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Assembling a DNA barcode reference library for the spiders (Arachnida: Araneae) of Pakistan

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Abstract

Morphological study of 1,795 spiders from sites across Pakistan placed these specimens in 27 families and 202 putative species. COI sequences >400 bp recovered from 1,782 specimens were analyzed using neighbor-joining trees, Bayesian inference, barcode gap, and Barcode Index Numbers (BINs). Specimens of 109 morphological species were assigned to 123 BINs with ten species showing BIN splits, while 93 interim species included representatives of 98 BINs. Maximum conspecific divergences ranged from 0–5.3% while congeneric distances varied from 2.8–23.2%. Excepting one species pair (*Oxyopes azhari–Oxyopes oryzae*), the maximum intraspecific distance was always less than the nearest-neighbor (NN) distance. Intraspecific divergence values were not significantly correlated with geographic distance. Most (75%) BINs detected in this study were new to science, while those shared with other nations mainly derived from India. The discovery of many new, potentially endemic species and the low level of BIN overlap with other nations highlight the importance of constructing regional DNA barcode reference libraries.

Introduction

With nearly 48,000 known species in 117 families [1], spiders are a major component of terrestrial ecosystems with important practical applications as biocontrol agents [2] and as bio-indicators [3,4]. Prior studies have documented 4,300 spider species in Europe [5] and a similar number (3,800) in the Nearctic [6]. By contrast, just 2,300 species have been reported from South Asia [7], suggesting that many species await detection in this region. Although studies on the spider fauna of Pakistan began nearly a century ago [8], work has recently intensified, but most of these studies have produced regional checklists (S1 Table). Unfortunately, these

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publications often employ invalid or incorrect species names or only identify specimens to a family [9], compromising their value [10–12]. It is likely that many species reported as new discoveries from Pakistan [13] await description. For example, in her dissertation research on spiders of Punjab, Parveen [13] reported the discovery of 33 new species but only one has been formally described [9]. Examination of prior taxonomic work (S1 Table) indicates that just 400 species of spiders have been documented from Pakistan. Considering the country's diverse ecosystems [14], this count must seriously underestimate the true diversity of its fauna given the much higher numbers reported for India (1686) [15] and Iran (528) [16]. The limited knowledge of the spider fauna of Pakistan is a particular example of the barrier to our general understanding of spider biodiversity in a global context, a factor compromising both scientific progress and conservation efforts [17].

The poor documentation of spider diversity of Pakistan reflects, in part, the paucity of taxonomic specialists working on the group [18]. Moreover, spiders pose a challenge for morphological approaches because cryptic species are common [19], and sexual dimorphism is often striking [20]. DNA barcoding [21] provides an alternate approach to identifications. It employs sequence diversity in a standard gene region (COI-5′) to discriminate both morphologically cryptic species and all life stages, even for species with sexual dimorphism [22,23]. Although concerns about the use of single marker [24,25] or discordance between the barcode and other gene regions [26] have been voiced [27], the advantages of employing a single standard gene region for DNA barcoding is now very well established [28]. Fifteen years after its introduction, this approach has demonstrated its effectiveness in discriminating species in diverse groups, including spiders [29–34].

The use of DNA barcoding for specimen identification and species discovery is greatly facilitated by BOLD, the Barcode of Life Data System (http://www.boldsystems.org). This informatics platform assembles specimen metadata and sequences and provides tools to facilitate data analysis and publication [35]. It also enables species discrimination by assigning each COI sequence cluster to a Barcode Index Number (BIN) [36], which is an analogue of Operational Taxonomic Unit (OTU). Because BINs have high congruence with species recognized through morphological analysis [37–40], they are now routinely used as a species proxy [41,42]. Consequently, they have gained wide adoption [41,43] for cryptic species recognition [40,43], species discovery [44], taxonomic revisions [45], and faunal assessments [46,47]. The DNA barcode reference libraries available for diverse animal groups [48–54] are helping to identify newly collected specimens [45,54] and to speed taxonomic progress [33]. By assigning sequences from unidentified specimens to a species proxy [44], the BIN system has greatly augmented the application of barcode data in groups where taxonomic knowledge is poor. These barcode libraries are, in effect, forming the foundation for a global "DNA library of life" [55].

At present, BOLD holds 6.8 million records derived from specimens representing 587,000 BINs (accessed 13 April, 2019). This total includes 117,000 records from spiders that have been assigned to more than 10,000 BINs. Past work on spiders has had varied motivations [39,56–60], but just two prior studies have aimed to construct a comprehensive DNA barcode library for a national fauna–Canada [61] and Germany [62]. The need for similar work in other regions is evident, particularly in south Asia. For example, barcode records are only available for 73 species of spiders from India [35,63] and for 41 species from Pakistan [64–66]. The current study aimed to develop a barcode library for the spider fauna of Pakistan and investigate the spider diversity overlap with other regions using BINs. The study addresses the gap for reference data in the country by expanding DNA barcode coverage for Pakistan to 202 species.

Materials and methods

Ethics statement

No specific permissions were required for this study. The study did not involve endangered or protected species.

Spider collection

From 2010 to 2016, 1,795 spiders were collected at 225 sites in Pakistan (Fig 1). Each spider was provisionally identified by collectors in Pakistan before it was sequenced for the barcode region of the mitochondrial COI gene [21]. GB subsequently validated and refined identifications by examining (including genitalic dissections) representative specimens from each barcode cluster. Generic and species assignments generally followed taxonomic publications on Asian spiders (S1 Table), but nomenclature was updated as required to follow the World Spider Catalog [1]. Collection data, a photograph, and a taxonomic assignment for each specimen are available in the public dataset, "DS-MASPD DNA barcoding spiders of Pakistan" (dx.doi.org/10.5883/DS-MASPD) on BOLD. The 1,795 specimens are held in four repositories: Centre for Biodiversity Genomics, University of Guelph, Guelph, Canada (585); National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan (1126); University of Sargodha, Sargodha, Pakistan (84). The location of any particular specimen is reported in the dataset.

Molecular analysis

DNA extraction, PCR, and Sanger sequencing were performed at the Canadian Centre for DNA Barcoding (CCDB) (http://ccdb.ca/resources/) using standard protocols. A single leg was removed from each specimen with a sterile forceps and transferred into a well in a 96-well microplate pre-filled with 30 μl of 95% EtOH. DNA was subsequently extracted by tissue lysis at 56˚C overnight followed by a column-based protocol [67]. PCR amplification of the COI-5′ barcode region employed the primer pair C_LepFolF and C_LepFolR (http://ccdb.ca/site/wp-content/uploads/2016/09/CCDB_PrimerSets.pdf). This primer cocktail includes equal volume of LepF1 [68] /LCO1490 [69] and LepR1 [68] /HCO2198 [69], respectively. The target COI region was amplified using 2 μL of DNA template in a 12.5 μL reaction containing standard PCR ingredients [30] employing the following PCR regime: 94˚C (1 min), 5 cycles of 94˚C (40 s), 45˚C (40 s), 72˚C (1 min); 35 cycles of 94˚C (40 s), 51˚C (40 s), 72˚C (1 min) and final extension of 72˚C (5 min). Amplicons were analyzed on a 2% agarose E-gel 96 system (Invitrogen Inc.) and were sequenced bidirectionally using the BigDye Terminator Cycle Sequencing Kit (v3.1) on an Applied Biosystems 3730XL DNA Analyzer. Sequences were assembled, aligned, and edited using CodonCode Aligner (CodonCode Corporation, USA) and validated in MEGA5 [70] to ensure they lacked a stop codon.

Data analysis

All sequences were submitted to BOLD (DS-MASPD) where those meeting required quality criteria (>507 bp, <1% Ns, no stop codon or contamination flag) were assigned to a BIN [36]. An accumulation curve, BIN discordance, genetic distance analysis, barcode gap analysis (BGA), and geo-distance correlation were determined using analytical tools on BOLD. The Accumulation Curve plots the rise in the number of BINs with increased sampling effort making it possible to ascertain if asymptotic diversity has been reached. The BGA determines if the maximum sequence divergence within members of a species or BIN is less than the distance to its Nearest-Neighbor (NN) species or BIN, a condition required for unambiguous identification [71,72]. The geo-distance correlation ascertains the correlation between geographic

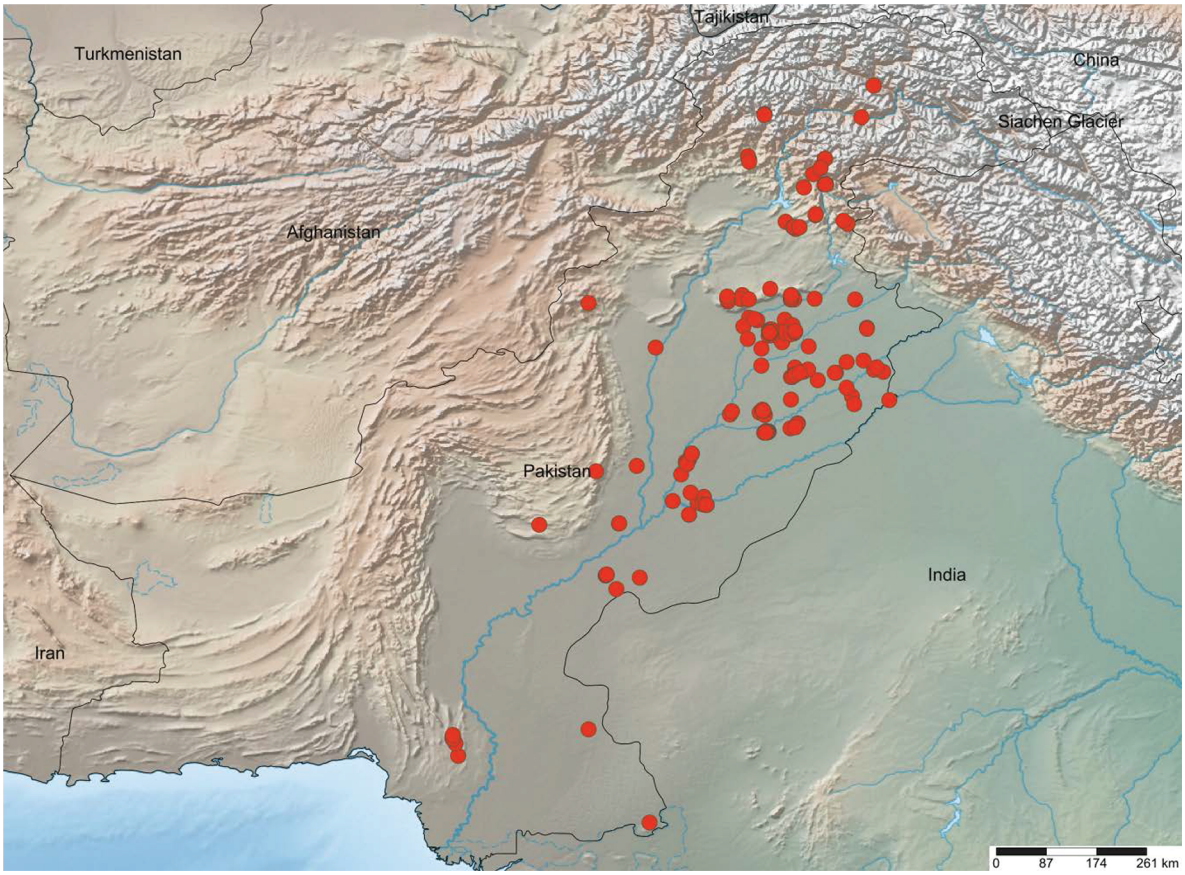

Fig 1. Map showing collection localities for the 1,795 spiders examined in this study. The map was developed using www.simplemappr.net. The author of SimpleMapper has waived all copyrights and no permission is needed to use. GPS coordinates (Latitude, Longitude) for the collection localities were: 24.45, 70.8; 25.488, 67.821; 25.681, 67.781; 25.756, 67.739; 25.757, 67.732; 25.759, 67.737; 25.76, 67.732; 25.801, 67.733; 25.812, 67.739; 25.9, 69.85; 28.083, 70.283; 28.261, 70.647; 28.293, 70.115; 28.304, 70.134; 28.306, 70.128; 28.308, 70.132; 28.308, 70.134; 28.309, 70.13; 28.309, 70.131; 28.309, 70.133; 29.083, 69.083; 29.103, 70.324; 29.104, 70.324; 29.105, 70.328; 29.24, 71.415; 29.242, 71.413; 29.39, 71.68; 29.393, 71.688; 29.393, 71.684; 29.394, 71.682; 29.396, 71.683; 29.401, 71.627; 29.429, 71.548; 29.454, 71.161; 29.518, 71.645; 29.584, 71.439; 29.868, 71.291; 29.9167, 69.9667; 30, 70.6; 30.026, 71.381; 30.053, 71.385; 30.065, 71.363; 30.105, 71.417; 30.189, 71.455; 30.189, 71.458; 30.189, 71.457; 30.191, 71.457; 30.516, 72.583; 30.518, 72.624; 30.519, 72.606; 30.52, 72.624; 30.522, 72.635; 30.523, 72.629; 30.525, 72.624; 30.529, 72.63; 30.531, 72.655; 30.531, 72.632; 30.533, 72.63; 30.534, 72.633; 30.534, 72.606; 30.537, 72.638; 30.538, 72.641; 30.54, 72.608; 30.585, 72.993; 30.6, 73.0667; 30.65, 73.1; 30.66, 73.1; 30.6612, 73.1086; 30.791, 72.594; 30.8, 72.05; 30.832, 72.512; 30.85, 72.083; 30.85, 72.544; 30.854, 72.538; 30.855, 72.54; 30.855, 72.539; 30.856, 72.572; 30.857, 72.542; 30.859, 72.566; 30.862, 72.56; 30.862, 72.554; 30.866, 72.555; 30.875, 72.557; 30.959, 73.984; 31.024, 74.531; 31.033, 73; 31.0833, 73.95; 31.2167, 73.8667; 31.3333, 73.4167; 31.3833, 73.0167; 31.3833, 73; 31.393, 73.027; 31.394, 73.026; 31.4167, 73.05; 31.4167, 73.0667; 31.45, 73.7; 31.45, 73.6833; 31.45, 73.1333; 31.463, 74.436; 31.4667, 73.2; 31.496, 74.294; 31.5, 73.2667; 31.532, 73.063; 31.5333, 74.3333; 31.56, 72.54; 31.6167, 73.8667; 31.64, 74.13; 31.825, 72.541; 31.8424, 70.8952; 31.86, 73.276; 31.924, 72.863; 31.965, 72.867; 31.976, 72.328; 31.986, 72.832; 32.027, 72.653; 32.034, 72.703; 32.05, 73; 32.055, 72.946; 32.059, 73.011; 32.063, 73.042; 32.0667, 72.6667; 32.0667, 72.6833; 32.067, 73.05; 32.074, 72.684; 32.077, 72.671; 32.077, 72.67; 32.078, 72.672; 32.08, 72.9; 32.081, 72.667; 32.082, 72.675; 32.083, 73.067; 32.0837, 72.6719; 32.084, 72.68; 32.088, 72.673; 32.093, 72.684; 32.1, 73.067; 32.102, 72.957; 32.109, 72.846; 32.11, 72.655; 32.119, 72.679; 32.122, 72.681; 32.125, 72.693; 32.1333, 74.1833; 32.15, 74.1833; 32.17, 72.26; 32.19, 73.025; 32.267, 72.476; 32.275, 72.904; 32.287, 72.43; 32.3054, 72.3482; 32.5333, 69.85; 32.56, 72.02; 32.59, 72.999; 32.59, 72.008; 32.59, 73.049; 32.59, 73.999; 32.591, 73.008; 32.591, 72.999; 32.5916, 72.3446; 32.592, 73.011; 32.592, 72.999; 32.593, 72.999; 32.594, 73.02; 32.594, 72.999; 32.595, 72.999; 32.5964, 72.217; 32.597, 73.041; 32.601, 73.369; 32.601, 73.038; 32.603, 73.042; 32.624, 73; 32.629, 73.009; 32.63, 73.005; 32.632, 73.013; 32.637, 73.008; 32.637, 72.008; 32.652, 73; 32.656, 73.005; 32.657, 73.004; 32.658, 73.003; 32.6581, 73.0034; 32.659, 73.008; 32.6592, 72.2433; 32.755, 72.677; 33.686, 73.076; 33.714, 73.132; 33.714, 73.133; 33.714, 73.13; 33.715, 73.132; 33.716, 73.129; 33.7167, 73.0333; 33.7167, 73.05; 33.7667, 73.8833; 33.8, 72.9167; 33.8167, 73.8167; 33.9, 73.3833; 33.9167, 73.3833; 34.333, 73.204; 34.334, 73.201; 34.38, 73.52; 34.38, 73.54; 34.385, 73.544; 34.386, 73.546; 34.386, 73.545; 34.541, 73.348; 34.543, 73.348; 34.546, 73.349; 34.638, 73.461; 34.639, 73.461; 34.639, 73.462; 34.7333, 72.35; 34.7667, 72.35; 34.776, 73.527; 34.777, 73.526; 34.778, 73.528; 34.78, 73.53; 34.78, 73.531; 34.8167, 72.3333; 35.426, 74.098; 35.461, 72.588; 35.465, 72.584; 35.4667, 72.5833; 35.478, 72.588; 35.918, 74.29; 35.918, 74.289.

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distance and genetic distance in each species or BIN employing two methods. The Mantel Test [73] examines the relationship between the geographic distance (km) and genetic divergence (K2P) matrices. The second approach compares the spread of the minimum spanning tree of collection sites and maximum intra-specific divergence [61]. The relationship between geographic and intraspecific distances was analyzed for each species with at least one individual from three or more sites. The analysis included all the conspecific records public on BOLD.

A neighbor-joining (NJ) tree was generated in MEGA5 using the Kimura-2-Parameter (K2P) [74] distance model along with pairwise deletion of missing sites. Nodal support on the NJ tree was estimated by 1000 bootstrap replicates. Bayesian inference (BI) was calculated by MrBayes v3.2.0 [75] using representative sequences of the 221 BINs and employing *Phalangium opilio* (Arachnida: Opiliones) and *Galeodes* sp. (Arachnida: Solifugae) as outgroups. The data was partitioned in two ways; i) a single partition with parameters estimated across all codon positions, ii) a codon-partition in which each codon position was allowed different parameter estimates. Sequence evolution was modelled by the GTR+Γ model independently for the two partitions using the "unlink" command in MrBayes. Analyses were run for 10 million generations using four chains with sampling every 1000 generations and the BI trees were obtained using the Markov Chain Monte Carlo (MCMC) technique. Posterior probabilities were calculated from the sample points once the MCMC algorithm converged. Convergence was determined when the standard deviation of split frequencies was less than 0.022 and the PSRF (potential scale reduction factor) approached 1, and both runs converged to a stationary distribution after the burn-in stage (the first 25% of samples were discarded by default). The resultant trees were visualized in FigTree v1.4.0. The NJ and Bayesian analyses were employed to assess support for the BINs detected in this study, not to reconstruct the phylogeny of Araneae.

Results

Coupling of the DNA sequence results with detailed morphological analysis made it possible to assign 1,574 of the 1,795 barcoded specimens to one of 109 species, but the other 221 specimens could only be placed into one of 93 interim species. Collectively, these specimens included representatives of 27 families, 113 genera, and 202 species (Table 1). Most species were only represented by a single sex, usually females. Two-thirds (1,256) of the specimens were immatures that lacked the diagnostic characters required for species assignment. However, their DNA barcodes allowed them to be linked to adults whose identification was established through morphology. Four families (Amaurobiidae, Atypidae, Ctenidae, Segestriidae), 43 genera, and 74 species identified here represent first records for Pakistan (Tables 1 and S1). As adults from 12 of the 93 interim species possessed clear morphological differences from any known species in their genus, they are likely new to science (Table 1).

As the accumulation curve failed to approach an asymptote (Fig 2), it is certain that more species await detection. Although one species (*Artema transcaspica*) failed to qualify for a BIN assignment because its only sequence was too short, the other 108 morphological species were assigned to 123 BINs with 10 species showing a split to two or more BINs (Table 1 and Fig 3). The 93 interim species were allocated to 98 BINs with three showing BIN splits (Table 1), making the total BIN count 221 –with 94 of them singletons. NJ clustering (Fig 3) and Bayesian inference (Fig 4), supported the monophyly of all 221 BINs. Barcode distances (K2P) varied for differing taxonomic ranks with conspecific values ranging from 0.0–5.3% (mean = 0.8%), congenerics from 2.8–23.2% (mean = 8.8%), and confamilials from 4.3–26.7% (mean = 15.1%) (Table 2). Excepting 14 species, maximum intraspecific divergences did not exceed 2% in the 90 species that were represented by two or more specimens (Table 1). The barcode gap analysis

Table 1. Species, maximum barcode divergence (K2P), nearest neighbor distance (NN), and BIN assignment of 1,795 spiders collected in Pakistan.

	No.	Taxa	*N*	K2P	NN	BINs
		Agelenidae C. L. Koch,1837				
	1	*Draconarius* sp. 1GAB_PAK	2	0	8.8	BOLD:AAO2052
	2	*Draconarius* sp. 2GAB_PAK	2	0	8.8	BOLD:AAO2053
*NP	3	*Tegenaria domestica* (Clerck, 1757)	1	N/A	19	BOLD:AAF1312
NP		**Amaurobiidae** Thorell,1870				
*NS	4	*Himalmartensus* cf. *martensi* Wang & Zhu, 2008	1	N/A	14	BOLD:ACB2928
		Araneidae Clerck, 1757				
NP	5	*Araneus affinis* Zhu, Tu & Hu, 1988	2	0.6	12	BOLD:AAV7611
	6	*Araneus mitificus* (Simon, 1886)	20	1.7	13	BOLD:AAV1598
	7	*Araniella* sp. 1GAB_PAK	4	0.8	10	BOLD:AAV1625
	8	*Argiope aemula* (Walckenaer, 1841)	8	1.4	10	BOLD:ACG0732
	9	*Argiope anasuja* Thorell, 1887	1	N/A	9.5	BOLD:ACB2926
NP	10	*Argiope lobata* (Pallas, 1772)	2	0.5	12	BOLD:ACI8559
NP	11	*Argiope pulchella* Thorell, 1881	6	0.8	9.5	BOLD:ACG0576
	12	*Argiope trifasciata* (Forsskål, 1775)	15	1.1	10	BOLD:AAQ2634
NP	13	*Chorizopes wulingensis* Yin, Wang & Xie, 1994	2	0.5	12	BOLD:ABX7347
U	14	*Cyclosa confraga* (Thorell, 1892)	8	0.8	18	BOLD:ADF2726
	15	*Cyclosa hexatuberculata* Tikader, 1982	4	0	11	BOLD:ADD8756
NP	16	*Cyclosa moonduensis* Tikader, 1963	9	1.1	11	BOLD:ACZ2455
	17	*Cyrtophora citricola* (Forsskål, 1775)	66	1.6	13	BOLD:AAO2032
	18	*Eriovixia excelsa* (Simon, 1889)	40	1.1	16	BOLD:AAQ0105
	19	*Gea subarmata* Thorell, 1890	1	N/A	10	BOLD:ACG0733
*NS	20	*Hypsosinga* cf. *alboria* Yin, Wang, Xie & Peng, 1990	4	0	10	BOLD:ABX7344
*NP	21	*Hypsosinga wanica* Song, Qian & Gao, 1996	17	1.6	10	BOLD:AAQ0134
	22	*Larinia phthisica* (L. Koch, 1871)	5	0.9	10	BOLD:AAO2160
	23	*Larinia* sp. 1GAB_PAK	1	N/A	11	BOLD:ABX7407
*	24	*Leviellus* sp. 1GAB_PAK	2	0	14	BOLD:AAV1590
NP	25	*Neoscona polyspinipes* Yin, Wang, Xie & Peng, 1990	20	0.8	4.9	BOLD:AAO1983
NP	26a	*Neoscona scylla* (Karsch, 1879)	13	1.9	7.8	BOLD:ACI8762
	26b	*Neoscona scylla* (Karsch, 1879)	16			BOLD:AAO1997
	27	*Neoscona* sp. 1BAG_PAK	1	N/A	7.6	BOLD:ACI2573
	28	*Neoscona* sp. 2BAG_PAK	1	N/A	9	BOLD:ADD4537
NP	29	*Neoscona subfusca* (C. L. Koch, 1837)	1	N/A	8.6	BOLD:AAV3851
	30	*Neoscona theisi* (Walckenaer, 1841)	160	2.5	7.6	BOLD:ACM3489
	31	*Neoscona vigilans* (Blackwall, 1865)	38	1.5	4.9	BOLD:AAO2202
	32	*Plebs himalayaensis* (Tikader, 1975)	2	0	17	BOLD:ACI8675
NP		**Atypidae** Thorell, 1870				
*NS	33	*Calommata* sp. 1GAB_PAK	1	N/A	21	BOLD:ACP9624
		Cheiracanthiidae Wagner, 1887				
NP	34	*Cheiracanthium inornatum* O. Pickard-Cambridge, 1874	5	2.3	7.4	BOLD:ACC4872
NP	35	*Cheiracanthium insulanum* (Thorell, 1878)	20	3.3	6.9	BOLD:AAQ0110
	36	*Cheiracanthium* sp. 1GAB_PAK	2	0.2	11	BOLD:ACA7676
	37	*Cheiracanthium* sp. 2GAB_PAK	2	1.1	4.9	BOLD:ABW2880
	38	*Cheiracanthium* sp. 3GAB_PAK	2	0.2	4.9	BOLD:AAU6055
		Clubionidae Wagner, 1887				
	39	*Clubiona drassodes* O. Pickard-Cambridge, 1874	28	0.9	13	BOLD:AAV1620
	40	*Clubiona filicata* O. Pickard-Cambridge, 1874	18	0.9	13	BOLD:AAV1603

(*Continued*)

Table 1. (Continued)

	No.	Taxa	*N*	K2P	NN	BINs
	41	*Clubiona* sp. 1GAB_PAK	1	N/A	8.8	BOLD:AAV1602
	42	*Clubiona* sp. 2GAB_PAK	1	N/A	8.8	BOLD:AAO2055
		Corinnidae Karsch, 1880				
	43	*Castianeira* sp. 1GAB_PAK	1	N/A	16	BOLD:ACP7698
NP		**Ctenidae** Keyserling, 1877				
*	44	*Anahita* sp. 1GAB_PAK	1	N/A	12	BOLD:ADF5307
*	45	*Ctenus* sp. 1GAB_PAK	1	N/A	9	BOLD:AAV1591
*	46	*Ctenus* sp. 2GAB_PAK	1	N/A	9	BOLD:ABW2888
		Filistatidae Ausserer, 1867				
	47	*Kukulcania* sp. 1GAB_PAK	1	N/A	22	BOLD:ABX7408
		Gnaphosidae Pocock, 1898				
	48	*Berlandina afghana* Denis, 1958	1	N/A	14	BOLD:AAV1613
	49	*Drassodes* sp. 1GAB_PAK	1	N/A	12	BOLD:AAV1404
*NP	50	*Drassyllus coreanus* Paik, 1986	2	0	14	BOLD:AAV0899
	51	*Gnaphosa jodhpurensis* Tikader & Gajbe, 1977	2	1.2	15	BOLD:ACR0656
*NP	52	*Haplodrassus signifer* (C. L. Koch, 1839)	1	N/A	13	BOLD:ACB2432
	53	*Micaria* sp. 1GAB_PAK	1	N/A	13	BOLD:ACP3811
*	54	*Phaeocedus* sp. 1GAB_PAK	2	1.2	14	BOLD:AAV1605
*NP	55	*Scopoides maitraiae* (Tikader & Gajbe, 1977)	2	0	16	BOLD:ACZ1655
*NP	56	*Trachyzelotes kulczynskii* (Bösenberg, 1902)	1	N/A	13	BOLD:AAQ2633
NS	57	*Zelotes* cf. *puritanus* Chamberlin, 1922	2	0.8	12	BOLD:AAQ0137
NP	58	*Zelotes shantae* Tikader, 1982	1	N/A	12	BOLD:ADD7482
	59	*Zelotes* sp. 1GAB_PAK	1	N/A	12	BOLD:ACZ4032
*NP	60	*Zimiris diffusa* Platnick & Penney, 2004	1	N/A	14	BOLD:AAV1616
		Hersiliidae Thorell, 1870				
	61	*Hersilia savignyi* Lucas, 1836	16	1.1	17	BOLD:AAP4789
		Linyphiidae Blackwall, 1859				
	62	*Gnathonarium dentatum* (Wider, 1834)	5	0	14	BOLD:AAQ0150
*	63	*Mermessus* sp. 1GAB_PAK	1	N/A	14	BOLD:ACP3810
*NP	64	*Neriene emphana* (Walckenaer, 1841)	3	0.8	14	BOLD:ACI8558
		Lycosidae Sundevall, 1833				
*	65	*Alopecosa* sp. 1GAB_PAK	1	N/A	9.2	BOLD:AAV1615
NS	66	*Arctosa* cf. *serrulata* Mao & Song, 1985	1	N/A	9.4	BOLD:ACB2931
	67	*Arctosa* sp. 1GAB_PAK	1	N/A	13	BOLD:AAV1608
	68	*Draposa oakleyi* (Gravely, 1924)	19	1.6	5.8	BOLD:ABX7398
NS	69	*Evippa* sp. 1GAB_PAK	5	1.4	8.3	BOLD:ABX7397
	70	*Evippa* sp. 2GAB_PAK	1	N/A	8.3	BOLD:ABW2890
	71a	*Hippasa pisaurina* Pocock, 1900	16	4.1	5.8	BOLD:AAO2058
	71b	*Hippasa pisaurina* Pocock, 1900	1			BOLD:ADF3448
	72	*Hippasa* sp. 1GAB_PAK	1	N/A	5.8	BOLD:ADE8277
*	73	*Hogna* sp. 1GAB_PAK	5	0.6	10	BOLD:AAQ0158
*	74	*Hogna* sp. 2GAB_PAK	1	N/A	11	BOLD:ADF5080
	75	*Lycosa poonaensis* Tikader & Malhotra, 1980	5	0.6	10	BOLD:ABW2889
	76	*Lycosa* sp. 1GAB_PAK	1	N/A	10	BOLD:AAO2168
E	77	*Lycosa terrestris* Butt, Anwar & Tahir, 2006	45	0.9	4.3	BOLD:AAO2150
NP	78	*Pardosa mionebulosa* Yin, Peng, Xie, Bao & Wang, 1997	3	1.6	5.3	BOLD:ACZ3882
	79	*Pardosa pseudoannulata* (Bösenberg & Strand, 1906)	5	0.6	5.9	BOLD:AAO2149

(*Continued*)

Table 1. (Continued)

	No.	Taxa	*N*	K2P	NN	BINs
	80	*Pardosa* sp. 1GAB_PAK	3	0.2	5.9	BOLD:AAO2146
	81	*Pardosa* sp. 2GAB_PAK	1	N/A	4.9	BOLD:AAO2148
	82	*Pardosa* sp. 3GAB_PAK	1	N/A	5.2	BOLD:AAV1588
	83	*Pardosa* sp. 4GAB_PAK	13	2.4	4.6	BOLD:AAO2147
	84	*Pardosa* sp. 5GAB_PAK	4	0.8	5.2	BOLD:AAV1589
NP	85	*Pardosa sutherlandi* (Gravely, 1924)	7	0.2	4.6	BOLD:ABX7411
NP	86	*Trochosa aquatica* Tanaka, 1985	17	0.6	5.9	BOLD:AAV3200
	87	*Trochosa* sp. 1GAB_PAK	3	0.3	5.9	BOLD:ADF4175
*NP	88	*Wadicosa fidelis* (O. Pickard-Cambridge, 1872)	75	1.9	7.2	BOLD:AAG7456
		Oecobiidae Blackwall, 1862				
	89	*Oecobius putus* O. Pickard-Cambridge, 1876	10	0.4	14	BOLD:AAV1624
		Oxyopidae Thorell, 1870				
E	90	*Oxyopes azhari* Butt & Beg, 2001	112	3.6	3.6	BOLD:AAO1991
E	91	*Oxyopes chenabensis* Mukhtar, 2017	5	0.9	6.4	BOLD:ABX7410
NP	92	*Oxyopes heterophthalmus* (Latreille, 1804)	8	0.3	4.9	BOLD:AAD0599
	93	*Oxyopes hindostanicus* Pocock, 1901	123	3	5.6	BOLD:AAO1990
NP	94	*Oxyopes macilentus* L. Koch, 1878	8	1.5	1.3	BOLD:AAF9665
NP	95a	*Oxyopes matiensis* Barrion & Litsinger, 1995	3	2.1	1.3	BOLD:ACX5149
	95b	*Oxyopes matiensis* Barrion & Litsinger, 1995	5			BOLD:ABX7414
E	96	*Oxyopes oryzae* Mushtaq & Qadar, 1999	52	1.9	3.6	BOLD:AAO1989
	97	*Oxyopes* sp. 1GAB_PAK	1	N/A	6.7	BOLD:ACZ2323
NS	98	*Oxyopes* sp. 2GAB_PAK	3	1.2	5.7	BOLD:ACZ4097
	99	*Oxyopes* sp. 3GAB_PAK	1	N/A	11	BOLD:ACP4193
NP	100	*Peucetia ranganathani* Biswas & Roy, 2005	14	0.8	11	BOLD:ACB4190
	101	*Peucetia* sp. 1GAB_PAK	1	N/A	13	BOLD:ACB4188
		Philodromidae Thorell, 1870				
	102	*Philodromus* sp. 1GAB_PAK	1	N/A	12	BOLD:ADD8987
	103	*Philodromus* sp. 2GAB_PAK	3	2	13	BOLD:ABX7412
*NP	104	*Pulchellodromus mainlingensis* (Hu & Li, 1987)	2	0	12	BOLD:ACB4189
NS	105	*Rhysodromus* cf. *xinjiangensis* (Tang & Song, 1987)	4	0	13	BOLD:AAO2159
NP	106	*Thanatus vulgaris* Simon, 1870	2	0.3	15	BOLD:AAQ0111
		Pholcidae C. L. Koch, 1850				
	107	*Artema* sp. 1GAB_PAK	1	N/A	19	BOLD:ABW2886
NP	108	*Artema transcaspica* Spassky, 1934	2	1	19	-
	109	*Crossopriza lyoni* (Blackwall, 1867)	2	0.3	16	BOLD:AAG2795
	110a	*Crossopriza maculipes* (Spassky, 1934)	4	5.3	16	BOLD:ACN4846
	110b	*Crossopriza maculipes* (Spassky, 1934)	7			BOLD:AAU5412
	110c	*Crossopriza maculipes* (Spassky, 1934)	1			BOLD:ACB2929
		Pisauridae Simon, 1890				
*NP	111	*Pisaura mirabilis* (Clerck, 1757)	4	0.5	10	BOLD:AAE4245
	112	*Pisaura* sp. 1GAB_PAK	1	N/A	12	BOLD:AAO2059
		Salticidae Blackwall, 1841				
NP	113	*Bianor albobimaculatus* (Lucas, 1846)	21	0.7	12	BOLD:AAP4728
	114	*Bianor* sp. 1GAB_PAK	1	N/A	13	BOLD:ACI8750
NP	115	*Epocilla sirohi* Caleb, Chatterjee, Tyagi, Kundu, Kumar, 2018	7	1.9	11	BOLD:ADD4346
	116	*Euophrys* sp. 1GAB_PAK	1	N/A	13	BOLD:ADD1307
*NS	117	*Evarcha* sp. 1GAB_PAK	3	0.8	9	BOLD:AAV1614

(*Continued*)

Table 1. (Continued)

	No.	Taxa	*N*	K2P	NN	BINs
	118	*Hasarius adansoni* (Audouin, 1826)	2	0	13	BOLD:AAW0165
NP	119	*Hyllus dotatus* (Peckham & Peckham, 1903)	3	0.7	11	BOLD:AAV1597
NP	120	*Menemerus brevibulbis* (Thorell, 1887)	3	1.4	7.7	BOLD:AAO2155
	121	*Menemerus marginatus* (Kroneberg, 1875)	1	N/A	11	BOLD:AAV1611
	122	*Menemerus nigli* Wesolowska & Freudenschuss, 2012	12	1.1	7.7	BOLD:AAQ0156
*NP	123	*Modunda staintoni* (O. Pickard-Cambridge, 1872)	3	0.8	14	BOLD:AAV0387
NP	124	*Mogrus cognatus* Wesolowska & van Harten, 1994	12	1.4	8.1	BOLD:AAV1599
	125	*Mogrus* sp. 1GAB_PAK	1	N/A	8.1	BOLD:ACZ1977
	126	*Mogrus* sp. 2GAB_PAK	6	0.8	10	BOLD:AAQ2635
	127	*Myrmarachne melanocephala* MacLeay, 1839	1	N/A	6.7	BOLD:AAV1609
	128	*Myrmarachne robusta* (Peckham & Peckham, 1892)	5	1.4	6.7	BOLD:ACS0377
*NP	129	*Philaeus chrysops* (Poda, 1761)	1	N/A	9.8	BOLD:ACE4347
*	130	*Philaeus* sp. 1GAB_PAK	1	N/A	9.8	BOLD:AAV0574
	131	*Phintella vittata* (C. L. Koch, 1846)	11	0	9.9	BOLD:ACR1776
	132a	*Plexippus paykulli* (Audouin, 1826)	34	5	8.8	BOLD:AAO2152
	132b	*Plexippus paykulli* (Audouin, 1826)	4			BOLD:AAO2151
	132c	*Plexippus paykulli* (Audouin, 1826)	1			BOLD:ACU8433
	132d	*Plexippus paykulli* (Audouin, 1826)	1			BOLD:ABX7409
	132e	*Plexippus paykulli* (Audouin, 1826)	1			BOLD:ACZ4027
	133	*Plexippus* sp. 1GAB_PAK	2	0.2	8.8	BOLD:AAV1604
	134a	*Pseudicius admirandus* Logunov, 2007	8	1.4	9.4	BOLD:AAQ0115
	134b	*Pseudicius admirandus* Logunov, 2007	2			BOLD:ADD4534
NP	135	*Rhene albigera* (C. L. Koch, 1846)	1	N/A	5.9	BOLD:AAV5815
NP	136	*Rhene flavigera* (C. L. Koch, 1846)	4	0	5.4	BOLD:ADD7823
	137	*Rhene* sp. 1GAB_PAK	1	N/A	5.4	BOLD:ACU6737
*NS	138	*Sonoita* cf. *lightfooti* Peckham & Peckham, 1903	1	N/A	13	BOLD:ADD9560
	139	*Stenaelurillus arambagensis* (Biswas & Biswas, 1992)	3	0.3	11	BOLD:ABX7343
*	140	*Talavera* sp. 1GAB_PAK	1	N/A	12	BOLD:ACZ2472
	141	*Telamonia dimidiata* (Simon, 1899)	17	1.4	10	BOLD:ACG1123
	142	*Thyene imperialis* (Rossi, 1846)	56	3.5	9	BOLD:AAO2153
	143	*Thyene* sp. 1GAB_PAK	1	N/A	11	BOLD:AAV1607
*NS	144	*Trite* sp. 1GAB_PAK	13	0.5	11	BOLD:AAO2154
NP		**Segestriidae** Simon, 1893				
*	145	*Ariadna* sp. 1GAB_PAK	1	N/A	20	BOLD:AAO2054
		Sparassidae Bertkau, 1872				
NP	146	*Heteropoda maxima* Jäger, 2001	20	0.3	4.3	BOLD:ACB5077
	147a	*Heteropoda* sp. 3GAB_PAK	1	2.3	5.4	BOLD:ABW2881
	147b	*Heteropoda* sp. 3GAB_PAK	1			BOLD:AAO2057
	148	*Heteropoda* sp. 4GAB_PAK	1	N/A	4.3	BOLD:ACB5549
	149	*Olios* sp. 1GAB_PAK	1	N/A	3.9	BOLD:ADD6859
	150	*Olios* sp. 2GAB_PAK	10	0.5	3.9	BOLD:ADD7417
	151	*Olios* sp. 3GAB_PAK	4	0.3	4.1	BOLD:ACB4191
	152	*Olios* sp. 4GAB_PAK	4	1.1	7.2	BOLD:AAQ0159
	153	*Olios* sp. 5GAB_PAK	15	2.2	7.2	BOLD:AAQ0157
	154a	*Olios tener* (Thorell, 1891)	4	1.9	11	BOLD:AAQ0107
	154b	*Olios tener* (Thorell, 1891)	1			BOLD:ADK3497
	154c	*Olios tener* (Thorell, 1891)	1			BOLD:ADJ7965

(*Continued*)

Table 1. (Continued)

	No.	Taxa	*N*	K2P	NN	BINs
	155	*Pseudopoda prompta* (O. Pickard-Cambridge, 1885)	4	0.9	13	BOLD:AAO2056
	156a	*Spariolenus tigris* Simon, 1880	1	4.1	12	BOLD:ADF5077
	156b	*Spariolenus tigris* Simon, 1880	1			BOLD:ABW2878
		Tetragnathidae Menge, 1866				
*NP	157	*Glenognatha tangi* (Zhu, Song & Zhang, 2003)	3	1.2	18	BOLD:AAQ0147
	158	*Guizygiella indica* (Tikader & Bal, 1980)	8	1.1	14	BOLD:ABX7345
	159	*Leucauge celebesiana* (Walckenaer, 1841)	7	0.2	11	BOLD:AAO2068
	160	*Leucauge decorata* (Blackwall, 1864)	30	0.5	11	BOLD:AAG8516
*	161	*Metleucauge* sp. 1GAB_PAK	1	N/A	19	BOLD:AAV1600
NP	162	*Tetragnatha boydi* O. Pickard-Cambridge, 1898	3	0	15	BOLD:ACB2930
NP	163	*Tetragnatha cavaleriei* Schenkel, 1963	2	0.5	16	BOLD:AAT8904
	164	*Tetragnatha javana* (Thorell, 1890)	43	2.8	17	BOLD:AAO2174
	165	*Tetragnatha mandibulata* Walckenaer, 1841	1	N/A	15	BOLD:AAK2567
NP	166	*Tetragnatha maxillosa* Thorell, 1895	4	0.3	15	BOLD:AAK2560
NP	167	*Tetragnatha nitens* (Audouin, 1826)	6	0.8	15	BOLD:AAD3790
	168	*Tetragnatha* sp. 1GAB_PAK	1	N/A	16	BOLD:ABW2885
		Theraphosidae Thorell, 1869				
*	169	*Chilobrachys* sp. 1GAB_PAK	1	N/A	4.3	BOLD:ADD5278
*	170	*Chilobrachys* sp. 2GAB_PAK	1	N/A	4.3	BOLD:AAQ0160
		Theridiidae Sundevall, 1833				
*NP	171	*Emertonella taczanowskii* (Keyserling, 1886)	1	N/A	12	BOLD:AAV1610
	172	*Enoplognatha* sp. 1GAB_PAK	1	N/A	12	BOLD:ACI8909
	173	*Enoplognatha* sp. 2GAB_PAK	1	N/A	15	BOLD:ACP4208
*	174	*Euryopis* sp. 1GAB_PAK	1	N/A	12	BOLD:AAQ0155
	175	*Latrodectus* sp. 1GAB_PAK	1	N/A	19	BOLD:AAV1732
	176	*Latrodectus* sp. 2GAB_PAK	1	N/A	16	BOLD:AAO3347
*	177	*Meotipa* sp. 1GAB_PAK	2	2	12	BOLD:AAQ0152
	178	*Phylloneta* sp. 1GAB_PAK	11	0.3	11	BOLD:AAV3043
*NP	179	*Steatoda cingulata* (Thorell, 1890)	2	0	14	BOLD:ABW2877
NP	180	*Theridion melanostictum* O. Pickard-Cambridge, 1876	1	N/A	11	BOLD:AAV1617
	181	*Theridion* sp. 1GAB_PAK	1	N/A	11	BOLD:ACB2932
	182	*Theridion* sp. 3GAB_PAK	1	N/A	12	BOLD:AAV1623
		Thomisidae Sundevall, 1833				
*NP	183	*Coriarachne melancholica* Simon, 1880	1	N/A	7.9	BOLD:ACI8639
*NP	184	*Ebelingia kumadai* (Ono, 1985)	3	0	12	BOLD:AAV1619
	185	*Henriksenia hilaris* (Thorell, 1877)	1	N/A	11	BOLD:AAV1618
NP	186	*Lysiteles kunmingensis* Song & Zhao, 1994	3	0	9.9	BOLD:ACI8899
*	187	*Misumenoides* sp. 1GAB_PAK	1	N/A	11	BOLD:AAV1594
*	188	*Misumenops* sp. 1GAB_PAK	2	1.9	11	BOLD:AAV1596
*	189	*Ozyptila* sp. 1GAB_PAK	1	N/A	11	BOLD:ADF5201
	190a	*Runcinia insecta* (L. Koch, 1875)	40	4.9	11	BOLD:AAI0997
	190b	*Runcinia insecta* (L. Koch, 1875)	2			BOLD:AAQ0108
*NP	191	*Tharpyna indica* Tikader & Biswas, 1979	1	N/A	12	BOLD:AAV1606
NP	192	*Thomisus onustus* Walckenaer, 1805	1	N/A	8.6	BOLD:AAD7031
E	193a	*Thomisus zaheeri* Parveen, Khan, Mushtaq, Ahmad & Rana, 2008	30	4.3	11	BOLD:AAP4819
	193b	*Thomisus zaheeri* Parveen, Khan, Mushtaq, Ahmad & Rana, 2008	1			BOLD:AAQ0153
	194	*Tmarus dostinikus* Barrion & Litsinger, 1995	13	0.2	11	BOLD:ABX7413

(*Continued*)

Table 1. (Continued)

	No.	Taxa	*N*	K2P	NN	BINs
NS	195a	*Tmarus* sp. 1GAB_PAK	3	2.9	11	BOLD:ABX7346
	195b	*Tmarus* sp. 1GAB_PAK	5			BOLD:ADJ6297
	195c	*Tmarus* sp. 1GAB_PAK	4			BOLD:ADK4624
	195d	*Tmarus* sp. 1GAB_PAK	1			BOLD:ADK4625
NP	196	*Xysticus joyantius* Tikader, 1966	1	N/A	13	BOLD:ADF4849
	197	*Xysticus* sp. 1GAB_PAK	3	0.6	7.9	BOLD:ACI8898
	198	*Xysticus* sp. 2GAB_PAK	1	N/A	12	BOLD:ADF4647
		Uloboridae Thorell, 1869				
*	199	*Hyptiotes* sp. 1GAB_PAK	1	N/A	15	BOLD:AAQ2632
	200a	*Uloborus* sp. 1GAB_PAK	4	4	14	BOLD:AAW8359
	200b	*Uloborus* sp. 1GAB_PAK	1			BOLD:ABW2879
		Zodariidae Thorell, 1881				
*	201	*Zodarion* sp. 1GAB_PAK	1	N/A	14	BOLD:AAV1621
*	202	*Zodarion* sp. 2GAB_PAK	1	N/A	14	BOLD:ACG0983
		Total	**1795**			**221**

N = number of individuals; K2P = maximum Kimura 2-parameter distance; NN = distance to Nearest Neighbor species; BIN = Barcode Index Number; NP = new species or family to Pakistan; * = new genus to Pakistan; E = endemic species to Pakistan; U = undescribed opposite sex; NS = putative new species to science.

https://doi.org/10.1371/journal.pone.0217086.t001

showed that maximum intraspecific distance for all but one of the 90 species with two or more records was less than its NN distance (*Oxyopes azhari* was the exception, overlapping with *Oxyopes oryzae*) (Fig 5). The Mantel test was non-significant (P>0.01) for 60 of the 69 species and the regression line for all species showed a weak positive relationship ($R^2 = 0.08$; $y = 0.0003x + 2.62$) (Fig 6).

The similarity between the spider fauna in Pakistan and that of other nations was calculated by examining BIN overlap. Less than a quarter (52/221) of the BINs from Pakistan were

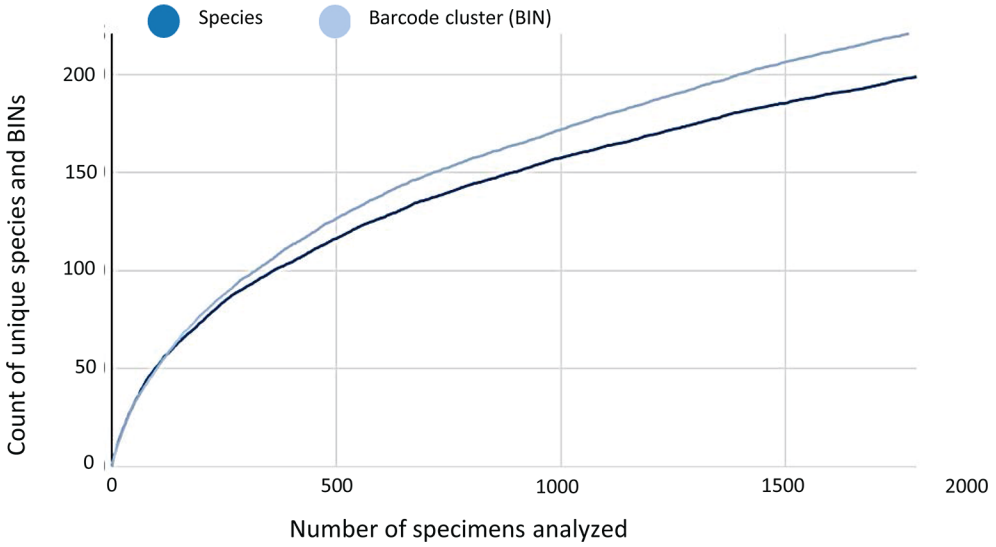

Fig 2. Accumulation curve for morphological species and barcode index numbers (BINs) for 1,795 spiders from Pakistan.

https://doi.org/10.1371/journal.pone.0217086.g002

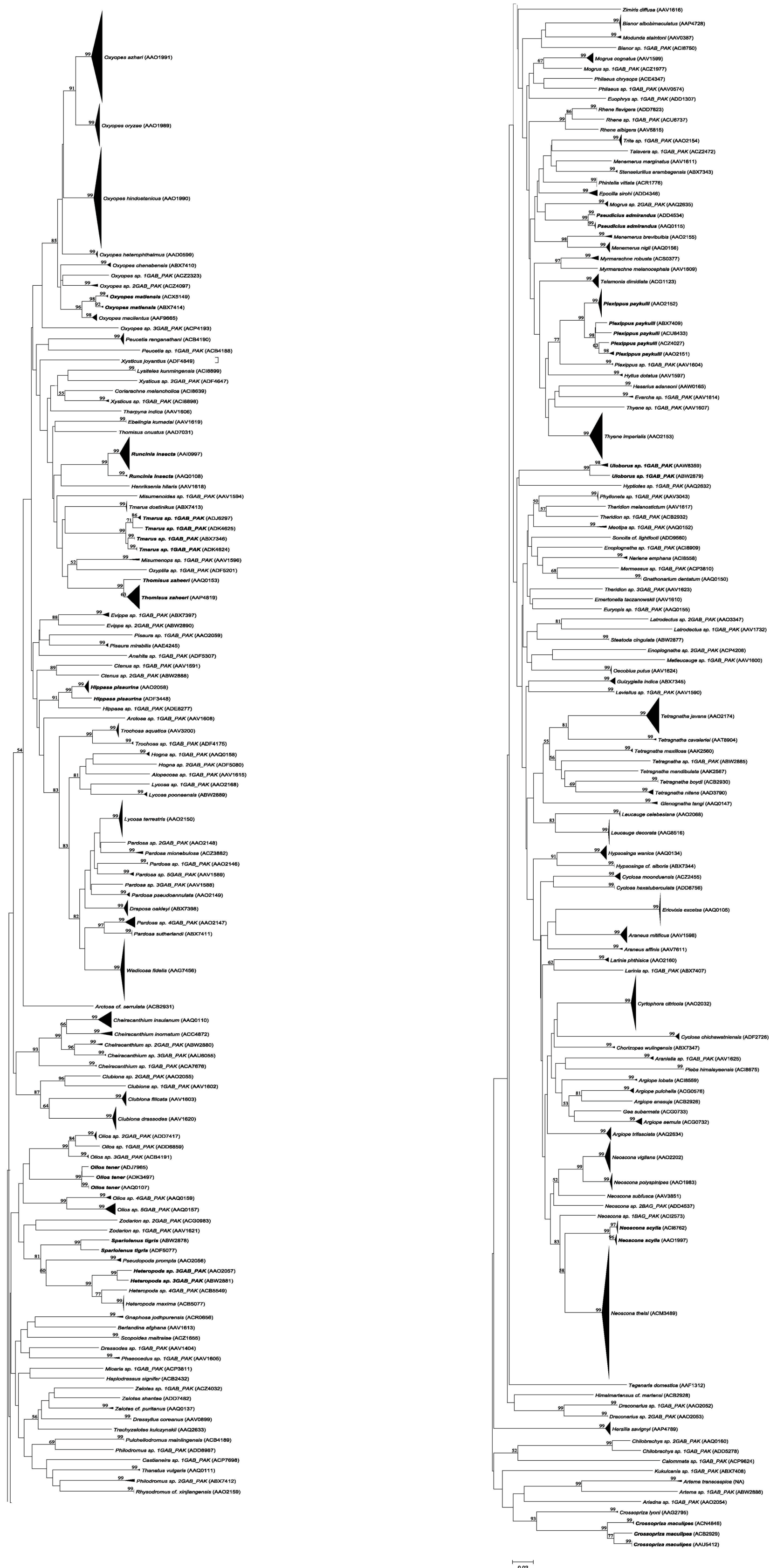

Fig 3. NJ analysis of spider species based on the analysis of 1,782 COI sequences. Bootstrap values (50% or higher; 1000 replicates) are shown above the branches. The scale bar shows K2P distances. The node for each species with multiple specimens is collapsed to a vertical line or triangle, with the horizontal depth indicating the level of intraspecific divergence. Species assigned to multiple BINs are indicated in **bold**. The tree is presented in two parts.

https://doi.org/10.1371/journal.pone.0217086.g003

represented among the 10,229 spider BINs reported in prior studies. As expected, the highest overlap (23%) was with India, but the proportion of shared BINs was far lower for the other 43 countries (Fig 7).

Discussion

Most prior work on the spider fauna of Pakistan has had a regional focus and only employed morphological approaches. For example, 157 species were reported from the province of Punjab [9], 56 from the district of Sargodha [76], 23 from Peshawar [11], and 13 from Buner [77].

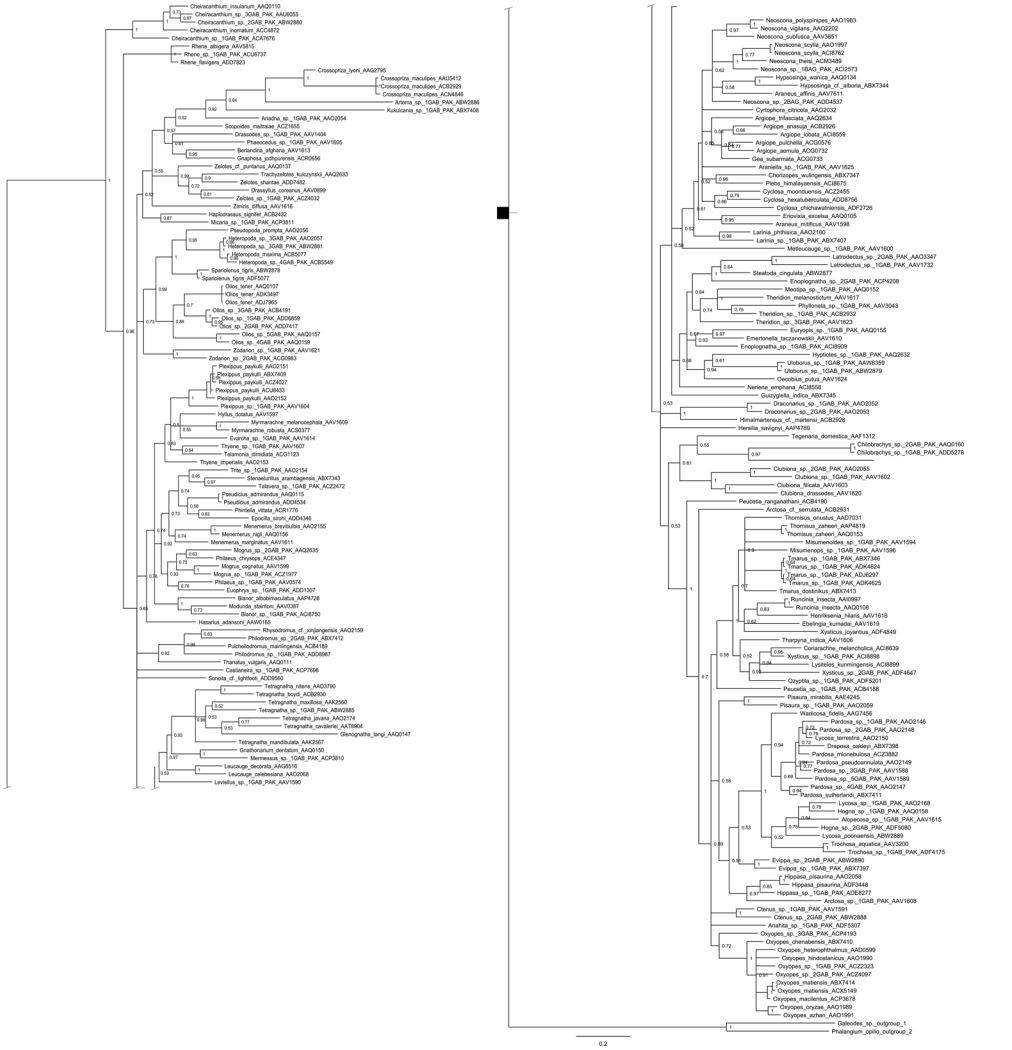

Fig 4. Bayesian phylogenetic analysis of spiders from Pakistan based on COI sequences. Posterior probabilities are indicated at the nodes. Taxa are followed by the BINs. *Phalangium opilio* (Arachnida: Opiliones) and *Galeodes sp.* (Arachnida: Solifugae) were employed as outgroups. Due to its large size, the tree is presented in two parts.

https://doi.org/10.1371/journal.pone.0217086.g004

Table 2. Sequence divergences (K2P) for differing levels of taxonomic affinity for the COI-5′ gene region for the spiders from Pakistan. Analysis was restricted to sequences >400 bp.

Distance class	*n*	Taxa	Comparisons	Min (%)	Mean (%)	Max (%)
Intraspecific	1702	122	44347	0	0.8	5.3
Congeners	1338	44	56792	2.8	8.8	23.2
Confamilial	1662	15	137164	4.3	15.1	26.7

https://doi.org/10.1371/journal.pone.0217086.t002

A recent checklist for the spiders of Pakistan [10] included records for 239 species, but the present study has substantially increased this total by adding first records for 84 described species and another 93 that could not be assigned to a known taxon. Most importantly, this study generated a DNA barcode reference library for 202 species, facilitating their future identification.

Because the spider fauna of Pakistan has seen such limited study, the discovery of new species was not unexpected, and follows a pattern seen for spiders in other regions. For example, the analysis of 80 species of Salticidae from Papua New Guinea revealed 34 species and five genera new to the country [78]. Likewise, 6% of the 136 spider species recovered from the Northern Cape Province, South Africa were new [79]. This study employed a mix of methods for spider collection, including beating, sweeping, and pitfalls. The choice of sampling method impacts species detection [80] and extensive sampling is critical to generate comprehensive species coverage [81]. Although the present study involved collections at 225 sites, the resultant species accumulation curve did not reach an asymptote, indicating that many more species await detection.

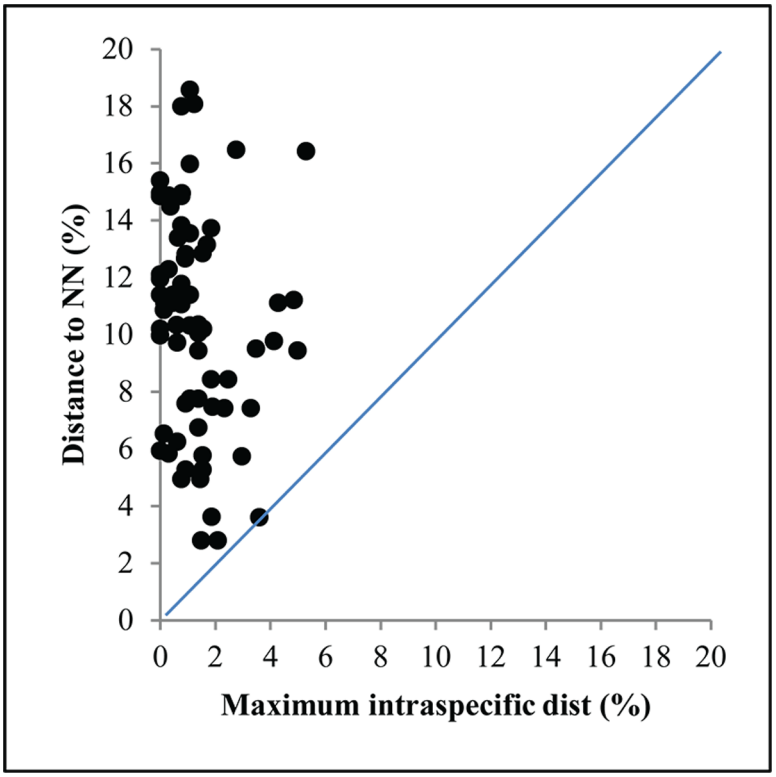

Fig 5. Barcode gap analysis for spider species represented by three or more records. Points that fall above the 1:1 line (blue) indicate the presence of a local barcode gap. NN = Nearest-Neighbor species.

https://doi.org/10.1371/journal.pone.0217086.g005

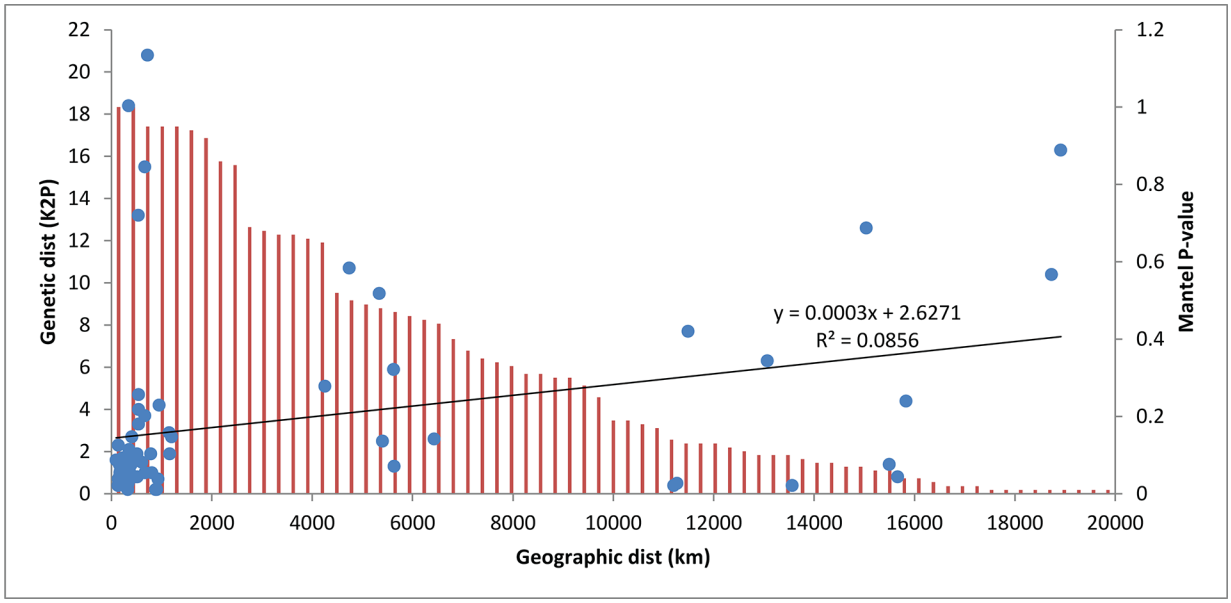

Fig 6. Intraspecific sequence divergence (K2P) for the COI gene (blue dots) versus geographic distance (km) for spider species from Pakistan with data from other regions. The relationship between genetic and geographic distances is indicated by a regression line. P-values for the Mantel Test are indicated by red vertical lines.

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The present study revealed a close correspondence (93%) between BINs and morphospecies as 188 of the 202 species were assigned to a unique BIN, reinforcing a pattern seen in other groups [37,38,40]. For example, the concordance between BINs and species was 78% in a study that examined 30,000 Canadian spiders representing 1,018 species [61] with most

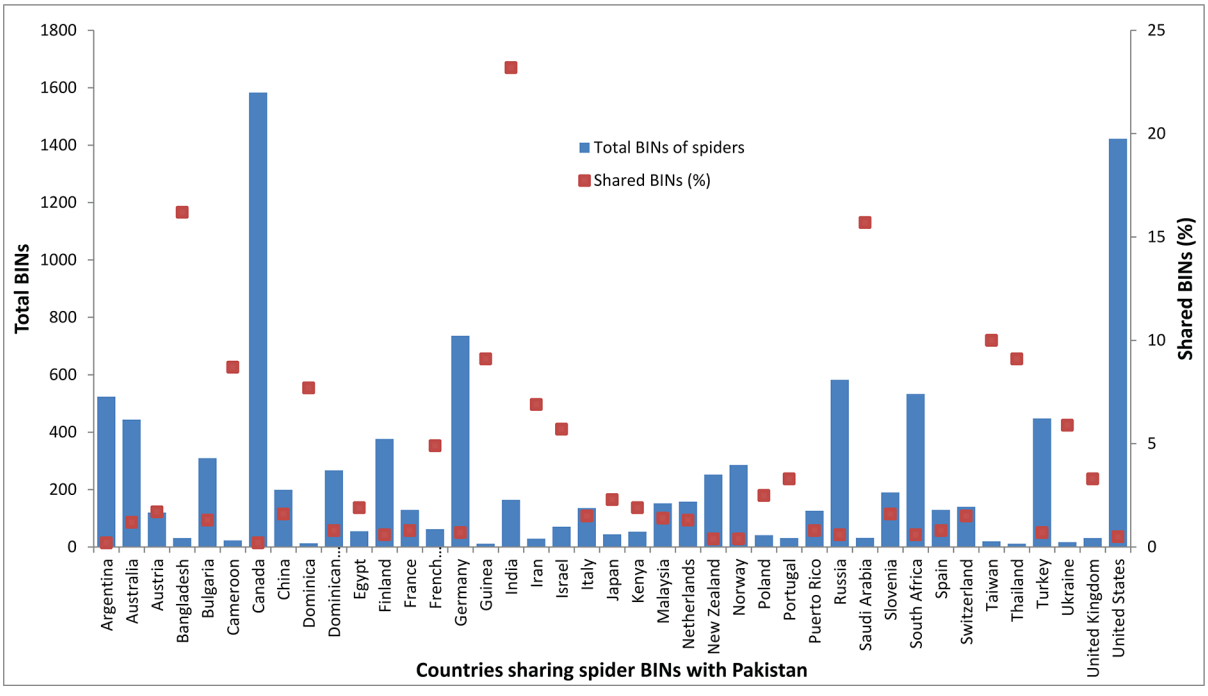

Fig 7. Percentage of spider BINs shared between Pakistan and 41 other nations.

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discordances reflecting BIN splits suggestive of overlooked species. Stronger species-BIN correspondence has been reported in several insect groups; 96% for Erebidae (Lepidoptera) from the Iberian Peninsula [38], 94% for tiger moths from Brazil [82] and 92% for beetles from central Europe [40]. However, some arthropod groups have shown relatively low level of species-BIN concordance; for example, orthopterans in Central Europe (76%) [83], waterstriders in Germany (82%) [84] and katydids in China (75%) [85]. Thirteen (6%) species in this study were assigned to two or more BINs (BIN splits), and one species (*Plexippus paykulli*) was assigned to five. BIN splits often indicate the presence of a species complex [43]. For example, 13% of 1,018 species of Canadian spiders [61], 13% of 1,541 Canadian Noctuoidea [86], 5.7% of 1,872 Finnish beetles [87], and 20% of 62 global mealybugs [88] possessed BIN splits. Although in most cases the subsequent morphological investigation has revealed overlooked species [89], other factors can cause BIN splits/mergers, such as hybridization [90], incomplete lineage sorting [83], or rapid speciation [91].

K2P divergences >2% were found in 14 of the 202 spider species from Pakistan with a maximum value of 5.3%. There was, however, no significant relationship between intraspecific divergence and the number of specimens analyzed. For example, 12 specimens of *Crossopriza maculipes* (3 BINs) showed 5.3% divergence and were assigned to three BINs while 160 specimens of *Neoscona theisi* possessed a maximum divergence of 2.5%. High COI divergence is not uncommon in spiders. For example, the maximum intraspecific divergence in 561 spider species from Germany was 10.1%, but it was below 2.5% in 95% of the cases with an arithmetic mean of 0.7% [62]. The divergence could depend on several factors such as the number of specimens analyzed, the number of localities, the geographic distance between them and the dispersal capabilities of the particular species [92,93]. With the exception of a single species (*Oxyopes azhari*), high conspecific distances did not impede the capacity of DNA barcodes to discriminate the species encountered in our study. However, species with BIN splits and high divergences are likely to represent a cryptic species complex. Preliminary morphological analyses including genitalic dissections of specimens from taxa with BIN splits in this study reinforced this conclusion.

Correlation analysis revealed only a weak relationship between the geographic range of the species examined in this study and their intraspecific divergence value. The Mantel test was significant for a few (13%) species, but species identification was not impeded as maximum intraspecific distances were nearly always less than NN distances. Similar results have been reported for Lepidoptera from Europe [94], Pakistan [32] and Central Asia [95]. Although a study that examined a single tribe, Agabini, of aquatic beetles in Europe [96] argued that regional divergences were so great as to obscure species assignments, this result is clearly not the rule [72].

Because BINs are generally an effective species proxy [41], we used them to assess faunal overlap. This work revealed that most (76%) BINs detected in this study were first records. Just 52 BINs have records from other nations and 13 of these were shared only with India. The BIN overlap with other nations was considerably lower for the spiders (24%) of Pakistan than for its Lepidoptera (42%) [42], but this difference almost certainly reflects the intensive barcode studies on the latter group. Although DNA barcoding has been used to assess regional biodiversity [41,47] and to ascertain species connections [42], the limited data availability complicates interpretation. Although further sampling will add new BINs, it is also likely to raise BIN overlap with other regions, improving our understanding of faunal overlap. Such efforts to better document local biodiversity are also certain to reveal new species as evidenced by the discovery of 93 taxa in this study that could not be assigned to a known species.

Supporting information

S1 Table. Taxonomic publications consulted for this study.
(DOCX)

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