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MORPHOLOGICAL AND GENETIC CHARACTERIZATION OF VARIOUS *Apis* SPECIES CAPTURED FROM SELECTED SITES OF PUNJAB

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ABSTRACT

The specimens of Apis mellifera, A. dorsata and A. florea were collected during field surveys during July, 2018 to August, 2020 from selected sites of Punjab, Pakistan. These sites included Bhawalpur, Khaniwal, Kasur, Lahore, Chakwal and Rawalpindi. The captured specimens were identified first on the basis of morphology and then identified using mitochondrial DNA genes. A. mellifera workers, queens and drones were black in color. Apis dorsata workers were yellow, however, queens and drones were brown. Apis florea queens and workers were yellow banded and the drones were black. The phenol chloroform technique was used to extract whole genomic DNA from preserved honey bee tissues. The quality of the DNA was determined using gel electrophoresis, and the quantity was determined using a thermal scientific Nano-drop. COI primers were used for PCR amplification. All of the acquired specimens' DNA sequences revealed reliable and obvious species identification. GenBank was contacted when appropriate amplified DNA samples were uploaded, and accession numbers were acquired (MZ433243.1, MZ433277.1 and MZ433289.1). After removing problematic bases, the Apis mellifera, Apis dorsata, and Apis florea COI gene fragments were 670, 680, and 675, respectively. Closely comparable Apis sequences were collected from NCBI in blast queries and used in phylogenetic studies. Neighbor-Joining tree, Maximum Likelihood tree and Minimum Evolution tree of the genus Apis was constructed based on p-distance using MEGA X. The overall, genetic divergence of genus Apis was 0.098 ± 0.022. It can be concluded that the morphological characteristics provide information as an indicator for estimating genetic variations between honey bee populations. Further work is needed to explore more areas of Pakistan and molecular identification is required to report any new or subspecies from country.

KEYWORDS:
Apis dorsata, Apis florea, Apis mellifera, Genetic characterization, Molecular identification

INTRODUCTION

Pakistan covers an area of 796096 square kilometer and located from 24° and 37° N latitude to 61° and 78° E longitude. The terrain of the country is varied and unique as out of six zoogeographic regions of the world, elements of Palearctic, Oriental and Ethiopian regions are represented in it, making Pakistan a land of many lands. Climate of the country is continental type with extreme variations of temperatures during summer and winter seasons leading to floods and droughts during different seasons of the year. Pakistan has approximately 3.7% area covered with forests which are further degrading day by day resulting in an increase in desert areas [1].

Bees are a specialized kind of flying insect that contributes much too global biodiversity. They resemble ants and wasps but they are famous for providing royal jelly, honey, bee wax, and also propolis which can be very useful against various diseases [2]. Honey bees are valuable insects for humanity in various aspects. They play their role in honey production and pollination of crops and wild plants for overall ecosystem maintenance. They are an important component of ecosystems because they pollinate one-third of all food consumed by humans, promote plant variety, maintain genetic variation within plant communities, enhance crop production, and therefore facilitate biodiversity at all levels of the food web [3]. Biological diversity is studied at three levels i.e. genetics, ecosystems and species in a given geographical region. Estimated diversity of planet earth is represented by 5 to 100 million species while 1.5 million species have been categorized

yet. The direct values of the diversity include provision of food, medicine, shelter and clothing while indirectly the humans largely depend on biodiversity for provision of clean air and water. Regardless of these values, the diversity plays significant role in pollination, seed dispersal and recycling of nutrients and this type of harmony is only possible if different components of biosphere are interlinked and maintained [4].

Honeybees, which are members of the Apidae family, are inhabitants of Africa, Europe, and Western Asia [5]. Within the endemic area, 28 different subspecies have been found, with morphology used to classify them [6]. Most were vital pollinators in both agricultural and forest settings [7]. The four well known honey bee species present in Pakistan are *Apis florea, Apis cerana, Apis dorsata, and Apis mellifera*, on the other side, had been introduced to the nation for commercial beekeeping in 1977. Regardless of the fact that Apis dorsata adds very little honey production because of its genetically reduced focused nature, its ecological relevance in pollination cannot be overstated. Fabricius initially described Apis dorsata in 1793, and Maa reintroduced it in 1953. Finally, Ruttner observed that it looked to be relatively homogenous in 1988. Throughout its geographical habitat in Southeast and East Asia, the Asian honey bee (Apis dorsata) is an important pollinator and honey producer [8]. In both shape and behaviour, this species differs from European honey bees (*Apis mellifera*). Asian bees have a life span that is completed in 21 days and is quite comparable to Apis mellifera. The colony is made up of a single fertile female (the queen), thousands of worker bees, and, depending on the season, male drones. Mitochondrial genomes are known for mutagenesis or adaptive substitutions, which distinguishes the genome and leads to phylogenetic divergence for different genes [9]. In mammals, the average variation in mitochondrial genome is 5-10 times considerably greater than that of the nuclear genome [10].

Several Apis florea subspecies, variations, and nations have been described during the last two centuries, and samples of Apis andreniformis have been recorded as *Apis florea*. The correctness of *Apis florea Fabricius* (1787) and Apis andreniformis Smith's dwarf honeybee identifications (1858). Apis florea is extremely widespread. Over the previous two centuries, several Apis florea subspecies, variants, and nationalities have been documented, and samples of Apis andreniformis have been recognized as *Apis florea* (Maa 1953). The identifications of *Apis florea Fabricius* (1787) & Apis andreniformis Smith's dwarf honeybees are valid (1858). Apis florea is highly widespread, extending over 7000 kilometers from its eastern part end in Vietnam and southeastern China, across continental Asia throughout and beneath the southern ends of the Himalayas, west to the Iranian Plateau, and south into Oman. This region is around 70 degrees longitude (40 degrees east–110 degrees east) and approximately 30 degrees latitude (6 degrees north–34 degrees north) spreading 7000 kilometers from its easternmost extreme in Vietnam and southeastern China, across mainland Asia along and beneath the southern sides of the Himalayas, west to the Iranian Plateau, and south into Oman. This region is around 70 degrees longitude (40 degrees east–110 degrees east) and approximately 30 degrees latitude (6 degrees north–34 degrees north) [11]. For up to two thousand years, indigenous cultures in Asia have raised the Asian honeybee Apis cerana [12]. According to Abrol [13] and Atwal [14], in the early twentieth century, Asian beekeepers began importing the Apis mellifera, prefer living in colonies of 30,000 to 50,000 bees.

Apis dorsata is solely found in Asia, unlike Apis mellifera, which is now distributed all over the world [15]. Apis dorsata is significantly bigger than *Apis mellifera*, and its individuals are approximately longer. Unlike other honey bees, Apis dorsata has little change in body size across castes (Oppenheim et al., 2020). It grows in open areas at elevations ranging from 1000 m to 3000 m, forming huge combs that are generally 3-4 feet long and 2-3 feet broad. [16-17]. Due to its aggressive behavior this species cannot be domesticated. Because its hives are a vital source of income for the community, traditionally, honey was obtained by removing the bulk of the comb off the tree [18].

Bees are generally classified based on their morphological characteristics, although genetic differences across races can be distinguished using mitochondrial DNA gene segments [5], single nucleotide polymorphism [19], and allozymes [20]. The use of molecular research to determine the level of genetic variation across and among honey bee species is a convincing strategy [21]. On Asian and western Apis species inhabiting the country, there is virtually little research on morphological and molecular analyses. Although some genetic analyses of A. mellifera have been done using the mitochondrial gene segment i.e., cytochrome c oxidase I [22], there is no molecular evidence on other Asian Apis species. Despite advances in molecular methods for species identification, honeybees such as Apis dorsata, Apis cerana, Apis florea, and Apis mellifera have not been thoroughly investigated in Pakistan, which is vital to understanding, geographical distribution, and colonization. Its conservation is particularly significant from the perspective of biodiversity, as the preservation of indigenous honey bee races in the Southeast Asian region is a top priority. Environmental factors play a big role in limiting gene flow, which leads to genetic differentiation [23].

MATERIALS AND METHODS

Study area. The current investigation was carried out in several locations in Punjab, Pakistan.

These sites include Bhawalpur, Khaniwal, Kasur, Lahore, Chakwal and Rawalpindi (Figure 1). The specimens of Apis mellifera, Apis dorsata and Apis florea were collected during field surveys extended from July 2018 to August, 2020. The captured specimens were identified on the basis of morphology and bring to the lab, Department of Wildlife and Ecology, UVAS, Ravi Campus for molecular characterization.

Sampling strategy. Each selected study area was further divided into three different microhabitat types for collection of specimens.

DNA extraction and amplification. The phenol chloroform technique was used to extract whole genomic DNA from conserved honey bee tissues (Ali et al., 2020). The quality of the DNA was determined using gel electrophoresis, and the quantity was determined using a thermal scientific Nano-drop. The primers listed in table 3 were used for PCR amplification. PCR amplification was carried out in a 25µL volume reaction with 2-5 µl of DNA. The PCR amplification consisted of 94 °C for 3 minutes, 40 cycles of 94 °C for half minute, 42–56 °C for another half minute (depending on the annealing temperature of the primers), and 72 °C for 60 Seconds, and a final 10 minutes at 72 °C. The DNA sequences acquired were uploaded to GenBank for accession numbers.

Phylogenetic analysis. Closely matched sequences of genus Apis were retrieved from NCBI in blast searches and incorporated in phylogenetic analysis. Neighbor-Joining tree, Maximum Likelihood tree and Minimum Evolution tree of genus Apis was constructed based on p-distance using MEGA X to check the evolutionary history. Genetic diversity within and between the species was calculated using Bioedit Software 7.0.

RESULTS

The specimens of Apis mellifera, Apis dorsata and Apis florea were gathered from several locations in Punjab, Pakistan (Figure 1) during studies conducted from July 2018 to August, 2020. These sites include Bhawalpur, Khaniwal, Kasur, Lahore, Chakwal and Rawalpindi. The GPS coordinates of study area are mentioned in Table 1.

Morphology and body measurements. A total of 10 specimens from each species were used to take morphological measurements. The morphological characteristics provide information as an indicator for estimation of genetic variations in the characteristic of honey bee. Workers, queens and drones of the Apis mellifera are of black color, Apis dorsata workers are yellow; queens and drones are of the brown form. The Asian Apis florea queens and workers are yellow banded and the drones are black. The average proboscis length for Apis florea workers was 3.18 mm. The length of fore wing of Apis dorsata is 1.24 ± 0.11 to 1.26 ± 0.11 and width is 0.43 ± 0.10 to 0.46 ± 0.10 and the length of hind wing varies from 0.80 ± 0.05 to 0.83 ± 0.05 and width was 0.21 ± 0.00 to 0.23 ± 0.00 (P>0.05). The total length of leg of Apis dorsata varied during the present study. Femur varies 0.30 ± 0.01 to 0.34 ± 0.01, length of tibia is 0.35 ± 0.03 to 0.39 ± 0.03 and the length of metatarsus is 0.51 ± 0.01.

Apis florea had a mean front wing length of 6.24 mm, a front wing width of 2.30 mm, a posterior wing length of 4.32 mm, and a posterior wing width of 1.49 mm. ± Apis mellifera had a minimum forewing length of 8.88 ± 0.08 mm and a minimum hindwing length of 6.20 ± 0.06 mm. The average forewing width was 3.00± 0.08 mm. The breadth of the hind wing varies amongst Apis mellifera populations, ranging from 1.78± 0.02 to 1.82 ± 0.02 mm. The shortest femur length measured was 2.41 ± 0.04 mm, while the longest tibial length measured was 2.91 ± 0.13 mm. The mean value of metatarsus length for Apis mellifera was 1.92 ± 0.07 mm.

The description of captured species from study area is as in Figure 1.

Genus *Apis* (Linnaeus, 1758)Honey bees (genus Apis) are social insects belonging to the Apidae family of the Hymenoptera order. They are classified as Aculeata (i.e., those having stingers). There are 11 species in the genus *Apis*. *A. dorsata* and *A. florea* are cavity-nesting tropical species. *A. mellifera* and *A. cerana* are two species that can survive outside of the tropics. *A. mellifera* is the most significant species for humans and has been introduced all over the world for use in beekeeping. *A. mellifera* produces the majority of the world's honey. Excluding the north part, it is native to Africa, the Middle East and Europe. Asia is home to all other *Apis* species, *A. cerana,* which is kept in hives in both the temperate and tropical zones is smaller and produces fewer colonies than *A. mellifera*. Other Asian species form multiple combs.

Apis mellifera (Linnaeus, 1758). The color of *Apis mellifera* varied between red to brown and have orange to black rings on abdomen. Apis mellifera have more hair on their thorax as compared to their abdomen and also have a pollen basket. *Apis mellifera* is a species native to Europe, Western Asia, and Africa. Humans originally brought Apis mellifera to other continents in the 17th century, but it's now found throughout the world.

Apis dorsata (Fabricius, 1793). The giant honey bee (*Apis dorsata*) is a honey bee native to South and Southeast Asia, primarily found in forested areas such as Nepal's Terai. They are usually

17 – 20 mm (0.7 – 0.8 in) in length. Nests are typically constructed in exposed locations high above the ground, such as on tree limbs, under rock overhangs, and even on buildings. When disturbed, these social bees are famous for their strong defense tactics and violent behavior. Indigenous peoples have traditionally used this species as a source of honey and beeswax, a technique known as honey hunting, while not domesticating it.

Apis florea (Fabricius, 1787) [21]. *Apis florea* is referred as dwarf honey bee Due to its small size in comparison to other honeybees. A worker's body length is normally 7–10 mm, and its overall coloring is red-brown. A colony normally constructs a single exposed comb on tree branches or plants. Honey is produced by Apis florea, which is gathered and consumed. They are great pollinators, thus they play a vital ecological role in the areas where they live. The basitarsus, which runs two-thirds of the length of the tibia, is a thumb-like bifurcation carried by drones. *Apis florea's* fimbriate lobe features three protrusions that sting with two stylet barbs. Apis florea's range is mostly restricted to tropical ranges. The species reported from Pakistan, Iran, Oman and Sri Lanka. Although it can be found as far east as Indonesia, it is primarily distributed in Southeast Asia.

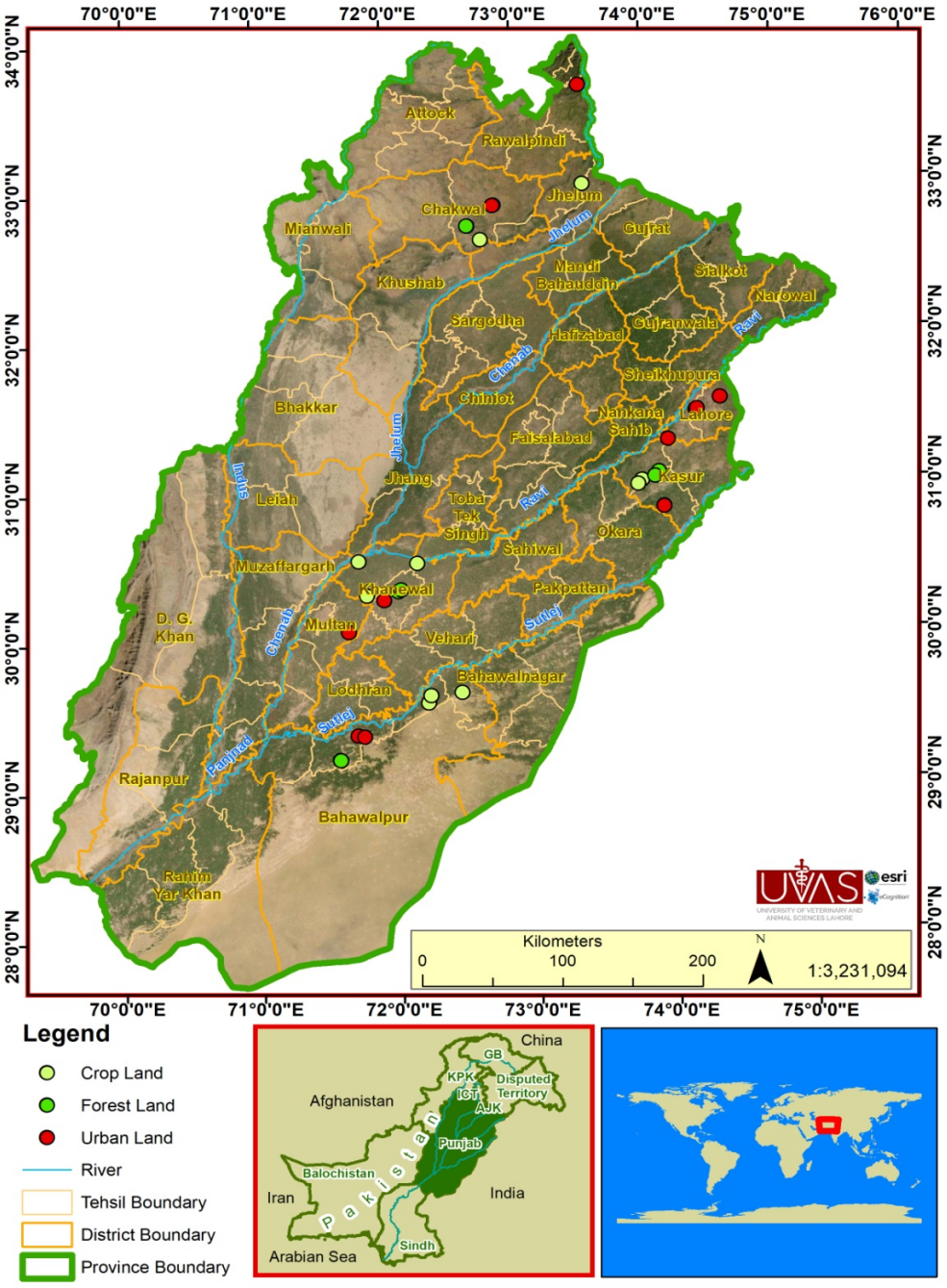

FIGURE 1
Map of study area

TABLE 1
GPS coordinates of samples collected from study area

Species	Collection Sites	GPS location
Apis mellifera n=25	Bahawalpur	29.660421, 72.483632
	Khanewal	30.531216, 72.178043
	Kasur	31.043064, 73.875854
	Lahore	31.497580, 74.29679
	Chakwal	32.780136, 72.628868
	Rawalpindi	---
Apis dorsata n=150	Bahawalpur n=30	29.22302, 71.57793 ; 29.22046, 71.57932 ;29.384738, 71.710050 29.375868, 71.758408; 29.594307,72.236664; 29.643673,72.256470
	Khanewal n=30	30.345037, 72.033357; 30.357852, 72.054797; 30.551282,71.743112 30.319722, 71.798545; 30.287108, 71.926776; 30.079554, 71.659665
	Kasur N=30	31.094229, 74.003663; 31.067959, 73.973808; 31.043064,73.875854, 31.016537, 73.845367, 30.863940, 74.030354, 30.863298, 74.031353
	Lahore n=25	31.497580, 74.29679; 31.50103, 74.30746; 31.309029, 74.079277 31.505322, 74.305497; 31.575902, 74.484181
	Chakwal n=30	32.780136, 72.628868 ; 32.780090, 72.627937; 32.914321, 72.833233 32.911679, 72.829670; 33.03687, 73.5194994; 32.684650, 72.729676
	Rawalpindi n=5	33.6985907, 73.5194994
Apis Florea n=150	Bahawalpur	29.22302, 71.57793; 29.22046, 71.57932; 29.384738, 71.710050 29.375868, 71.758408; 29.594307,72.236664; 29.643673,72.256470
	Khanewal	30.345037, 72.033357; 30.357852, 72.054797; 30.551282, 71.743112 30.319722, 71.798545; 30.287108, 71.926776; 30.079554, 71.659665
	Kasur	31.094229, 74.003663; 31.067959, 73.973808; 31.043064, 73.875854, 31.016537, 73.845367; 30.863940, 74.030354; 30.863298, 74.031353
	Lahore	31.497580, 74.29679; 31.50103, 74.30746; 31.309029, 74.079277 31.505322, 74.305497; 31.575902, 74.484181
	Chakwal	32.780136, 72.628868; 32.780090, 72.627937; 32.914321, 72.833233 32.911679, 72.829670; 33.03687, 73.5194994; 32.684650, 72.729676
	Rawalpindi	33.6985907, 73.5194994

Amplification and sequencing. During present study, DNA of *Apis mellifera, Apis dorsata* and *Apis florea* were extracted through phenol chloroform method. The purity of DNA was check through gel electrophrosis (Figure 2). DNA quantification was done on Thermo Scientific NanoDrop 2000 (Table 2). The COI gene was amplified using universal primer set as mentioned in Table 3. The obtained DNA sequences have shown reliable and clear species identification of all the captured specimens. Table 4 summarizes the successful amplified DNA samples and the GenBank accession numbers. After trimming ambiguous bases, the obtained COI gene fragment of *Apis mellifera, Apis dorsata* and *Apis florea* were 670, 680 and 675 respectively.

Phylogenetic analysis. The related DNA sequences of *Apis mellifera, Apis dorsata* and *Apisflorea*were available at NCBI database. Closely matched sequences of genus Apis were retrieved from NCBI in blast searches and incorporated in phylogenetic analysis.

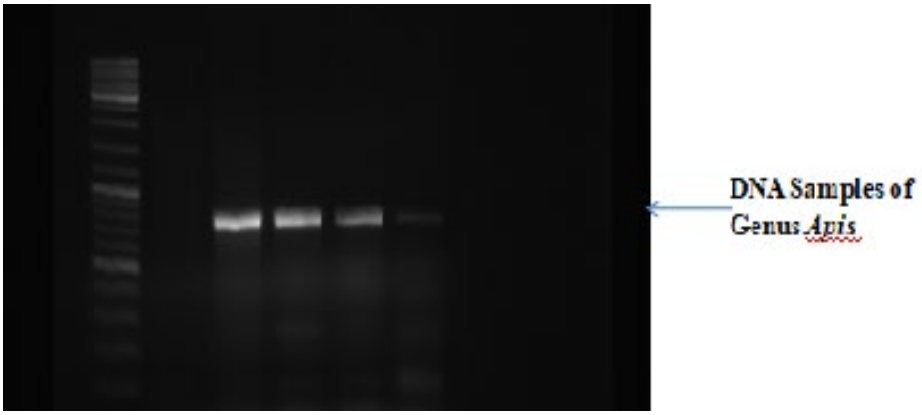

FIGURE 2
Successfully amplified DNA samples of genus*Apis*

TABLE 2
DNA quantification was done on Thermo Scientific Nano-Drop

Species	Nucleic Acid(ng/uL)	A260/A280	A260/A230	A260	A280
Apis mellifera	429.046	1.643	0.602	8.581	5.222
n=5	322.333	1.723	0.83	6.447	3.741
	8.095	1.281	0.555	0.162	0.126
	16.815	1.241	0.419	0.336	0.271
	65.933	1.374	0.861	1.319	0.96
Apis dorsata	106.899	1.47	1.533	2.138	1.455
n=5	52.384	1.394	1.03	1.048	0.752
	152.997	1.708	1.064	3.06	1.792
	36.772	1.119	0.275	0.735	0.657
	128.059	1.304	0.649	2.561	1.964
	52.436	1.127	0.393	1.049	0.93
Apis florea	817.462	1.736	1.172	16.349	9.419
n=5	310.506	1.521	1.063	6.21	4.083
	468.601	1.618	0.934	9.372	5.791
	114.824	1.128	0.335	2.296	2.035

TABLE 3
Primers set for COI gene used during present study

Marker	Primer Pair	Sequence (5′–3′)	Annealing condition	Amplification length
16S rRNA	16SA-L	CGCCTGTTTATCAAAAACAT	50 °C for 30 seconds	560 bp fragment
	16SB-H	CCGGTCTGAACTCAGATCACGT		
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	42°C for 30 seconds	710 bp fragment
	HCO2198	TAAACTTCAGGGTGAC-CAAAAAATCA		
Cytb	CYTB1	CCATCCAACTCTCAGCATG ATGAAA	55°C for 30 seconds	360 bp fragment
	CYTB2	GCCCCTCAGAATGATATTTGTCCTCA		

TABLE 4
The details of successful amplified DNA samples and GenBank accession numbers

Genus	Family	Species	Voucher number	GenBank accession number
Apis	Apidae	*Apis mellifera*	ZMUVAS1	MZ433243.1
		Apis dorsata	ZMUVAS2	MZ433277.1
		Apis florea	ZMUVAS4	MZ433289.1

Neighbor-Joining tree of genus Apis was constructed based on p-distance using MEGA X (Figure. 3). Bootstrap values are mentioned at the node of the tree. The analysis included 22 nucleotide sequences. For each sequence pair, all ambiguous points were removed.

Similarly, Maximum Likelihood tree (Figure 4) of genus Apis was constructed based on p-distance using MEGA X to check the evolutionary history. Bootstrap values are mentioned at the node of the tree. The analysis included 22 nucleotide sequences. For each sequence pair, all ambiguous bp were removed. The final dataset contained 375 positions in total. The overall, genetic divergence of genus *Apis* was 0.098 ± 0.022.

DISCUSSION

The discrimination of all species is difficult and only few taxonomists have exactly identified 0.01% of total estimated 15 million species approximately. Identification on morphological basis still required a team of 15000 taxonomists in perpetuity to identify all species of globe [24]. The existence of humans is directly linked with biodiversity for provision of food, shelter, health and clean water. However, the anthropogenic activities have led many species to extinction while others are striving for their survival. This in turn has put human survival at stake as humans also depend on biodiversity for their survival. Despite knowing importance of biodiversity, human pressure on biodiversity increased and loss of biodiversity is the major concern of the conservation biologists [25].

Species identification on morphological basis is still considered authentic but this approach has

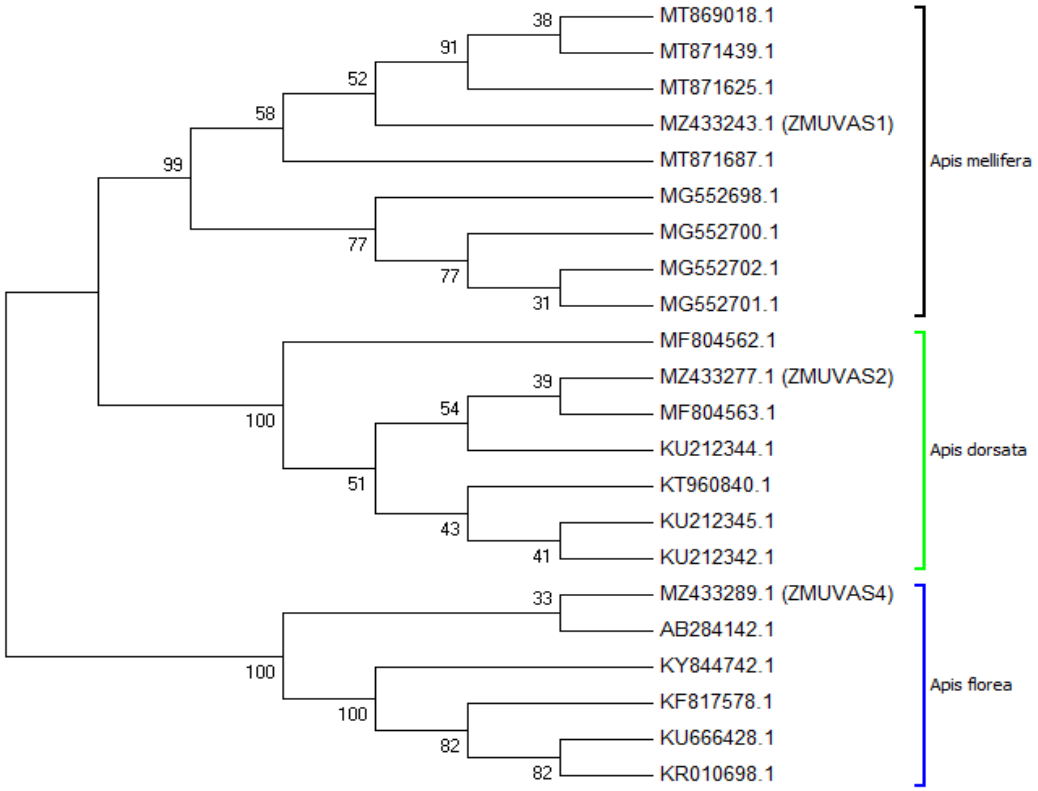

FIGURE 3
Neighbour-joining tree of genus *Apis* based on p-distance using MEGA 10.1.X

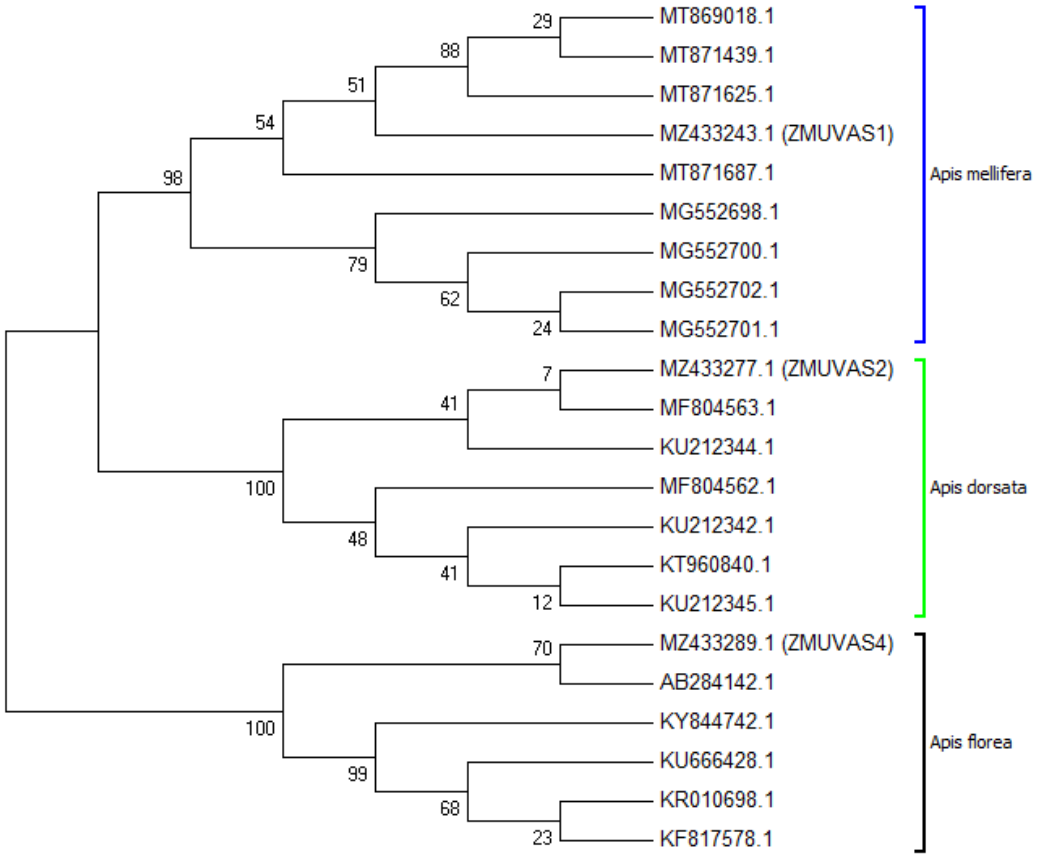

FIGURE 4
Maximum Likelihood tree of genus *Apis* based on p-distance using MEGA X.

four major problems. Phenotypic characters and genetic variability can lead to incorrect identification. Moreover, this technique is not appropriate for morphologically cryptic taxa and often these keys are used for particular life stage or gender, making identification difficult. Finally, the use of morphological keys often required expertise and misdiagnoses are common [26]. Morphological identification of species requires confirmation through molecular analysis as many resembling taxa have been identified as separate species. There is no short formula to identify species based on DNA sequence as the rate of molecular evolution varies. Previously, phylogenetic studies focused on mitochondrial genes such as ribosomal12S and 16S DNA, but occurrence of insertions and deletions in comprehensive taxonomic studies is major constrained [27-28]. The partial sequence of the Apis cerana cytochrome oxidase subunit I gene (COI) and its evolutionary connection (GenBank Accession No. KM230116). The Apis cerana COI gene sequence differed significantly from that of other Apis species [29].

Many species' biological and environmental characteristics during larval stages have been discovered using molecular based identification approaches [30]. Mitochondrial DNA (mtDNA) markers have been frequently used in the A. mellifera honey bee colonies and evolutionary studies. It includes areas with varying evolutionary rates. In theory, several genetic investigations employing molecular methods have supported the basic pattern of subspecies distribution [31].

Khan [32] reported DNA sequences of the COI gene from two Asian honey bee species (Apis cerana and Apis dorsata) and one European honey bee species (Apis mellifera) from Pakistan. A total of 20 honey bee species (Apis cerana), 18 (Apis dorsata), and 23 (Apis mellifera) were Sanger sequenced, and existing NCBI data was used to assess genetic diversity. The mitochondrial 16S rDNA gene from the recovered Apis mellifera subspecies to give tools for the quick identification and phylogenetic analysis of honey bees in Saudi Arabia. This resulted in a distinct genomic sequence that verifies their taxonomic position within the Apidae family [33]. The results revealed considerable genetic variation and diversity within and between species based on ND5 gene as compared to COI gene. The mitochondrial ND5 gene sequences from the genus Apis indicated the potential to resolve species relationships.

Intraspecific divergence of a COI gene partial coding segment is particularly useful for species identification [34]. The overall nucleotide differences based on the COI gene in A. mellifera was comparable to those reported by Rizwan et al. [22] for Apis population in Pakistan, which was 7.772. Both the A. mellifera selected for this research and the A. mellifera investigated by Rizwan et al. [22] have a lot of genetic interaction and the least amount of diversity. Martimianakis et al.[35] discovered this type of genetic interaction when studying the evolutionary relationship of the Greek Apis population based on sequencing of the COI and ND5 genes' mtDNA regions. They stated that distinct subspecies of A. mellifera from various locations create a relationship. These previous investigations support our findings of A. mellifera population genetic interaction based on the COI gene. Within and across Apis species, the overall amount of nucleotide differences based on the ND5 gene was greater. The average number of nucleotide differences between adjacent related segments was notably greater, especially for ND5. This indicated that there was diversity within the Apis genus.

Since the discovery of DNA Barcoding, it is being utilized in many research projects like records of uncontrived regions of biodiversity specie recognition using databases for barcodes, pest management Regulation of species and health impacts The use of DNA barcoding up in a particular region for a preparatory molecular characterization of a given organism group would be amongst the most popular examples in biodiversity studies. These can extent from very confined representative groups to variety of species in wide areas. Hence, DNA barcoding or DNA taxonomy has gained attention of the biologists to exactly identify a species. Clear cut species identification is carried out through sequence of a molecular marker such as a section of mitochondrial 16S rDNA gene, extensively sequenced for most of vertebrates for identification using DNA barcodes [24]. Molecular analysis is used to identify the cryptic taxa as morphological similarities are not always paralleled with genetic information [36]. Advancements in molecular biology have led scientific community to quantify interspecific and intraspecific variations within and among different populations. The techniques which are routinely applied to document genetic diversity in vertebrates involve levels of variability at mini- and microsatellite loci.

Under the specifications of barcoding of DNA or DNA taxonomy, the usage of sequences of short DNA for the purpose of standard organism identification has gathered much recognition. The assignment of undiscovered life-history phases to adult organisms, the wide-scale identification of organisms in research either related to ecology or genomics and the most contentious exploratory surveys to identify possibly undiscovered "target" species are some of the favorable and dominant uses of this technique [37].

The Sundaland lineage is reported from Indonesian and Malaysian [38]. The mitochondrial divergences between species of genus Apis and their subspecies are thought to occur during the Pleistocene [39]. Throughout the middle Holocene, African weather became more humid, and the great Sahara desert practically vanished. This change most likely aided gene transfer between Maghreb and Sahel honeybee species. Mitochondrial DNA sequence data

FIGURE 5
Minimum Evolution tree of genus *Apis* based on p-distance using MEGA X

provided support to this theory [34]. Some of the traditional Apis subspecies are supported by mitochondrial lineages within each species and their geographic ranges, but not all. Differences between subspecies within a lineage are more difficult to detect [40]. This might demonstrate the variations between the mitochondrial DNA of A. m. litorea and A. m. scutellata, according to Meixner et al.[41]. Furthermore, mitochondrial DNA markers cannot discern the difference between the naturally separated subspecies A. m. monticola and A. m. scutellata. This might be owing to periodic introgression between the two subspecies in Malawi's northern high tableland.

The honeybee subspecies Apis m. adansonii, Apis m. monticola, Apis m. scutellata, Apis m. capensis, and Apis m. unicolor differ very little at the molecular level. The major cause for the low molecular divergence across African subspecies, particularly Apis m. adansonii and Apis m. scutellat, is likely related to their migratory nature [17]. In contrast, European subspecies such as Apis m. mellifera and Apis m. ligustica have been widely introduced into North Africa [42]. Nonetheless, data from microsatellite allele sequences shows that some European alleles may have infiltrated Moroccan honeybees

CONCLUSION AND RECOMMENDATION

During the current investigation, DNA was extracted from Apis mellifera, Apis dorsata, and Apis florea using the phenol chloroform technique. When compared to commercially available DNA extraction kits, the quality of DNA extracted using the phenol chloroform technique is good. The DNA of these species successfully amplified using universal COI primer set. After trimming ambiguous bases, the obtained COI gene fragment of Apis mellifera, Apis dorsata and Apis florea were 670, 680 and 675 bp

respectively. The overall, genetic divergence of genus Apis was 0.098 ± 0.022. It can be concluded that the morphological characteristics provide information as an indicator for estimation of genetic variations between honey bee populations. Further work is needed to explore more areas of Pakistan and molecular identification is required to report any new or subspecies from country.

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