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Assessing the Diversity of Cursorial Spiders using DNA Barcoding

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ABSTRACT

Spiders are natural predators of many insect pests and preferred as they are carnivores. In the present study we identified the cursorial spiders of different families using DNA barcoding as it is very helpful in identifying species where morphological identifications can be difficult e.g., delimitation of juvenile stages. Standard barcode region of CO1 gene of 64 samples was amplified. The sequences of 658 base pairs were recovered from 62 samples, representing 7 families, 20 genera and 27 species. Araneidae was the most dominant family followed by Salticidae, Oxyopidae, Clubionidae, Tetragnathida, Thomisidae, Mitergidae and Lycosidae. The interspecific value of divergence was more than the intraspecific value of divergence for all seven families which described a clear barcode gap. No overlap was recorded in intraspecific and interspecific divergence value. Furthermore, distance to NN was higher than the maximum intraspecific value for all species. A barcode reference library of the cursorial spiders of Punjab University, Lahore and Soon Sakeser Valley Punjab was also established. It is concluded that CO1 has potentially enough information for fast and accurate identification of spiders. Although, morphological studies alone are working satisfactory for the identification of spiders, still its efficiency increased when combined with DNA barcoding.

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Authors' Contribution
SA performed experimental work and collected the data. HMT designed the study and did editing and proof reading. SN analysed the data.

Key words
Spiders, DNA barcoding, COI gene, Morphological identification, Cryptic species

INTRODUCTION

Spiders is a diverse group that act as natural predators of insect pests (Coddington and Levi, 1991; Carcamo *et al*., 2014). They are potentially very helpful in reducing different insect pest populations in ago-ecosystems (Jeyaparvathi *et al*., 2013). Before using them as bio-control agents in agro-ecosystem their true identification is needed (Maloney *et al*., 2003). Though spider catalog document 45,942 species of spiders but this is such a small number considering the total diversity and an enormous number is still to be discovered (world spider catalog version 17). Morphological characters are commonly used for identification but such identifications are unfavorably judged due to phenotypic and genetic variations (Hebert *et al*., 2003).

Identification at species level is very important as it is helpful in understanding the diversity of species, phylogenetic patterns and evolutionary histories. For taxonomists it is very difficult task to identify and classify different types of animals as they have distinct body forms at various life stages (Hebert *et al*., 2003). Moreover, it is difficult to distinguish cryptic (mysterious) species on the base of morphometric characters (Hajibabaei *et al*., 2007; Bickford *et al*., 2007).

True morphological identification of numerous species of spiders is not only a complex phenomenon but is time taking and intricate (Barret and Herbert, 2005). Morphological identifications are done through keys which rely on the careful study of adults only, so it is tough to identify various life stages. Many spider species show sexual dimorphism, therefore different procedures are used to identity male and female specimens. Some type of technical help is required to the scientists for the challenging number of identification (Godfray, 2002; Blaxter, 2003).

From a few decades scientists have taken help form the molecular methods to identify species. Molecular methods overcome the complexities in spider identification (Navajas and Fenton, 2000). DNA barcoding is one of the well-known and most often used molecular method for species identification (Nagoshi *et al*., 2011; Van der Bank *et al*., 2012). It is preferred as it helps to get complete set of information even from one specimen regardless of the life stage (Hebert *et al*., 2003a, 2004).

DNA barcoding is considered as an advance technique which is commonly being used taxonomic identifications (Nagoshi *et al*., 2011; Doña *et al*., 2015; Xu *et al*., 2015). It uses short standardized COI (cytochrome C Oxidase subunit I) gene region of mitochondrial DNA for identification of species (Herbert *et al*., 2003). This specific sequence (658 base pairs) is called as "DNA barcode". This sequence is used as species tag (Jinbo *et al*., 2011).

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COI is considered the best target for animal barcoding as it is found in all invertebrates and vertebrates. Furthermore, insertions (additions) and omissions in this area are quite rare. Finally, it bears sufficient sequence divergence that is adequate for distinguishing closely linked species (Hebert *et al*., 2003).

In DNA barcoding of animals some other mitochondrial genes are also used frequently and known as universal markers. These genes include 16S, DNA, 12 DNA (Vences *et al*., 2004; Kappner and Bieler, 2006; Aliabadian *et al*., 2009) and cytochrome b (Desalle *et al*., 2005). They are preferred as they are maternal genes and no recombination is found (Birky, 2001). Mutation rate in mitochondrial genes is very high that causes the variations within the species called as intraspecific variations (Hlaing *et al*., 2009). Other reason for using the mitochondrial gene is huge number of mitochondria in all cells when comparing to nuclear DNA (nDNA) (Randi *et al*., 2000) so even a small body part is enough to extract the mitochondrial DNA (Stoeckle and Hebert, 2008).

DNA barcoding being an innovative tool is equally significant for the discovery of novel undescribed species beside its function of identification (Hebert *et al*., 2003a, b; Hebert *et al*., 2004). Unlike other conventional methods DNA barcoding has huge capacity of application in identification as it is convenient, efficient and an economical procedure (Hebert *et al*., 2004; Smith *et al*., 2007; Padial and De La Riva, 2007; Kerr *et al*., 2009). Therefore, it has been widely considered for the determination of new species from diverse group of animals at any of the life stage development (Hebert *et al*., 2004). Furthermore, DNA Barcoding has successfully dealt with unfavorable judgments and it furnishes effective procedure for species level identifications (Sun *et al*., 2012).

Present study has been designed to identify the spiders of different families collected from Soon Sakeser Valley using DNA barcoding and to compare the performance of this method to assess the species diversity. Barcode sequences that were generated during present study were compared to the available sequences of spiders in the GeneBank to validate our morphological identifications.

MATERIALS AND METHODS

Live spiders were collected from different habitats i.e., trees, crops and grasses using hand picking and jarring method. Sampling was done from August, 2016 to October, 2016. Spiders were collected from Punjab University, Lahore and Soon Sakeser Valley Punjab. The GPS locations of Lahore and Soon Sakeser Valley were 31.344 N, 74.17 E and 32.9 9 N, 71.44 E respectively.

The spiders collected from fields were brought to the laboratory, in the Department of Zoology, GC University, Lahore. The spiders were washed with alcohol in the laboratory and preserved in 95% ethanol and stored at -20ºC till the DNA extraction was done. Samples were properly labelled with their date of collection, collection site and collector's name before preservation.

PCR amplification and sequencing

Partial mitochondrial COI DNA fragment was amplified in multiple individuals of same species. Universal primers (HCOOUTOUT and LCO1490) were used for the PCR amplification. A standard PCR was carried out in 25µL. The reaction mixture contained 12.5µl reaction mixture, 1µl of each forward and reverse (10µM) primers, 8.5µl of water and 2µl of sample DNA per reaction. The temperature conditions used for PCR were as following:

The initial denaturation was done at 94°C for 1 min; 35 cycles were completed each involving incubation at 94°C for 45 sec., then annealing at 48°C for 45 sec. and 72°C for 30 sec. and a final elongation or extension step at 72°C for 5 min in the thermocycler. The verification of PCR product was done on 1% agarose gel electrophoresis.

Sequencing of these purified samples was done in collaboration with Centre For Biodiversity and DNA Barcoding, University Of Guelph, Canada.

Data analyses

MAFFT, a multiple sequence alignment program was used for sequence alignment. Later on phylogenetic tree, based on the genetic data, was constructed using TNT software. Maximum likelihood estimation (MLE) method was used for tree construction. Genetic distances among the sequenced spider were calculated using MEGA 6.0.6 software. The Automatic barcode Gap Discovery (ABGD) was used to compute barcode gap. Finally, sequenced data was submitted to BOLD (Barcode of Life Database) databases.

RESULTS

Out of 64 PCR products, barcode sequences of 658 base pairs were recovered from 62 samples, representing 7 families, 20 genera and 27 species (Table I). All identified spider species are listed in Table II. Most of the arboreal spiders for study belonged to the family Aranaidae. Family Araneidae was followed by Salticidae, Oxyopidae, Clubionidae, Tetragnathida, Thomisidae and Mitergidae. Family Araneidae was represented by seven species. However, family Salticidae, Thomisidae and Oxyopidae were represented by 5, 5 and 4 species respectively. Each of the family Clubionidae, Tetragnathidae and Mitergidae were represented by 2 species.

Table I. Family names with number of genera, species and specimens.

Family name	No. of genera	No. of species	No. of specimens
Araneidae	5	7	23
Salticidae	5	5	10
Thomisidae	4	5	8
Oxyopidae	2	4	8
Clubionidae	1	2	5
Tetragnathidae	2	2	5
Mitergidae	1	2	3
Total	20	27	62

An overlap of maximum and mean intraspecific distances versus the intraspecific (nearest neighbour) distance was observed. The minimum distance to the nearest neighbour was higher than the maximum intraspecific distance for all species (Table III). Similarly, the minimum distance to the nearest neighbour was also higher than the maximum interspecific distance for all species. The Automatic barcode Gap Discovery (ABGD) showed a clear gap between intraspecific and interspecific distance (Fig. 1). Figure 2 demonstrates the phylogenetic tree for seven studied families of spiders. Detail of pair wise genetic distances is included as Supplementary data.

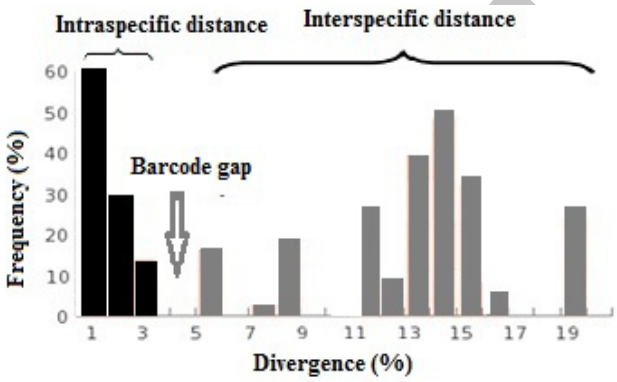

Fig. 1. Mean Intra and Interspecific distance.

An overlap of maximum and mean intraspecific distances versus the intraspecific (nearest neighbour) distance is observed. The minimum distance to the nearest neighbour was higher than the maximum intraspecific distance for all species. Similarly, the minimum distance to the nearest neighbour was also higher than the maximum interspecific distance for all species. The Automatic barcode Gap Discovery (ABGD) showed a clear gap between intraspecific and interspecific distance (Fig. 1).

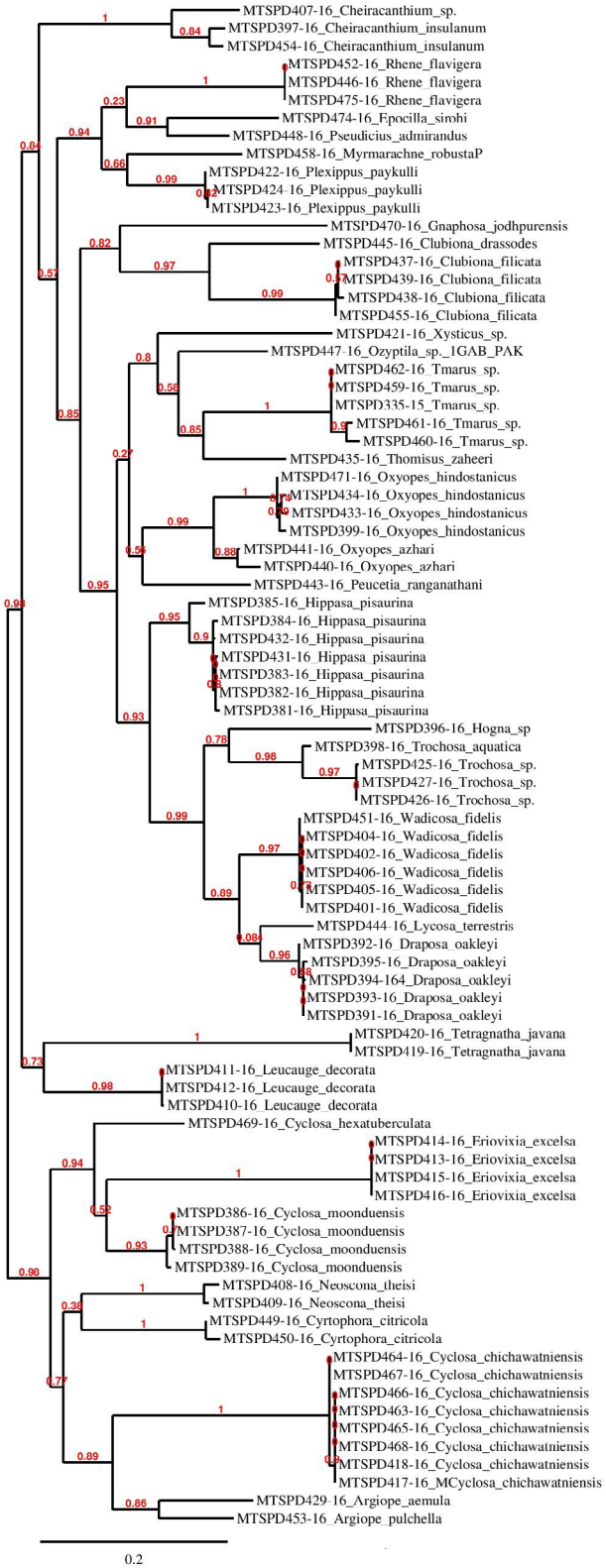

Fig. 2. Phylogenetic tree of seven studies famalies.

Table II. List of spider species identified during the present study along with their distribution.

Family name	Species name	Worldwide distribution
Araneidae	*Argiope aemula* (Walchenaer, 1841)	India to China, Philipine, Australia
	Argiope pulchella (Walchenaer, 1841)	India to China
	Cyclosa chichawatniensis (Mukhtar and Mushtaq, 2005)	Punjab Pakistan
	Cyclosa hexatuberculata (Tikader, 1982)	India, Pakistan
	Cyclosa moonduensis (Tikader, 1963)	India, Pakistan
	Cyrtophora citricola (Simon, 1864)	Tropical areas of Asia, Africa, Australia and Mediterranean areas of Europe
	Neoscona theisi (Walckenaer, 1841)	(India, China to Pacific)
Salticidae	*Epocilla* sp.	
	Myrmarachne robusta (Peckham and Peckham, 1892)	Asia
	Plexippus paykulli (Audouin, 1826)	Cosmopolitan
	Pseudicius admirandus (Logunov, 2007)	Europe to central Asia
	Rhene Flavigera (Koch, 1848)	Cosmopolitan
Thomisidae	*Thomisus zaheeri* (Parveen *et al*., 2008)	Pakistan
	Tmarus sp.	
	Ozyptila sp..	
	Xysticus joyantius (Tikader, 1968)	Cosmopolitan
	Xysticus sp.	
Oxyopidae	*Oxyopes azhari* (Butt and Beg, 2001)	Pakistan
	Oxyopes hindostanicus (Pocock, 1901)	India, Pakistan and Srilanka
	Oxyopes oryzae	
	Olios sp.	
Clubionidae	*Clubiona drassodes* (Cambridge, 1874)	India, Bangladesh, China
	Clubiona filicata (Cambridge, 1874)	India, Bangladesh, Pakistan, Thailand, Myanmar, Laos, China
Tetragnathidae	*Leucauge decorata* (Blackwel, 1864)	Paleotropical
	Tetragnatha javana (Thorell, 1890)	Africa to Japan, Philippines, Indonesia
Mitergidae	*Cheiracanthium insulanum* (Thorell,1878)	India, Loas, Thailand
	Cheiracanthium sp.	

In the Accumulation Curve the number of species are increasing with the increase in sequence count.The curve is not platue which predicts that sampling efficiency was not enough to record all species of study area. Figure 2 is showing the neighbour joining trees for seven studied families. Neighbour joining tree separately for each family are given in supplementary file 1. The barcodes sequences generated during the study are 2.

DISCUSSION

Identification of spiders based on morphology is quite difficult especially in juvenile stages because juvenile stage lack true identifying characters such as the genitalia (Hubert, 2004), sexual dimorphism, especially in weavers (*Nephila* and *Micrathena*) that are the most reliable character for spider identification to taxonomists. Platnick (2009) added other challenges i.e. the least information about diagnostic characters for one sex. About 46% of spider descriptions just consider one sex, and moreover 1.5% identification is based on juveniles only, hence making it difficult to match life stages with the sexes. Infact identification of the known adult samples is also time taking, as the spider species need quite detailed examination and sometimes even dissection of sexual organs for firm identification (Locket and Millidge, 1951).

Table III. Comparison of the mean and maximum intra-specific values to the nearest neighbour distance.

Species	Mean Intra-Sp	Max Intra-Sp	Nearest neighbour	Nearest species	Distance to NN
Argiope aemula	N/A	N/A	MTSPD453-16	*Argiope pulchella*	12.08
Argiope pulchella	N/A	N/A	MTSPD429-16	*Argiope aemula*	12.08
Cyclosa chichawatniensis	0.32	0.77	MTSPD469-16	*Cyclosa hexatuberculata*	18.54
Cyclosa hexatuberculata	N/A	N/A	MTSPD389-16	*Cyclosa moonduensis*	12.17
Cyclosa moonduensis	0.64	1.08	MTSPD469-16	*Cyclosa hexatuberculata*	12.17
Neoscona theisi	1.39	1.39	MTSPD453-16	*Argiope pulchella*	15.04
Clubiona drassodes	N/A	N/A	MTSPD437-16	*Clubiona filicata*	13.37
Clubiona filicata	0.54	0.77	MTSPD445-16	*Clubiona drassodes*	13.37
Cheiracanthium insulanum	2.34	2.34	MTSPD407-16	*Cheiracanthium* sp.	7.93
Cheiracanthium sp.	N/A	N/A	MTSPD454-16	*Cheiracanthium insulanum*	7.93
Oxyopes azhari	2.03	2.03	MTSPD442-16	*Oxyopes oryzae*	4.92
Oxyopes hindostanicus	1	1.24	MTSPD441-16	*Oxyopes azhari*	6.73
Oxyopes oryzae	N/A	N/A	MTSPD441-16	*Oxyopes azhari*	4.92
Peucetia ranganathani	N/A	N/A	MTSPD442-16	*Oxyopes oryzae*	13.56
Epocilla sp.	N/A	N/A	MTSPD448-16	*Pseudicius admirandus*	10.84
Myrmarachne robusta	0	0	MTSPD422-16	*Plexippus paykulli*	11.39
Plexippus paykulli	0.21	0.32	MTSPD448-16	*Pseudicius admirandus*	10.14
Pseudicius admirandus	N/A	N/A	MTSPD422-16	*Plexippus paykulli*	10.14
Rhene flavigera	0	0	MTSPD448-16	*Pseudicius admirandus*	12.45
Olios sp.	N/A	N/A	MTSPD423-16	*Plexippus paykulli*	16.78
Leucauge decorata	0.1	0.15	MTSPD474-16	*Epocilla* sp.	15.2
Tetragnatha javana	0	0	MTSPD469-16	*Cyclosa hexatuberculata*	18.9
Ozyptila sp.	N/A	N/A	MTSPD435-16	*Thomisus zaheeri*	13.31
Thomisus zaheeri	N/A	N/A	MTSPD447-16	*Ozyptila* sp.	13.31
Tmarus sp.	1.94	2.95	MTSPD447-16	*Ozyptila* sp.	14.1
Xysticus joyantius	N/A	N/A	MTSPD433-16	*Oxyopes hindostanicus*	13.2
Xysticus sp.	N/A	N/A	MTSPD447-16	*Ozyptila* sp.	14.69

Where the species is a singleton, N/A is displayed for intra-specific values.

Under such circumstances, DNA barcoding is likewise a significant and trustworthy method (Hebert *et al*., 2004; Barrett and Herbert, 2005; Blagoev *et al*., 2013; Raso *et al*., 2014; Doña *et al*., 2015; Xu *et al*., 2015). For the first time DNA Barcoding was discovered by Hebert *et al*. (2003), now this method is used in biosecurity (Armstrong and Ball, 2005), consumer protection (Lowenstein *et al*., 2010), ecology (Jurado-Rivera *et al*., 2009), conservation (Francis *et al*., 2010), biodiversity assessment (Janzen *et al*., 2009) and taxonomy (Benziger *et al*., 2011). DNA barcoding is used for evaluating the accuracy of identification and sequence variability in various taxa (Ward, 2009).

To check the validity of DNA barcoding in species identification as an authentic molecular tool and the reliability of the available data on GeneBank, current study included arboreal spiders of 27 different species belonging to 20 genera and seven families. Araneidae family was found the most dominant family in foliage followed by Salticidae, Thomisidae, Oxyopidae, Clubionidae, Tetragnathida and Mitergidae. However, Tahir *et al*. (2011, 2015) in his study on spiders of Sarghoda, Punjab found that Salticidae family is the most abundant family and Monzo *et al*. (2009) found Lycosidae family as the most dominant family in some type of foliage i.e. citrus orchards. This difference is because they remained restricted to a single type of foliage where as we have collected the spiders from different types of plantation. Furthermore climatic conditions, capturing time and efforts could be notable factors of this difference (Bukhari *et al*., 2012; Vetter *et al*., 2013).

The barcode gap is the key factor upon which the

accuracy of DNA barcoding depends. Barcode gap can be defined as the discontinuity in interspecific and intraspecific divergence values; more accurate results call for a high barcode gap (Hebert *et al*., 2004; Dasmahapatra and Mallet, 2006; Meier *et al*., 2008). Moreover, if the distance of a specie to its nearest neighbour sequence is more than the maximum intraspecific distance then the particular specie is different from its Nearest Neighbour (Ashfaq *et al*., 2014).

When the interspecific value of divergence is more than the intraspecific value of divergence it describes a clear barcode gap (Lipscomb *et al*., 2003; Stoeckle, 2003; Hebert *et al*., 2004; Meyer and Paulay, 2005) Overall a very clear barcode gap was noted between interspecific and intraspecific divergence values in all seven families of this study. Furthermore, distance to NN was recorded higher than the maximum intraspecific value for all species. Naseem and Tahir (2016) have reported similar results in their study for five spider families (Oxyopidae, Sparassidae, Salticidae, Tetragnathidae and Thomisidae). Slowik and Blagoev (2012) worked on family Clubionidae and Gnaphosidea and noted same results. While using barcoding for species identification the overlap among intraspecific and interspecific distances becomes problematic (Ward *et al*., 2009), but in our study no overlap was recorded in intraspecific and interspecific divergence value for the seven families of this study.

In the current study NN distance was recorded higher than the maximum intraspecific divergence of all species. Robinson *et al*. (2009) reported similar results for the Lycosidae family i.e. less than 3% maximum intraspecific divergence was recorded. Likewise, in many cases of spiders, maximum intraspecific sequence variation recorded was <1 % (Blagoev *et al*., 2013). In congeneric species pairs Barrett and Hebert (2005) recorded 3% sequence divergence. Moreover, no overlap among the mean nucleotide divergences at inter and intraspecific levels was recorded.

Mostly species are differentiated from each other when divergence value is more than 2% but in the above cases where the value of NN is less, such cases are well-lit by the scientists as young species or recent origins (Nazari *et al*., 2011; Mutanen *et al*., 2012). In fact some species vary by only a single base pair or even without any divergence (Herbert *et al*., 2004; Burns *et al*., 2007). The values obtained from the intraspecific divergences in our results were higher than the already reported values in many taxonomic groups. For example in a study on 300 aphid species, just 0.2% intraspecific divergence value was recorded (Foottit *et al*., 2008).

In spider morphospecies many cases of intraspecific divergence are revealed. In a single population of California, a divergence range of 6-12% was found in haplotypes of *Aptostichus simus* (trapdoor spider) (Bond *et al*., 2001). These results depict that the morphologically identified species could critically underestimate accurate evolutionary diversity meanwhile spider's genitalia do not advance as quickly and divergently as formerly it was believed (Bond *et al*., 2001; Hedin, 1997).

The accumulation curve is a curve that monitors and compares the efficiency of samples in different groups and shows the accumulation of sequence diversity. The curve of current study was not platue due to inefficient sampling (spider catch). One reason of this was that collection was not done from whole area. Another reason was arboreal spiders are usually nocturnal and collection at night is not that easy. Thirdly, some spiders like Leucage camouflage as it has different patterns on its skin. Lastly, the abundance of spiders may drop in winter so collection was only done in summer.

For the bio identification, CO1 gene is facing a lot of controversies (Will and Rubinoff, 2004; DeSalle, 2005; Hurst and Jiggins, 2005; Meier *et al*., 2006; Jansen *et al*., 2009; Sundberg *et al*., 2010). This is due to the overlapping between inter and intraspecific variations between CO1 gene (Meyer and Paulay, 2005; Meier *et al*., 2006; Jansen *et al*., 2009; Alexander *et al*., 2009). So other complementary markers should also be used for specie delimitation (Hebert and Gregory, 2005). Most of the times 16S rDNA (Aliabadian *et al*., 2009) and Cyt b (Bradley and Baker, 2001; DeSalle *et al*., 2005; Hajibabaei *et al*., 2007) are also recommended. Other genes like 12S, 18S rRNA, cyts and some mitochondrial protein coding genes can also be used (Blaxter, 2004; Nijman and Aliabadian, 2010; Nicolas *et al*., 2012). This can improvise DNA barcoding based species identification system.

It can be concluded from all above discussion that alone morphological identification is not reliable so DNA barcoding has become not only important but compulsory molecular tool for species identification. Collaboration of taxonomists is also needed as alone DNA barcoding may not give 100% efficiency. By DNA barcoding method quick identification of spiders is anticipated to become progressively correct, inexpensive, and achievable. So we suggest that the collaboration for building a worldwide community resource, comprising of skillful experts, identified specimens in permanent stable collections which must linked to online present specimen and sequence records, would be the supreme productive step to understand and enable the research on worldwide spider biodiversity.

Statement of conflict of interest

The authors have declared no conflict of interest.

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