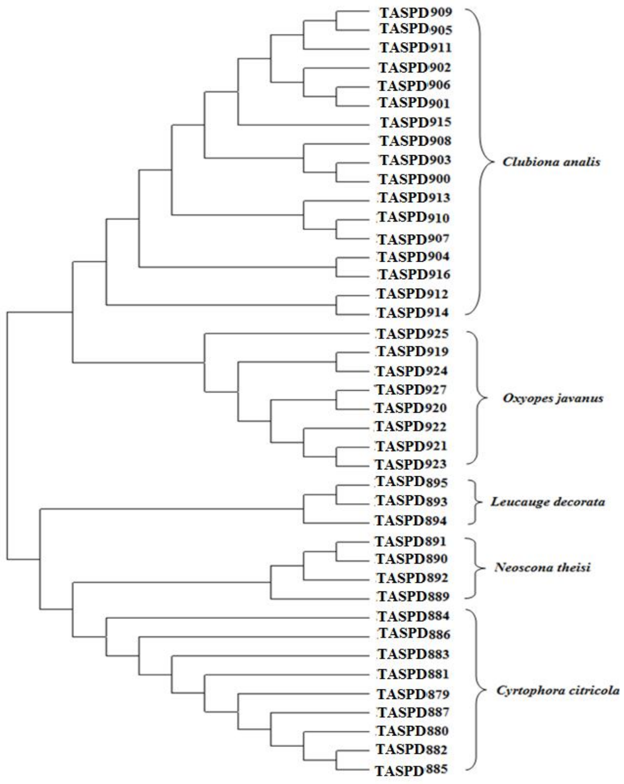

Fig. 5. Phylogenetic tree of five spiders species based on COI gene.

Species identifications based on DNA barcode are considered more reliable and accurate if large gaps exist in genetic distances of congenerics and conspecifics (Hebert *et al.*, 2004; Meyer and Paulay, 2005). Barcode gap can be detected by recording the overlap between the highest intraspecific and the lowest interspecific genetic distances (Meier *et al.*, 2008). Our results showed large 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 spiders 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%. Barrett 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 *C. 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).

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