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MOLECULAR PHYLOGENETIC STUDY ON *APIS MELLIFERA* SUBSPECIES INFERRED FROM CYTOCHROME OXIDASE I SEQUENCE

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ABSTRACT
The taxonomy of honey bee has been in chaos for the last many decades. COI (Cytochrome Oxidase I) is an ideal candidate to study the molecular phylogeny of organisms. We extracted the COI protein sequence of nine *Apis mellifera* subspecies from NCBI data bank. The manually curetted sequences are subjected for *In silico* analysis by using the best and most reliable software and online tools and servers. We observed that out of the nine subspecies of *Apis mellifera*, one subspecies *Apis mellfera syriaca* shown 76 amino acid differences out of 80 amino acids. The distance matrix, divergence matrix, Tajmas test, mutation study, charge difference of amino acid, compact pattern disparity index, and PNG site prediction shown that *Apis mellfera syriaca* diverged right from the beginning and by the mutation, inversion and deletion the species exhibit as a separate entity. By a further molecular level study including morphometric approach will substantiate the species status of the *Apis mellfera syriaca* in future.

Key Words*: Apis Mellifera Subspecies, COI, In Silico Study, Divergence Matrix, Mutation Matrix, PNG Site*

INTRODUCTION
Apis melifera includes 26 subspecies (Ruttner, 1988, 1992; Sheppard *et al*., 1997) and is native to Africa, Europe and Asia; however it is widely distributed around the world. These subspecies typically exhibit reduced gene flow with other such groups due to water, mountain or desert barriers and have been called "geographic races", to reflect their adaptation to specific geographic areas (Ruttner, 1988). Based on various phylogenetic parameters the speciation event that produced *Apis mellifera* has been estimated to have occurred between 0.7 to 1.3 million years ago (Ruttner, 1988; Cornuet and Garnery, 1991; Arias and Sheppard, 1996). The taxonomy of honey bee has been in chaos for the last many decades. By morphometric study (Ruttner, 1988, 1992; Sheppard *et al*., 1997) the available subspecies have been grouped into four lineages. As the morphometric studies are challenged continuously molecular level study was begins recently with the help of allozymes (Nunamaker and Wilson, 1982; Badino *et al*., 1988), nuclear DNA (Hall, 1990; Tarès *et al*., 1993), mitochondrial DNA (mtDNA) (Moritz *et al*., 1986; Smith *et al*., 1989, 1991; Hunt and Page, 1992; Garnery *et al*., 1993; Oldroyd *et al*., 1995; Arias and Sheppard, 1996; Pedersen, 1996; De la Rùa *et al*., 2000) and microsatellites (Estoup *et al*., 1993; Garnery *et al*., 1998). This type of molecular level study enables us to understand better the phylogeny and geographical distribution of the honeybee, particularly *Apis mellifera* and its subspecies in detail. The mitochondrial cytochrome oxidase c subunit 1 (COI) gene is one of the most popular markers for population genetic and phylogeographic studies across the animal kingdom (Avise, 1994). Subspecies level classification of honey bees points out an interpretation of the amount of accumulated genetic differences between postulated "subspecies" relative to the distribution of overall within species variation (Hepburn and Radloff, 1997; Sheppard, 1997).The selected subspecies of *Apis mellifera* are *Apis mellifera caucasica, Apis ligustica,Apis mellifera carnica, Apis mellifera sicula, Apis mellifera iberica, Apis mellifera adami, Apis mellifera macedonica, Apis mellifera anatolica* and *Apis mellifera syriaca.* The present investigation took COI protein sequences to find out the molecular relation between the selected subspecies of Apis mellifera. We performed multiple sequence alignment, distance finding between the species,sequence diversity study,estimate gamaparameter for site rate (ML), molecular clock

infer ancestral sequence, protein motif searching, studying various physic-chemical parameters, and phylogenetic tree construction study by neighbor joining and UPGMA methods.

MATERIALS AND METHODS

The NCBI databank (http://www.ncbi.nlm.nih.gov/)search with the word"COI of Apis" extracted 217 results. Out of these results we manually searched the different subspecies of *Apis mellifera*. The manual search obtained nine subspecies of *Apis mellifera* having sequence of COI (Table1). The sequences of all the nine subspecies of *Apis mellifera* were extracted and stored in our computer for further analysis. These sequences were manually curated to remove unwanted sequence symbols. The final sequence was used for the present study. Multiple sequence alignment was studied by clustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The aligned sequences in Clustal format is converted into nexus format by using sequence multiformat conversion tool. The nexus format sequences were used to automated maximum likelihood phylogenetic analyses with the help of online tool DIVEIN. DIVEIN is a web service that performs automated maximum likelihood phylogenetic analyses of nucleotide and amino acid sequences. Starting with a set of aligned sequences, DIVEIN can calculate phylogenetic trees via a variety of evolutionary models, reconstruct the consensus, Most Recent Common Ancestor (MRCA) and Center of Tree (COT) sequences, re-root trees at COT, compute genetic distance distributions, diversity, and divergence from the consensus, MRCA, COT and any sequence in the alignment, graphically represent the inferred tree and plots of divergence, diversity, and distance distribution histograms and detect, visualize and numerically summarize phylogenetically informative sites as well as private mutations (Deng, *et al*, 2010). The various parameters regarding the amino acid composition distance, pattern disparity index (Kumar and Gadakar, 2001), Estimates of Base Composition Bias Difference between Sequences (Kumar and Gadakar, 2001, Tamura *et al*, 2011), Estimates of Evolutionary Divergence between Sequences, Estimates of Average Evolutionary Divergence over all Sequence Pairs, Evolutionary relationships of taxa (Saitou and Nei,1987, Zuckerkandl and Pauling,1965, Tamura), Tajima's test and Maximum Likelihood Estimate of Gamma Parameter for Site Rates(Schwarz and Dayhoff, 1979, Tamura *et al*, 2007, 2011) were performed by using MEGA software. MEGA is an integrated tool for conducting automatic and manual sequence alignment, inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses (Tamura *et al*, 2007, 2011). The Tajmas tests were performed by combination of three sequences at a time. The results were later correlated to obtain a better understanding of the evolutionary relationship (Tajima, 1993, Tamura, 2011). The tree was also predicted by MOBYEL@Pasteur site (http://mobyle.pasteur.fr/data/jobs/protein_distance_phylogeny/ E25997390158892).

Table 1: Different subspecies of *Apis mellifera* selected for the study

Genus	Species	Subspecies	Sequence length In number of amino acids
Apis	*Mellifera*	As reference	219
Apis	*Mellifera*	*caucasica*	163
Apis	*Mellifera*	*ligustica*	163
Apis	*Mellifera*	*carnica*	163
Apis	*Mellifera*	*sicula*	415
Apis	*Mellifera*	*iberica*	415
Apis	*mellifera*	*adami*	415
Apis	*mellifera*	*macedonica*	415
Apis	*mellifera*	*anatolica*	415
Apis	*mellifera*	*syriaca*	105

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Figure 1: Multiple sequence alignment of *Apis mellifera*

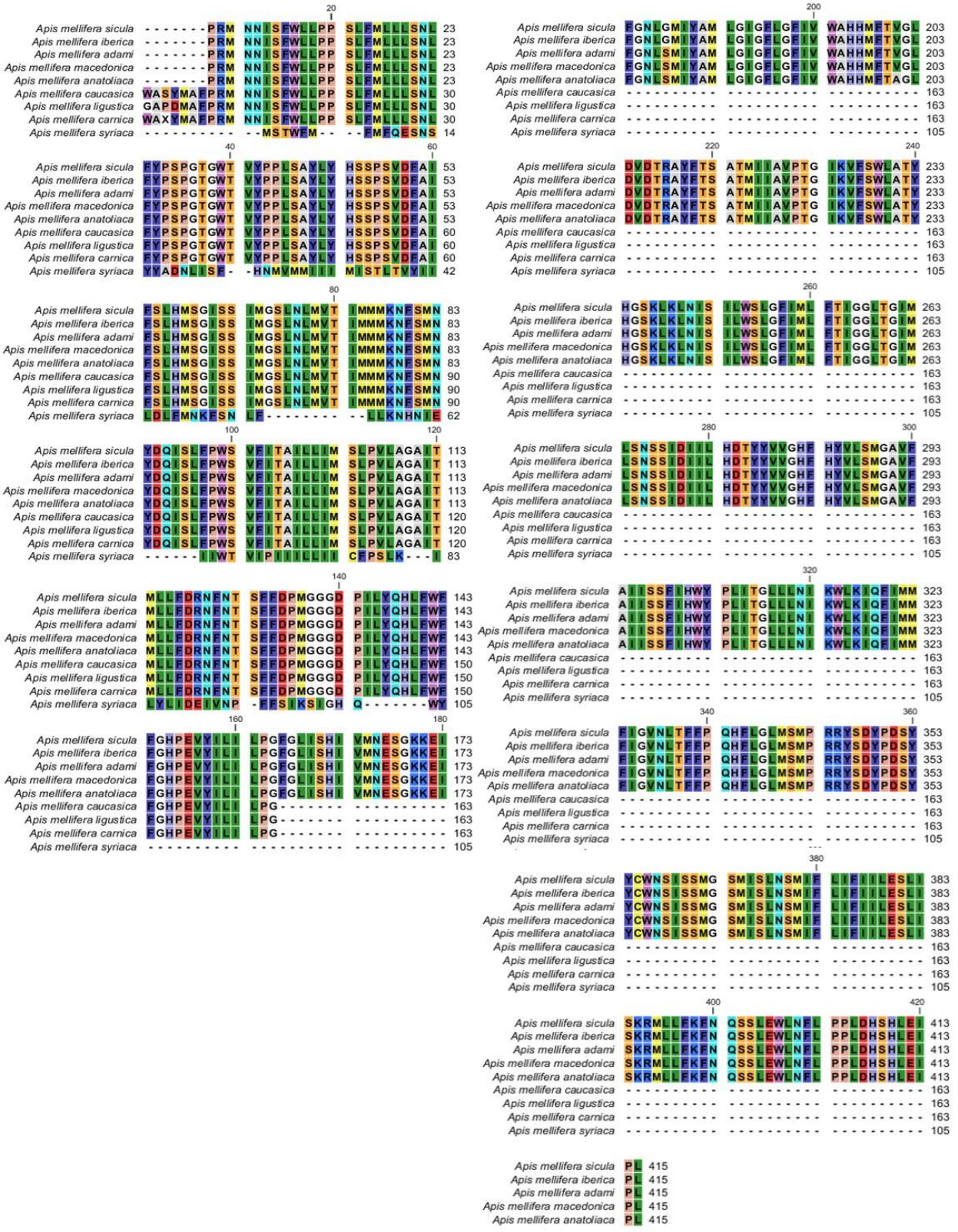


Figure 2: Phylogenetic relationship between nine subspecies of *Apis mellifera*

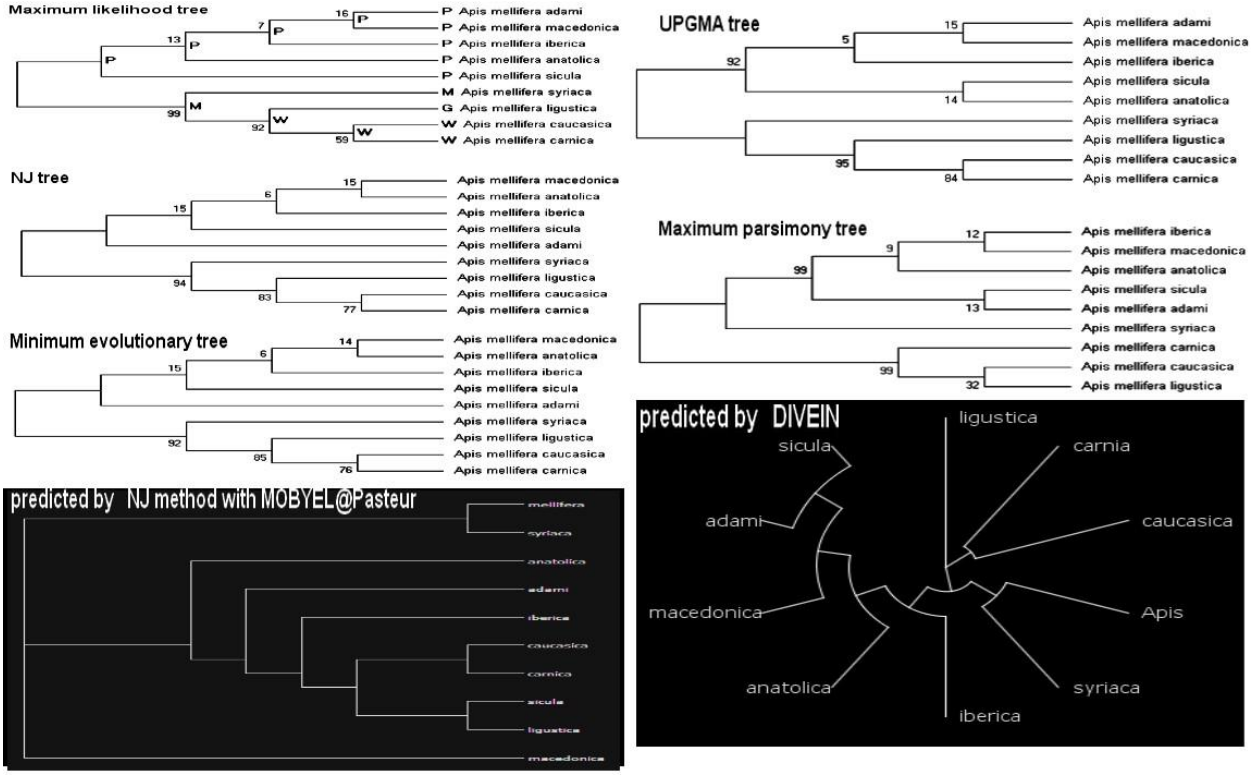


Table 2: Distance between the subspecies of *Apis mellifera*

1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
2	0.000							
3	2.424	0.000						
4	2.424	2.424	0.000					
5	2.424	2.424	2.424	0.000				
6	2.424	2.424	2.424	0.000	0.000			
7	2.424	2.424	2.424	0.000	0.000	0.000		
8	2.424	2.424	2.424	0.000	0.000	0.000	0.000	
9	2.578	2.578	2.578	2.578	2.578	2.578	2.578	0.000

1: Apis mellifera caucasica 2: Apis mellifera ligustica; 3: Apis mellifera carnica; 4: Apis mellifera sicula; 5: Apis mellifera iberica; 6: Apis mellifera adami; 7: Apis mellifera macedonica; 8: Apis mellifera anatolica; 9: Apis mellifera syriaca

Table 3: AA composition distance between thye nine subspecies of *Apis mellifera*

	1	2	3	4	5	6	7	8	9
2	0.00								
3	0.09	0.00							
4	0.09	0.09	0.00						
5	0.09	0.09	0.06	0.00					
6	0.09	0.09	0.06	0.00	0.00				
7	0.09	0.09	0.06	0.00	0.00	0.00			
8	0.09	0.09	0.06	0.00	0.00	0.00	0.00		
9	1.86	1.86	1.49	1.49	1.49	1.49	1.49	0.00	

1:Apis mellifera caucasica 2: Apis mellifera ligustica; 3: Apis mellifera carnica;
4: Apis mellifera sicula; 5: Apis mellifera iberica; 6: Apis mellifera adami;
7: Apis mellifera macedonica; 8: Apis mellifera anatolica;
9: Apis mellifera syriaca

Table 4: Computed pattern disparity index between nine subspecies of *Apis mellifera*

	1	2	3	4	5	6	7	8	9
2	0.00								
3	0.00	0.00							
4	0.00	0.00	0.00						
5	0.00	0.00	0.00	0.00					
6	0.00	0.00	0.00	0.00	0.00				
7	0.00	0.00	0.00	0.00	0.00	0.00			
8	0.00	0.00	0.00	0.00	0.00	0.00	0.00		
9	0.94	0.96	0.87	0.56	0.56	0.56	0.56	0.00	

1: Apis mellifera caucasica 2: Apis mellifera ligustica; 3: Apis mellifera carnica;
4: Apis mellifera sicula; 5: Apis mellifera iberica; 6: Apis mellifera adami;
7: Apis mellifera macedonica; 8: Apis mellifera anatolica;
9: Apis mellifera syriaca

Table 5: Estimates of Evolutionary Divergence between Sequences of Nine subspecies of *Apis mellifera*

	1	2	3	4	5	6	7	8	9
2	0.00								
3	2.40	0.03							
4	2.42	2.42	0.00						
5	2.42	2.42	2.42	0.00					
6	2.42	2.42	2.42	0.00	0.00				
7	2.42	2.42	2.42	0.00	0.00	0.00			
8	2.42	2.42	2.42	0.00	0.00	0.00	0.00		
9	2.58	2.58	2.58	2.58	2.58	2.58	2.58	0.00	

1: Apis mellifera caucasica 2: Apis mellifera ligustica; 3: Apis mellifera carnica;
4: Apis mellifera sicula; 5: Apis mellifera iberica; 6: Apis mellifera adami;
7: Apis mellifera macedonica; 8: Apis mellifera anatolica;
9: Apis mellifera syriaca

Table 6. Results from the Tajima's test for 9 Sequences in group of three

Configuration	Subspecies of *Apis mellfera*		
	1A ,2B, 3C	4A, 5B, 6C	7A, 8B 9C
Identical sites in all three sequences	77	80	6
Divergent sites in all three sequences	00	0	0
Unique differences in Sequence A	00	0	0
Unique differences in Sequence B	02	0	0
Unique differences in Sequence C	00	0	74

1A, 1B, 1C: *The equality of evolutionary rate between sequences* ***A*** *(Apis mellifera caucasica) and* ***B*** *(Apis mellifera ligustica), with sequence* ***C*** *(Apis mellifera carnica) used as an outgroup in Tajima's relative rate test . The χ^2 test statistic was 2.00 (P = 0.15730 with 1 degree[s] of freedom). P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages.* ***4A, 4B, 4C:*** *The equality of evolutionary rate between sequences* ***A*** *(Apis mellifera sicula) and* ***B*** *(Apis mellifera iberica), with sequence* ***C*** *(Apis mellifera adami) used as an outgroup in Tajima's relative rate test. The χ^2 test statistic was 0.00 (P = 1.00000 with 1 degree[s] of freedom). P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages.* ***7A, 7B, 7C:*** *The equality of evolutionary rate between sequences* ***A*** *(Apis mellifera macedonica) and* ***B*** *(Apis mellifera anatolica), with sequence* ***C*** *(Apis mellifera syriaca) used as an outgroup in Tajima's relative rate test. The χ^2 test statistic was 0.00 (P = 1.00000 with 1 degree[s] of freedom). P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineage.*

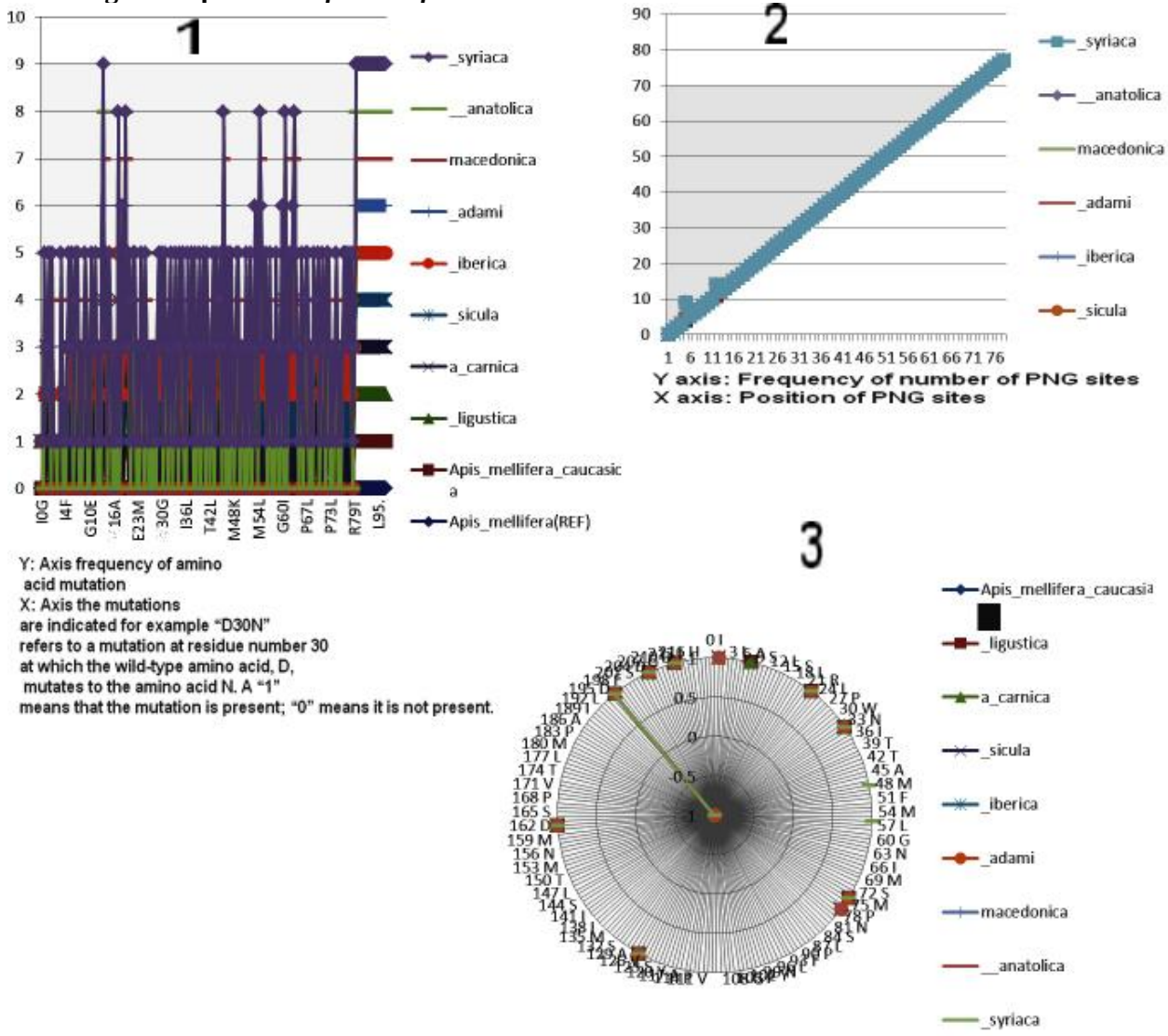
The presence of mutation was studied by using the tool Aminotrack. The sequence of COI from *Apis mellifera* was first submitted for FASTA then the most probable sequence from the list of alignment based on E value and similarity value selected the same Apis mellifera sequence. This sequence was used as reference sequence to study the mutation in selected nine subspecies of the *Apis mellifera*. The AminoTrack deducted mutation matrix, then the matrix was used to make amino acid sequence mutation graph using Microsoft excel statistical package. By the same facility we deducted the charge changes and PNG sites (Marshall, 1974, Kasturi, *et al* 1997).

RESULTS

The multiple sequences alignment shows that the first six amino acid is different and shows deletion in *Apis mellifera sicula, Apis mellifera iberica, Apis mellifera adami, Apis mellifera macedonica and Apis mellifera anatolica*. In the case of *Apis mellifera caucasica, Apis mellifera ligustica and Apis mellifera syriaca* the amino acid sequence after the 160th position is different and shows deletion. Thus based on the alignment it is observed that *Apis mellifera sicula, Apis mellifera iberica, Apis mellifera adami, Apis mellifera macedonica and Apis mellifera anatolica* and *Apis mellifera caucasica, Apis mellifera ligustica and Apis mellifera syriaca* forms two clads (Fig 1). The further study of the subspecies status was concentrated based on the MSA result. The multiple sequence alignment was used to produce phylogenetic trees with various software and with various methods (Figs. 2, 3). The phylogenetic tree by DIVEIN, MOBYEL@Pasture, maximum likelihood tree, minimum evolutionary tree, NJ tree, UPGMA tree, and maximum parsimony tree also reveals the same trend of grouping the nine subspecies into two groups. In all the phylogeny *mecedonica* subspecies appears to be the ancestor of *adami, anatolica* and *iberica*. The exciting observation is that the subspecies *syriaca* and mellifera species diverged at the same period, but the mellifera itself diverged in multidirectional and aroused, by mutation of deletion, inversion and substitution followed by natural selection, into different subspecies as *Apis mellifera sicula, Apis mellifera iberica, Apis mellifera adami, Apis mellifera macedonica and Apis mellifera anatolica* and *Apis mellifera caucasica* and *Apis mellifera ligustica*. The *Apis mellifera syriaca* remain unchanged over evolutionary stress and strains. This observation is reflected in the distance matrix study (Table2), amino

acid composition distance study (Table 3), Computed pattern disparity index between nine subspecies of *Apis mellifera* (Table4), evolutionary divergence matrix study (Table5), tajmas test (Fig3), mutmatrix study (Figure 3), PGN site study (Fig6) and charge changes in the amino acid sequence (Fig3). In the distance study between the nine subspecies involves the number of amino acid substitutions per site from between sequences. Analyses were conducted using the Dayhoff matrix based model (Schwarz, 1979).

Figure 3: MutMatrix, PNG site and Charge changes in the amino acid sequences in the eight subspecies of *Apis mellifera*



1: MutMatrix; 2: PNG site; 3: Charge changes

The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). All positions containing gaps and missing data were eliminated. There were a total of 79 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, 2011). In the study of Base Composition Bias Difference between Sequences the difference in base composition bias per site is

shown (Kumar, 2001). Note that even when the substitution patterns are homogeneous among lineages, the compositional distance will correlate with the number of differences between sequences. All ambiguous positions were removed for each sequence pair. There were a total of 80 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, 2011). In the study of Disparity Index per site all sequence pairs (Kumar, 2001). Values greater than 0 indicate the larger differences in base composition biases than expected based on evolutionary divergence between sequences and by chance alone. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5 (Tamura, 2011). The study of the number of amino acid substitutions per site from between sequences is done by using the Poisson correction model (Zuckerkandl and Pauling, 1965). The estimates of Average Evolutionary Divergence over all Sequence Pairs 1.58. The Tajmas test shown that the subspecies *syriaca* has 74 sequences different from the other eight subspecies sequence (Table 6). The other subspecies shown difference in amino acid sequence in Tajmas test is *ligustica*, but it may be technical artifacts as only two amino acid differ out of 79.

DISCUSSION AND CONCLUSION

The difference in the amino acid composition is due to the difference in the nucleotide sequence. The change in nucleotide sequence is occurring in nature spontaneously due to various reasons of intrinsic and extrinsic. The final result is the change in genome and consequently the formation of new species. Variations among offspring as a consequence of either the introduction of new genes via random changes called mutations or reshuffling of existing genes during sexual reproduction (Gregory, 2009). The common sequence between selected subspecies and *syriaca* is only six, another proof to show that by genetic drift, deletion or inversion followed by natural selection seeded the formation of *syriaca* subspecies. If a new subspecies exhibit more than 50% variation in genetic material, then it is good candidate for selecting and putting under the status of "species". Under this background it is sufficient to raise the *syriaca* subspecies to species level. We recommend in our study a further morphometric, genomic and proteomic study in support of our findings needed to finalize the status of *syriaca* subspecies as a separate "species" under the genera *Apis*. The phylogenetic position of honey bees is in dilemma for the last 100 years. The species status of many regional variety is questioned many taxonomists. But most of the regional varieties exhibit more genetical individuality and specificity to rise to the level of species status. The lack of proper study at molecular level lags behind many subspecies still in realm of subspecies. Molecular level study both wet lab and dry lab help us to understand more about the exact position of the honey bee subspecies in the taxonomic view point. Our study indicates that COI will help more to unify the above concept. COI is unchanged more than millions of years, hence used to study the phylogenetic relationship of closely related species and subspecies. The present study reveals that out of the nine subspecies of Apis *mellifera*, *Apis mellifera syriyaca* of Syria region has sufficient genetic background to rise to the level of species. But other species not shown much genetic diversity for the status of species. A further study incorporating more gentical markers is needed to substantiate the findings of this research work.

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