HIGH MITOCHONDRIAL DIVERSITY OF *APIS MELLIFERA* UNDER COI GENE FROM CHINA AND PAKISTAN

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Abstract. The genetic diversity of honeybee populations (240 individual) *Apis mellifera* from ten various locations of China and Pakistan was investigated by using COI (Cytochrome Oxidase Subunit I) gene. After screening of sequences, BioEdit was used for the alignment, and these aligned sequences were employed to MEGA-7 software, to find out the phylogenetic relationship with all available subspecies through Neighbor-Joining method. On the other hand, genetic divergence was calculated via Kimura-2-Parameter using MEGA7, whereas genetic diversity was calculated by DnaSP v5. Phylogenetic tree results divided all populations into seven subspecies within two groups and maximum genetic divergence was observed overall at 3.88%, followed by Pakistan at 2.65% while minimum divergence value (2.02%) was observed for China. Likewise, significant genetic diversity overall was observed with 23 mutations, confirmed with the probability value of Fu's *Fu* at 0.019. Country-wise observations revealed that Pakistan had much highly significant variability through Fu's *Fu* and *D* tests, along with 17 mutations whereas China had shown less diversity with ten mutations. Moreover, the calculation of nucleotide mismatch distribution had also confirmed genetic variability among all our sequenced data. Thus, our findings have revealed the strong genetic interaction and a significant genetic variation among all seven subspecies of *A. mellifera* from two different countries. These results could be more helpful to investigate the genetic relationship among other subspecies of *A. mellifera* from different ecological zones.
Keywords: *honeybee subspecies, mitochondrial DNA, A. m. ligustica, A. m. carnica, A. m. adami, A. m. macedonica, A. m. anatoliaca, phylogenetic analysis, ecological zones*

Introduction

Honeybee rearing is worldwide accepted for its commercial values, and Chinese people are dependent on reared animals, including honeybee, along the sides of various rivers in China. In earlier records of China, honeybee was known as Mi-feng (Li and Luo, 2003). Rearing of honeybees covers more than six million colonies across the country, and is dominated by two species, i.e., *Apis mellifera*, and *A. cerana*. Although, *A. cerana* is being replaced by *A. mellifera* in whole China due to transportation of *A. mellifera* (Yang, 1984), through natural mating and hybridization. Similarly, Pakistan is also involved in the rearing of *A. mellifera*, and has more than 0.3 million colonies along the entire country (Waghchoure-Camphor and Martin, 2008). Unfortunately, honeybees are facing mortality due to the random applications of pesticides worldwide (Husain et al., 2014; Williams et al., 2015), due to which all types of commercial products are being costly.

A wide range of subspecies of *Apis mellifera* inhabit in natural distribution and have differences on the basis of behavioral and morphological features (Koca and Kandemir, 2013; Tofilski, 2008). The evolutionary study for the characterization of the species of *Apis mellifera* (Linnaeus, 1758) was first demonstrated based on morphometric

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parameters (Ruttner et al., 1978). *A. mellifera* has almost 30 subspecies all over the world (Ruttner, 1988), and can be classified into four categories, i) lineage A covers African subspecies; ii) lineage C covers northern Mediterranean subspecies; iii) lineage M contains northern European subspecies and iv) lineage O includes subspecies from the Middle East (Garnery et al., 1992; Whitfield et al., 2006). The new sophisticated molecular techniques have made it easy to further confirm these groups more precisely (Wallberg et al., 2014). Furthermore, this classification has been expanded with the addition of two new lineages, e.g., lineage Y and Z cover Ethiopia (Franck et al., 2001) and Libya (Alburaki et al., 2013), respectively.

A number of studies have been carried out to identify *Apis mellifera* subspecies on the basis of geometric morphometrical features, using programs of automatic identification (Tofilski, 2008). Due to the importation of *A. m. carnica* and *A. m. ligustica* in Europe, the conservation of local subspecies or ecotypes has been damaged by gene flow and replacement over longer distances (De la Rúa et al., 2009; Jensen and Pedersen, 2005; Maul and Hähnle, 1994; Moritz et al., 2005; Peer, 1957). The two races of the Western honeybee are closely related and live in neighboring climatic regions, i.e., *A. m. carnica* Pollmann and *A. m. ligustica* Spinola (Ruttner, 1988). Mitochondrial DNA (mtDNA) is a commonly used approach to explore the genetic evolution and diversity of species due to its conserved gene content and nucleotide substitutions. The honeybees were being identified through the intergenic region of cytochrome oxidase subunit I and II (COI–COII), to find out the evolutionary relationships of honeybees (Chen et al., 2016; Magnus et al., 2014; Techer et al., 2017) under the length and sequence variations of mtDNA (Franck et al., 2000; Hall and Smith, 1991). Out of Hymenopteran subspecies, *A. m. ligustica* is first subspecies for which a complete mitochondrial genome was sequenced (Crozier and Crozier, 1993), and later on, many mitochondrial regions were selected and sequenced for the description of phylogenetic relationships (Cao et al., 2017; Magnus and Szalanski, 2010; Muñoz et al., 2012; Techer et al., 2017).

Even the mitochondrial genome information about the other regions is limited, but some particular regions are being employed regularly to differentiate evolutionary lineages and subspecies of honeybees, like 16S ribosomal DNA (rDNA) (Bouga et al., 2005; Kek et al., 2017), NADH dehydrogenase-2 (ND2) (Kandemir et al., 2006; Techer et al., 2017), and NADH dehydrogenase-5 (ND5) (Bouga et al., 2005; Martimianakis et al., 2011; Özdil and İlhan, 2012), as well as COI regions (Bouga et al., 2005; Kekeçoglu et al., 2009; Marino et al., 2002; Sheffield et al., 2009). As previous studies explored that mitochondrial DNA is transferred maternally and can face high range of polymorphism, therefore, this region of *A. mellifera* genome has been utilized for phylogenetic studies (Ilyasov et al., 2011; Kek et al., 2017; Radoslavov et al., 2017). Likewise, from mitochondrial study consisting two DNA segments, the highest genetic divergence values were observed in COI. For this purpose, different methods were employed to construct phylogenetic trees for COI region of mitochondrial DNA, such as Bayesian Inference; Maximum Parsimony and Neighbour-Joining (Kek et al., 2017; Martimianakis et al., 2011). The present study was designed to determine the mitochondrial diversity and phylogenetic relationships of *Apis mellifera* subspecies of China and Pakistan through COI gene.

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Materials and methods

Sample collection

We sampled a total 240 adult worker honeybees from three different ecological zones of China and Pakistan in year 2017 and immediately froze them in dry ice and brought the samples to the College of Bee Science of Fujian Agriculture and Forestry University, China (*Suppl. Table 1*).

Supplementary Table 1. *Sampling Sites from different Locations of China and Pakistan*

Country	Location	Colonies	Coordinates
China	JL *carnica*	24	43.1520°N,126.4371° E
	JL *ligustica*	24	43.1520°N,126.4371° E
	FZ	24	26.0745°N,119.2965°E
	FQ	24	25.7211°N,119.3843° E
	JM	24	31.0354°N,112.1994° E
	LY	24	29.0284°N,119.1722° E
	PH	24	30.702°N,121.0119° E
	TW	24	25.0330°N,121.5654° E
Pakistan	ID	24	33.7294° N, 73.0931° E
	FD	24	31.4187° N, 73.0791° E

JL = Ji-Lin, FZ = Fuzhou, FQ = Fu-Qing, JM = Jing- Men, LY = Long-You, PH = Ping-Hu, TW = Taiwan, ID = Islamabad, FD = Faisalabad

DNA extraction, PCR amplification and sequencing

Total nucleic acid was obtained from the thorax of honeybee samples by applying king Fisher^{tm}cell and tissue kit as per manufacturer's instructions. Several primers have been designed to amplify the COI gene. Polymerase Chain Reaction (PCR) amplification was conducted using the following primer, COI-F1: 5'-TTTGATTACTTCCTCCCTC-3' and COI-R1: 5'-GCACCTATTGAAAGAACA-3', designed on NCBI tool. The total volume 25 µL, containing, 2 µL DNA template, 1 µL each primer (forward and reverse), ddH_2O 8.5 µL and 12.5 µL 2X Easy Taq Super Mix Trans solution. The amplification program started with 94 °C for 4 min, followed by 36 cycles of 94 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s, and a final step of 72 °C for 5 min. The amplified PCR products were visualized in 1.5% agarose gel stained with ethidium bromide and purified with E.Z.N.A® Gel Extraction Kit. After purification of PCR products, all samples were sent to a local company (Biomarker Pvt, Ltd.) for bidirectional sequencing (Islam et al., 2018a). COI sequences were analyzed and edited using BioEdit 7 (Hall, 1999; Islam et al., 2018b). The sequences were preliminarily aligned using Clustal X (Thompson et al., 1997) and subsequently aligned manually. Unique COI sequences of all samples were deposited in the NCBI GenBank database via accession numbers MF416679–MF416918.

Phylogenetic and genetic distance analysis

Along with our 240 sequences, 67 COI sequences of fourteen subspecies were collected from NCBI database, to represent all available subspecies as well as one sequence of *Apis florea* (JX982136) as an out-group member. The portion (738bp to

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831bp) of the COI5′ gene sequence utilized in our analysis existed between nucleotide 298-1150bp (COI complete sequence 1566bp) (Arias et al., 2008; Crozier and Crozier, 1993). MEGA-7 was used to perform phylogenetic analysis and nucleotide identity percentage within and among other major subgroups (Jan et al., 2017; Thompson et al., 1997). Likewise, genetic distances were also calculated by MEGA-7 under Kimura-2-Parameter (K2P) model.

Genetic diversity

For mitochondrial diversity, all indices were performed in DnaSP v5 (Librado and Rozas, 2009), under two neutrality tests [Fu's *Fs* (Fu, 1997) and Tajima's *D* (Tajima, 1989)]. For all blasted sequences of *A. mellifera* in the present study, a phylogenetic tree was constructed to understand the species network with relation to out-group member.

Results

Two hundred and forty honeybee samples were collected during 2015–2017 from various localities of China and Pakistan. The 240 COI-5' sequences (GenBank MF416679–MF416918) were blasted to determine the similarity index and identification of each isolate and are represented in the phylogenetic trees depicted in this study. Overall, there was 98%-100% nucleotide identity when all sequences were blasted in the NCBI database, along with zero percent E value.

Phylogenetic analysis

The phylogenetic tree grouped the entire collection of *A. mellifera* into two groups, and biggest group consists of individuals from all localities, except ID, along with seven subspecies. On the other hand, second group had covered only ID-sequences, along with two sequences from FD location and *A. m. ligustica* subspecies. The biggest group had shown that honeybees from all localities have good genetic interaction and diversity among the various colonies. On the other hand, it was observed that honeybee colonies from ID had least genetic diversity, due to which they had grouped into a single group that have little interaction with the colonies from FD. While two sequences of *A. florea* were kept as out-group member (*Fig. 1*). From all blasted sequences, it was observed that honeybee colonies from ID (Pakistan) have strong genetic interaction with each other as well as two colonies from FD (Pakistan) (*Suppl. Fig. 1*). Moreover, this group had presented resemblance up to 95%, as shown in *Figure 1*.

Colonies from other locations have huge genetic interaction among various colonies, due to enough diversity. As we know that FD colonies have different eco-geography, along with certain range of diversity, however, it was observed that most of the colonies built intermingled relationship with all Chinese colonies (*Fig. 1*). Likewise, a huge genetic interaction was also observed among all other Chinese colonies on the base of COI-5' DNA. Moreover, it was also observed that colonies from all locations had strong and random relationship with all seven subspecies in biggest group, like *A. m. ligustica*, *A. m. carnica, A. m. adami, A. m. macedonica, A. m. anatoliaca, A. m. caucasica* and *A. m. meda* (*Suppl. Fig. 1*). In short, our COI-5' DNA sequences had revealed that colonies from ID have minimum genetic variation and strong interaction with each other while all other colonies had enough variation, leading towards huge genetic interaction with *A. m. ligustica*.

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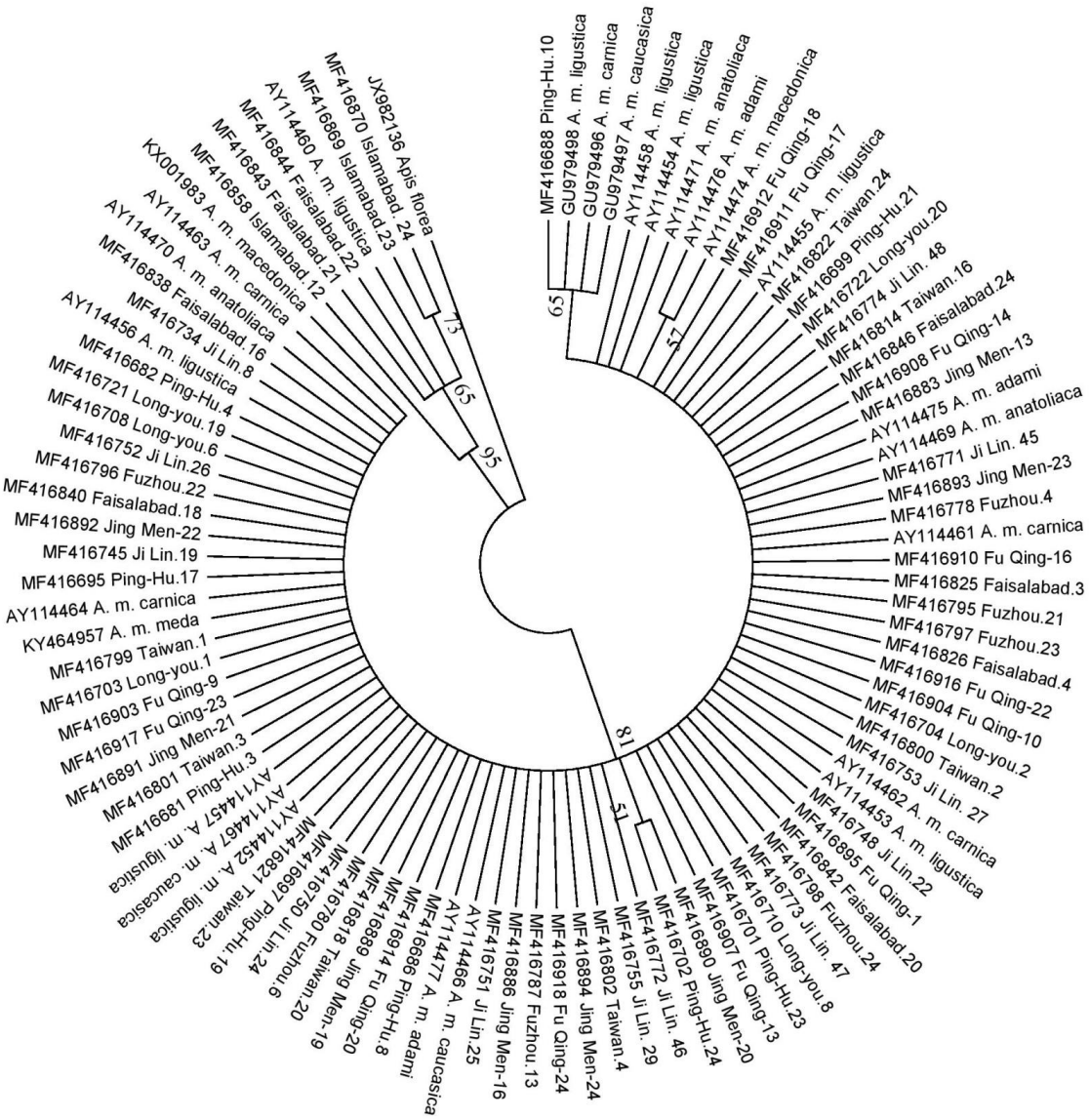

Figure 1. *Phylogenetic relationship of Apis mellifera sub-species in South-China and Pakistan. Phylogenetic tree was constructed through neighbor-joining method by using Kimura-2-Parameter model. For tree construction, we used 73 mtCOI-5' DNA sequences from our data collection, after choosing selected sequences, along with one complete mtCOI DNA sequence of A. florea (JX982136) as an out-group. Two main groups were developed after alignment. This tree shows percent similarity among different colonies from various locations. For both trees, bootstrap value was replicated 1000 times*

Genetic distance among subspecies

According to our sequenced database, pairwise maximum distances for our ten locations cumulatively range between 3.88%. As well, country-wise divergence was observed up to 2.65% and 2.02% for Pakistan and China, respectively. Moreover, maximum interspecific distance (2.36%) was observed for FD-location sequences, followed by PH-location (2.02%) whereas lowest value of maximum interspecific distance was observed for FQ-location and JL *carnica* sequences (0.26% for both). On

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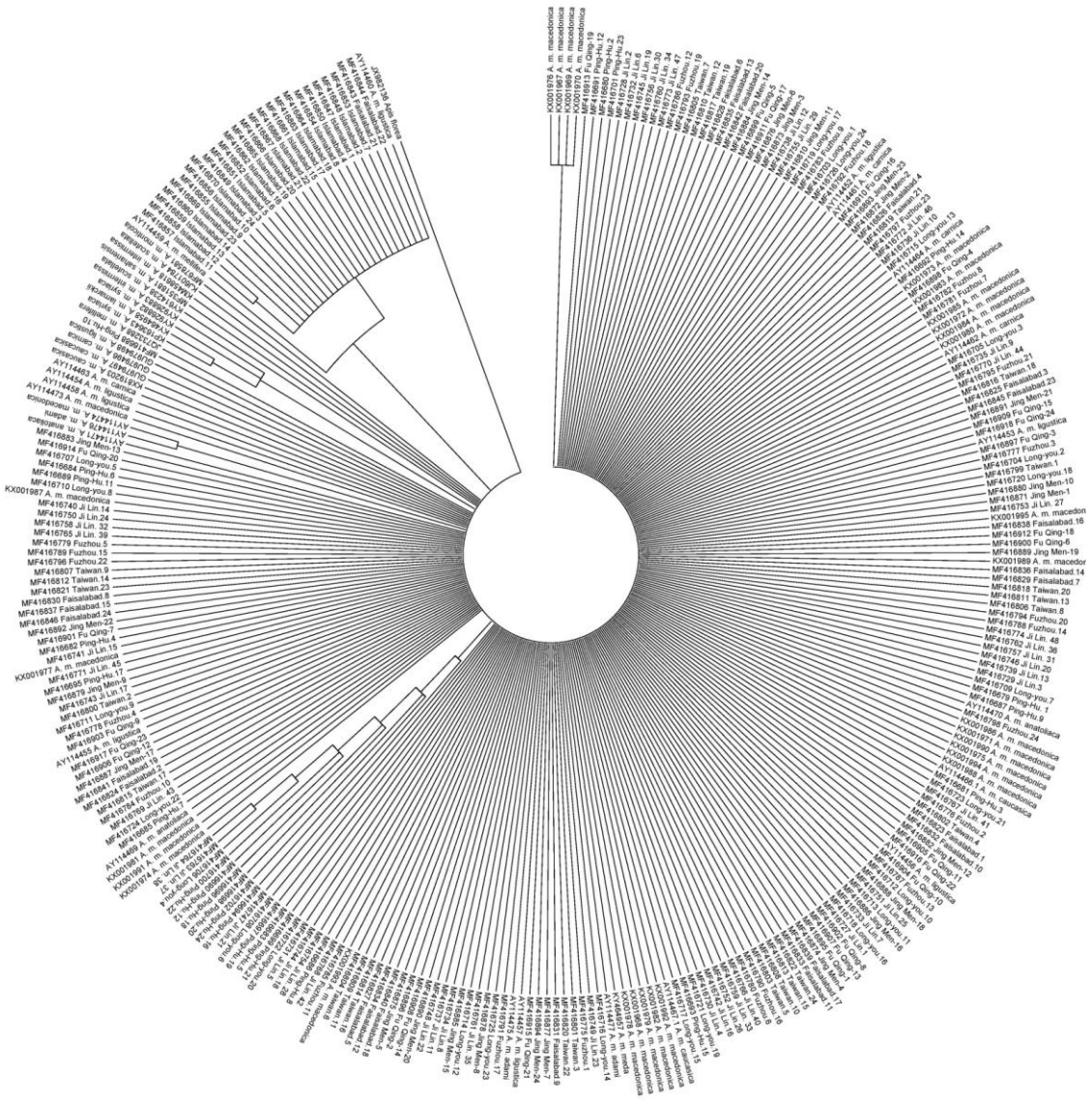
the other hand, maximum intraspecific distance was observed for FD-location as well as FQ-location/JL *carnica*, which was up to 2.10% (*Table 1*).



Supplementary Figure 1. *Phylogenetic tree construction by MEGA 7. Phylogenetic tree was constructed for our 240 sequences and 67 sequences, taken from NCBI, representing 14 subspecies of Apis mellifera. On the other hand, A. florea (JX982136) was taken as out-group member. Tree was constructed through Neighbor-Joining Method under K2P model, and bootstrap value was 1000*

Genetic diversity

As colonies were collected from different locations, therefore, there must be some genetic variation based on their geographical zones. All sequences were subjected to the genetic diversity analysis, and diversity indices were calculated through two neutrality tests, i.e., *D* and *Fs* tests, based on pairwise nucleotide diversity. Maximum genetic diversity, based on Eta value, was observed in FD colonies, showing up to 14 mutations within all colonies, followed by PH location (12 mutations). The significance of genetic

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diversity was confirmed by both neutrality tests, which shown that FD and PH locations had significant variation based on the *p*-value of Fu's *Fs* test (0.016) and *p*-value of Tajima's *D* test (0.01), respectively. On the other hand, most of the Chinese colonies from various locations (FQ, JL *ligustica*, JL *carnica*, LY and TW) presented least value of genetic diversity (*Table 2*).

Table 1. *Distance divergence (%) determination by MEGA7 by using K2P model under pairwise deletion for honeybee colonies*

Colony	Sample size	Mean dist.	Maximum dist.
Overall	**240**	**0.46**	**3.88**
China	**192**	**0.08**	**2.02**
Pakistan	**48**	**1.10**	**2.65**
FD	24	0.34	2.36
FQ	24	0.07	0.26
FZ	24	0.07	0.38
ID	24	0.15	0.65
JL *carnica*	24	0.04	0.39
JL *mellifera*	24	0.05	0.26
JM	24	0.07	0.51
LY	24	0.12	0.42
PH	24	0.15	2.02
TW	24	0.03	0.38

Table 2. *Genetic diversity calculation of honeybee different colonies by DnaSP v5 through polymorphism data analysis*

Colony	n	Nucleotide diversity					Neutrality tests			
		S	k	Eta	Θ	π	Fu's *Fs*	*p*-value	*D*	*p*-value
Total	**240**	**44**	**2.429**	**23**	**0.00852**	**0.00412**	**4.539**	**0.019**	**-1.49450**	**0.10**
China	**192**	26	0.114	10	0.00256	0.00071	-1.850	0.110	-2.18819	0.01
Pakistan	**48**	**24**	**7.772**	**17**	**0.00499**	**0.01018**	**11.805**	**0.000**	**3.23453**	**0.001**
FD	24	18	2.232	14	0.00588	0.00322	6.230	0.016	-1.63342	0.10
FQ	24	3	n/c	n/c	0.00098	0.00069	n/c	n/c	-0.75696	0.10
FZ	24	4	0.159	1	0.00129	0.00065	-0.249	0.335	-0.6811	0.10
ID	24	10	0.562	4	0.00329	0.00141	0.141	0.292	-1.90540	0.10
JL *carnica*	24	5	n/c	n/c	0.00164	0.00038	n/c	n/c	-2.22353	0.08
JL *mellifera*	24	2	n/c	n/c	0.00065	0.00045	n/c	n/c	-0.66674	0.10
JM	24	5	0.159	1	0.00165	0.00072	-0.249	0.335	-1.63539	0.10
LY	24	4	n/c	n/c	0.00128	0.00109	0.000	0.000	-0.40369	0.10
PH	24	21	1.000	12	0.00663	0.00138	3.135	0.156	-2.89855	0.01
TW	24	3	n/c	n/c	0.00098	0.00033	n/c	n/c	-1.65313	0.10

Abbreviations: Eta = Total number of mutations, n = number of sequences, k = Average number of nucleotide differences, S = Number of segregating sites, Θ = nucleotide substitution rate, π = nucleotide diversity, Fu's *Fs* = A negative value of *Fs* is evidence for an excess number of alleles, whereas a positive is related to deficiency of alleles, and *D* is the Tajima test statistic (both *D* and Fu's *Fs* are used to single nucleotide polymorphism)

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Country based cumulative genetic diversity was determined, and colonies from Pakistan had shown maximum variation, representing 17 mutations among all colonies, whereas China had presented only ten mutations. High significance was noted for Pakistani colonies cumulatively, representing *p*-value 0.000 and 0.001 for Fu's *Fu* and *D* tests, respectively. However, overall cumulative genetic diversity of all sequences was significant at the utmost level, presenting 23 mutations, and this diversity was confirmed by Fu's *Fs* test (*Table 2, Fig. 2*).

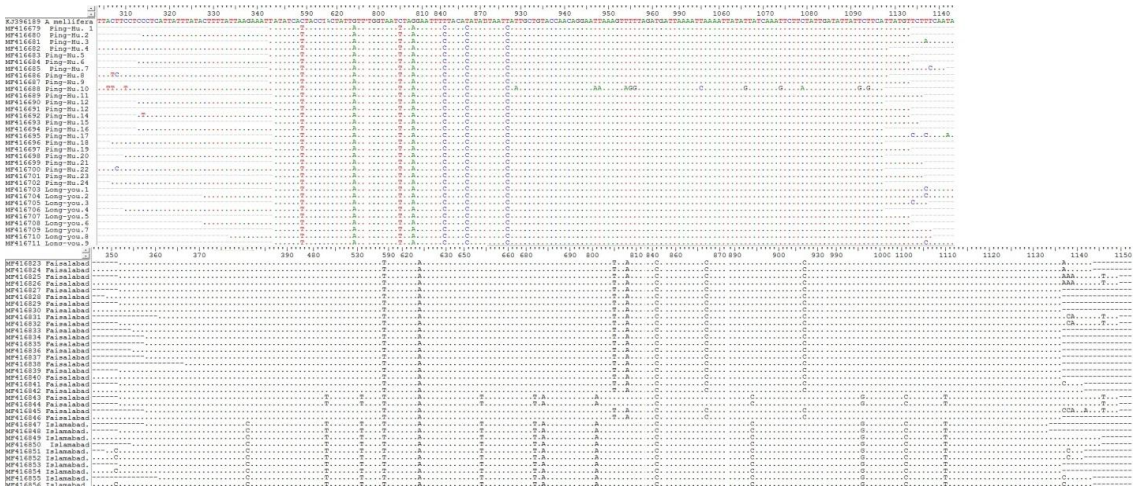

Figure 2. *Number of variable sites in COI region of our aligned sequences through BioEdit*

To confirm all the indices of genetic diversity, all blasted sequences were evaluated for population size changes, to calculate pairwise mismatch distribution for all sequences of subspecies. Overall and country-wise results had expressed a significant genetic variation for COI gene, but there was no population expansion of any subspecies in both countries (*Fig. 3*).

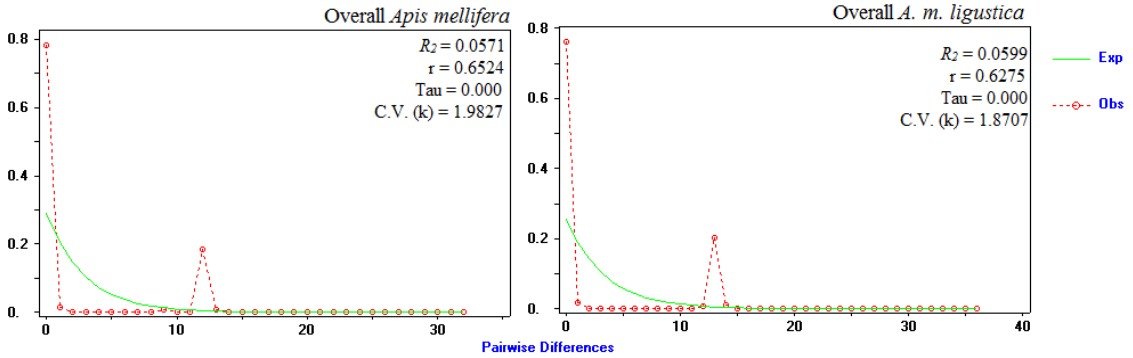

Figure 3. *Pairwise mismatch distributions for Apis mellifera sub-species, based on COI gene sequences by DnaSP v5. The X-axis shows the observed distribution of pairwise genetic variation, and the Y-axis represents the frequencies*

Discussion

The COI-5' DNA study was organized to characterize the genetic interaction and variability of *Apis mellifera* subspecies from two countries on a vast geographical zones.

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Therefore, we collected 240 colonies, and sequenced them to assess phylogenetic relationship among different colonies as well as their genetic diversity and interaction.

Phylogenetic relationship of all sequences has defined the genetic interaction among the colonies from various locations as well as diversity within the colonies of same location, based on COI DNA. The phylogenetic tree of *Apis mellifera* along with 14 referenced subspecies, presented two groups (*Suppl. Fig. 1*). The largest group has been consisted of populations from all localities, except ID, representing vast genetic interaction among all populations. However, *A. m. ligustica* and *A. m. carnica* were dominant in our sequences. On the other hand, populations from ID location also presented huge genetic interaction within locality and least diversity, represented strong relationship with *A. m. ligustica* (*Fig. 1*). Same trend of genetic interaction was observed by mitochondrial study of Martimianakis et al. (2011), in which they described that various subspecies of *A. mellifera* built relationship with each other from different localities. Such earlier studies support our results of genetic interaction among two different subspecies as well as different localities in this study. Furthermore, such phylogenetic relationship among different subspecies of *A. mellifera* was also described by other people across the world, based on the COI gene (Özdil and İlhan, 2012; Radoslavov et al., 2017) and other distinct genes (Ilyasov et al., 2016a, b; Techer et al., 2017).

In our study, all nucleotide substitutions were occurred between 298-1150bp. However, there were only seven main nucleotide substitution sites in our sequence whereas minor substitutions were observed at many sites of COI region. Due to such minor substitutions, colonies of both subspecies from all locations were clustered together in different groups (*Figs. 1* and *2*), except ID locations.

The genetic divergence and interaction for DNA sequences were explained by many researchers for different classifications of organisms, i.e., plant, animal, arthropod as well as prokaryotic species (Goraya et al., 2015; Islam et al., 2018a; Qasim et al., 2018a, b). Likewise, Radoslavov et al. (2017) explained the interaction and divergence of various *Apis* subspecies under various genes. Since, based on COI gene sequences, a comprehensive divergence was calculated for various localities as well as country wise, and all sequences were grouped into two groups, and determined their inter-specific maximum as well as interspecific mean distances by MEGA 7 (*Table 1*). The maximum interspecific distance (2.36%) was observed for FD colonies whereas maximum intraspecific distance was noted among FD and FQ colonies, also at 2.10%, because FQ colonies presented minor percent distance within the entire location. Likewise, maximum mean intraspecific distance was observed between FD and TW location, which was 0.31% only. However, overall highest mean value of divergence was observed for Pakistan (1.10%), while highest maximum value of divergence was observed for overall locations (3.88%) (*Table 1*).

Genetic variability indices were calculated up to various levels, like species, genus and family, to find out genetic diversity in the mitochondrial DNA. For this purpose, different genes were selected by several researchers, like ND5 (Ozdil and İIlhan, 2014) and COI-COII inter-genic region genes (Pinto et al., 2014; Rahimi, 2015). Therefore, for mitochondrial DNA diversity of our sampled *A. mellifera* colonies, mutations were calculated in the COI region of various locations (*Fig. 2; Table 2*). Most of the colonies from various locations had shown minor genetic diversity due to commercial rearing. However, FD location had shown maximum genetic variability within the colonies. Therefore, colonies from FD location have genetic interaction with all other colonies of whole collection (*Fig. 1*). A recent study of Özdil and İlhan (2012) confirms our results

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that there are many sequence mutations within and among the colonies. However, p-values of Fu's *Fu* and Tajima's *D* test for colonies of most locations are non-significant, which proves minor variation. On the other hand, genetic variability has shown significant impact for overall country wise colonies.

Conclusion

Conclusively, we can say that the honeybee colonies collected from all locations present a hybrid situation between *A. m. ligustica* and *A. m. carnica*, as both subspecies are capable of interbreeding and are dominant in our collection. Likewise, Wu et al. (2017) has described that *A. ligustica* has been originated from two different sources in Taiwan, which supports our hypothesis that there is some possibility of hybridization between different colonies. On the basis of hybridization, there are seven subspecies in our entire collection on the base of mitochondrial DNA. This mitochondrial characterization of honeybees from different localities of Asia further extends our understanding on the genetic diversity and interaction of honeybee based on COI gene, in spite of rearing in the confined environment, as compared to wild environment. Thus, on the base of our findings, we conclude that there could be many other interactions among honeybee colonies from various locations, based on more prominent traits, like honey production, royal jelly production, reproduction as well as behavior. Therefore, with respect of our results, we suggest the use of other genes to find out genetic interaction among different countries' colonies, to improve the potential of honey and royal jelly production, as well as to assess the genetic diversity based on other genes.

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