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Comparative Study of Mitochondrial Genomes using 16srRNA in Different *Apis sps.*

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ABSTRACT: We present a comparative analysis of select mitochondrial DNA (mtDNA) representing three *Apis sps* (*Apis mellifera Apis cerana* and *Apis dorsata*) order Hymenoptera in an effort to study a common set of genes and to understand the evolution of mitochondrial genome. A functional analysis of mitochondrial genomes was carried out using gene sequencing. Tocompare the similarity between closely related insect mitochondrial genome sequences phylogenetic comparisons of sequences were performed. LSU and SSU rRNA sequences were used to construct a phylogenetic tree to determine the relationship among four insect orders. LSU rRNA sequences yielded a tree with branching patterns reflecting the expected pattern as insect species belonging to different orders were put into separate clades. Based on the sequence similarity, insect species belonging to four different orders in general appear to be closely related. However, a comparative and functional analysis of Apis mitochondria sequences revealed differences in gene organization of mtDNA. Based on this study we conclude that, although the gene types are very similar across these species, significant differences in AT-GC content perhaps suggest multiple mitochondrial ancestors.

Key words: Mitochondria, genome analysis, gene content, gene order, phylogenetic analysis.

INTRODUCTION

We live in a world of insects with immense species diversity (insects representing more than 80% of the species). Insects belong to class-Insecta of phylum Arthopoda and constitute the largest group in the animal kingdom. Current trends in the application of DNA marker techniques in a diversity of insect ecological studies show that mitochondrial DNA (mtDNA), microsatellites, Random amplified polymorphic DNA (RAPD), expressed sequence tags (EST) and amplified fragment length polymorphism(AFLP) markers have contributed significantly to our understanding of the genetic basis of insects and honeybees diversity. Mitochondria are key energy generators in most eukaryotic cells. Research on mitochondria has primarily focused on the process of ATP generation, phylogeny and evolutionary origins. Unique sequence signatures from mitochondrial DNA (mtDNA) have been used not only to categorize species, but also to study animal, bird, and human migration as well as in diagnostics and forensics. With the increase in the whole genome sequencing of eukaryote genomes, mtDNA are inevitably sequenced and this has facilitated comparative studies. Mitochondria are believed to have evolved in eukaryotes through a process called serial endosymbiosis from an unknown microbial ancestor. An alternate theory proposed by Gray *et al.* (1999) suggests that mitochondria arose from a common ancestral extinct eukaryote, and evolved concurrently with the nucleus. Mitochondria are key energy generators in most eukaryotic cells. Research on mitochondria has primarily focused on the process of ATP generation, phylogeny and evolutionary origins. Unique sequence signatures from mitochondrial DNA (mtDNA) have been used not only to categorize species, but also to study animal, bird, and human migration as well as in diagnostics and forensics. With the increase in the whole genome sequencing of eukaryote genomes, mtDNAs are inevitably sequenced and this has facilitated comparative studies. Mitochondria are believed to have evolved in eukaryotes through a

process called serial endosymbiosis from an unknown microbial ancestor. An alternate theory proposed by Gray *et al.* (1999) suggests that mitochondria arose from a common ancestral extinct eukaryote, and evolved concurrently with the nucleus. Although the common mitochondrial ancestor is yet to be identified, several studies have suggested a very close relationship with endosymbionts belonging to:

Proteobacteria such as *Rickettsia* spp., *Anaplasma* spp. and *Ehrlichia* (Gray *et al.,* 1999; Gray *et al.,* 2001). Irrespective of their origins, mtDNA in general appear to have lost genes and have retained identical genes (coding and non-coding) by a process commonly referred to as reductive evolution. We selected these four Apis species for analysis based on prior behavioral genetic research of honeybees (Chandra *et al.,* 1998; Chandra *et al.,* 2000; Chandra *et al.,* 2001; Chandra and Singh, 2005; Rueppel *et al.,* 2006) which suggest strong similarities these species. In addition, species exhibit close evolutionary origins. It is then the purpose of this study to comparatively analyze the rRNA of mitochondrial DNA and regulatory non-coding sequences of the mtDNA of these three species, and to determine if conserved sequences across the four *Apis sps*. are suggestive of mtDNA evolutionary origin.

MATERIAL AND METHODS

Populations of *Apis sps* belonging to family-Apidae were collected from various districts of Jammu region (India).

A. DNA extraction

DNA was extracted from eight to fourteen single adult *Apis sps.* individuals in samples. For total DNA isolation, a single honey bee individual was thoroughly macerated with sterile micropestle in a 1.5ml eppendorfmicro centrifuge tube containing 25 µl lysis buffer (10 mMKCl, 2 mMTris-Cl pH 8.4, 60 µg ml^{-1} proteinase K (Merck >30 mAnson units/mg), 0.09 per cent Nonidet NP-40 and 0.09 per cent Tween 20). The buffer turns turbid on complete maceration. The mixture was incubated at 65°C for 45 min (for proteinase K action leading to cell lysis) and then heated at 95°C for 10 min (for denaturation of proteinase K and other cellular proteins). The turbid DNA extract was centrifuged (13,200 rpm) in a microcentrifuge for 3 min followed by two-fold dilution with autoclaved distilled water. Water used for dilution was of Mili-Q quality, and passed through 0.2 µm filter to remove any unwanted DNA harboring contaminant before autoclave sterilization. All DNA extract were stored at – 20°C until used as template in PCR analysis. The concentration of DNA was determined by spectrophotometric method using UV visible scanning spectrophotometer.

DNA Amplification by PCR - The DNA was amplified by using16Scb-LRprimer. PCR amplifications of 16srRNA (mitochondrial DNA) genes were carried out in a reaction volume of 20 µl.

DNA cloning and sequencing PCR fragments from each distinct restriction pattern were cloned using the 'INST/ACLONE^{TM} PCR Product Cloning Kit' (Fermentas Life Sciences) using manufacturer's protocol and used to transform competent Escherichia coli DH-5a cells. Positive clones were selected. The recombinant vectors were recovered and sequenced following the protocols suggested by Applied Biosystems (www.appliedbiosystems.com). The natural orientation of each sequence was determined by aligning of each sequence with the reported sequences (in GenBank database, *www.ncbi.nlm.nih.gov/pubmed/*) for honey bees and related strains in the literature using 'Gene align function' of the DNA software program 'CLC Free Workbench ver 3.2.4. of CLC Bio A/S. The multiple alignment of the all honeybee nucleotide sequences, both amongst themselves as well as with the available sequences of the previously reported *Apis* strains/ genotypes from different countries (available in GenBank database) was performed using 'CLC Free workbench software, ver 3.2.4' of CLC Bio A/S. These alignments were used both for the identification of polymorphic nucleotides and for deriving genetic relatedness amongst different honeybee samples. Based upon the information generated on the polymorphic bases in the aligned sequences, genetic relatedness dendrograms (phylogenetic tree) were developed for the same using 'tree function' of the above software. This function utilizes UPGMA (Unweighed pair group method using arithmetic averages) of Sneath and Sokal (1973). Additional information on level of genetic phylogenetic relatedness of the 16s mitochondrial sequences of *honey bee*samples under study was obtained using 'Nucleotide blast tool' of '*National Center for Biotechnology Information' available at* www.ncbi.nlm.nih.gov.med.

RESULTS AND DISCUSSION

During the present course of study, *Apis mellifera, Apis cerana* and *Apis dorsata* of family Apidae were investigated. The observations included the molecular genetical analysis of above given species along with their sequencing of 16Scb ribosomal RNA gene of mitochondrial DNA was presently worked out. The present studies on 'Molecular genetic analysis on *Apis species* was carried out under laboratory conditions using various standardized protocols. Custom sequencing of cloned 16Scbr-RNA gene fragments and processing of raw sequence data into corrected sequences was done and Locating

the vector sequences and cloned *Apis* species. sequences in corrected sequences was done under present study. In order to determine phylogenetic relation of the *Apis* species.under study, the 16Sr-RNA nucleotide sequences were aligned with GenBank database for previously reported *Apis* species./ genotypes from different countries was done. Multiple alignment of 16S rDNA sequences from the three species of *Apis* under study identified nucleotide differences at 78 nucleotide positions. In this respect *A. mellifera* and differed from *A cerana* at 58 nucleotide positions, *A. mellifera* and *A. dorsata* at 42 positions and *A. dorsata* and *A. cerana* at 65 nucleotide positions. These differences which were primarily substitution mutations also included one nucleotide deletions in *A. mellifera* (position 270), *A. cerana* (position 265) and *A. dorsata* (position 269) in the multiple alignments. Most of the nucleotide differences were concentrated in the second half of the 16S rDNA sequence under study (Fig. 1). The standard format of genetic relatedness dendrogram (Fig. 2(a) showing quantified genetic divergence amongst these species suggested that whereas the two *A. cerana* samples were closely related to each other, these have a genetic divergence of around 0.16 nucleotide difference/ nucleotide or 16% from *A. dorsata* and 10 % from *A. mellifera*. Though such a higher genetic divergence amongst two species of the same genera appears too high, but keeping in view the high mutational rates of mitochondrial genome during evolutions appear reasonable for differentiating amongst two related species (Saccone *et al.,* 2000).

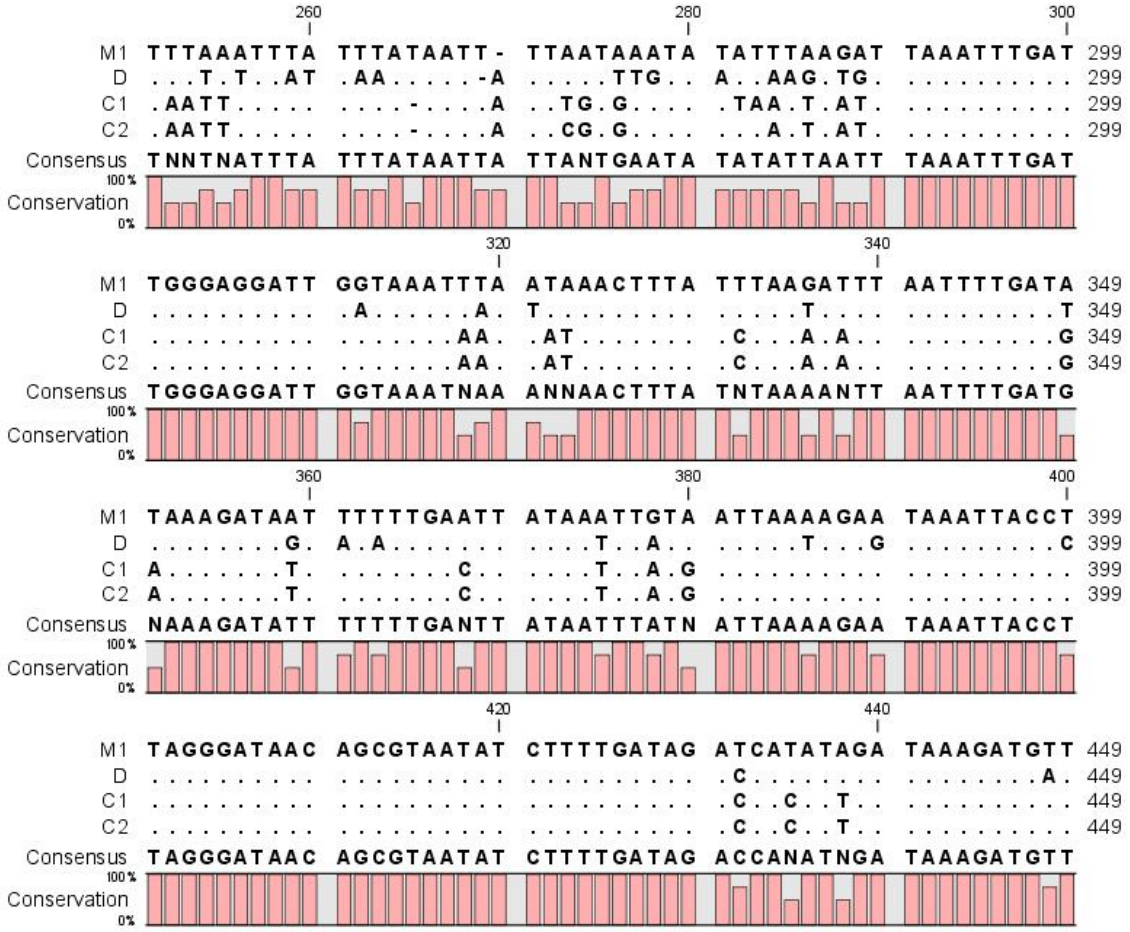

Fig. 1. Multiple alignment of nucleotide sequences of *Apis* mellifera. A. cerana and A. dorsata samples showing a portion of the polymorphic 16S rRNA region.

The three species different differed from each other as shown by existence of many polymorphic nucleotides all over the DNA length. This figure shows a portion of the multiple alignment with polymorphic bases.

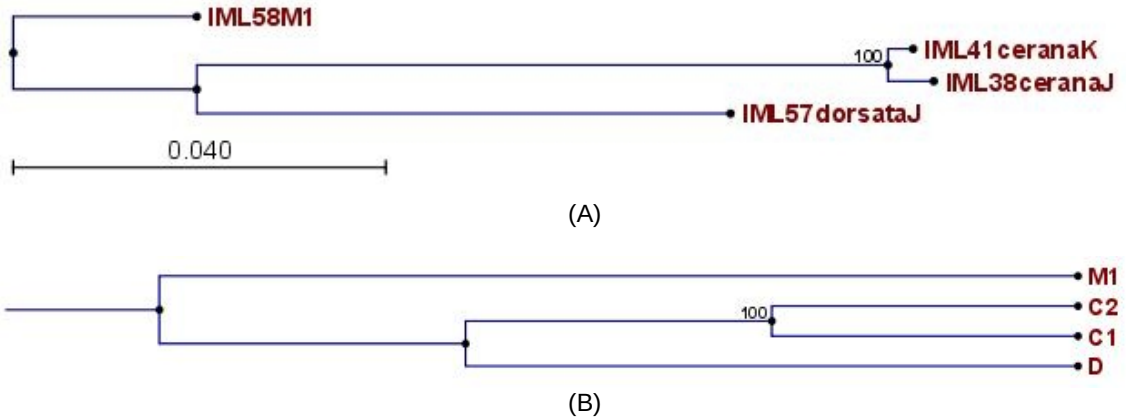

Fig. 2. Genetic relatedness dendrogram based upon 16 S rDNA sequences amongst different Apis species under study. A. Standard format; B Topology format.

The topological format of the data (Fig. 2(b)) suggests the possible course of evolution of the three *Apis* species from an unknown common ancestor. Thus, whereas *Apis mellifera* has possibly evolved directly from this common ancestor, *Apis dorsata* and *Apis cerana* appear to have evolved from another secondary common ancestor from the primary common ancestor. In this context, whereas *Apis mellifera* can be considered as an older ancestral species, *Apis cerana* appears to be relatively younger species with *Apis dorsata* occupying the intermediary position in this evolution. A close scrutiny of the whole sequence alignment resulted in identification of followings:

(i) A large number of nucleotide substitutions, deletions and additions that are distributed all over the length of alignment

(ii) A number of polymorphic nucleotides which though were distributed all over the length of alignment were more concentrated in the middle region (between nucleotide positions 255-390).

(iii) A three nucleotide deletion (262-264) in all *A. mellifera* samples than *A. cerana* and *A. dorsata*; a single base deletion (281) in all *A dorsata* samples than *A. mellifera* and *A. cerana*; a single base addition (257) in *A cerana* samples than other species; a three base addition (TTT at 66-68 position) in *A. mellifera* and *A. dorsata* than in *A cerana*.

(iv) Presence of AA at position 171-172 in *A mellifera* and *A. dorsata* instead of TT in case of A cerana; presence of T and A at positions 200 and 209, respectively in *A. mellifera* and *A. dorsata* than A and T in *A. cerana*.

The mtDNA region sequenced showed both a high A+T content (mean of 88.45%), as has been previously reported for the whole mtDNA of *A. mellifera* (Crozier and Crozier 1993), and more transitional events, as occurs in other organisms (DeSalle, 1992). These *A. cerana* populations have shown a high level of genetic variability, which agrees with the great variation previously found in other studies of the geographical (Ruttner, 1988, 1992), morphometric (Damus and Otis, 1997) and molecular (Deowanish *et al.,* 1996; Smith and Hagen, 1997) variation in *A. Cerana* populations. . In this sense, similar studies should be extended to other mitochondrial gene loci and morphologically characterized samples of the different subspecies of *Apis melllifera* to reconstruct this evolutionary history. The present and previous studies are therefore indicative of the fact that 16S ribosomal-RNA of mitochondrial DNA serve as a best marker for the identification of species and sub-species, thus helpful in genetic diversity studies. This study is extremely useful for identification of genetic polymorphisms in various insect groups.

REFERENCES

Chandra BCS, Hosler JS, Smith BH (2000). Heritable Variation for Latent Inhibition and its Correlation with Reversal Learning in Honeybees (*Apis mellifera*). *J. Comparative Psychol*. **114**: 86-97.

Chandra BCS, Hunt GJ, Cobey S, Smith BH (2001). Quantitative TraitLoci (Qtl) Associated with Reversal Learning and Latent Inhibition inHoney Bees (*Apis Mellifera*). *Behavior Genet*. **31**: 275-285.

Chandra BCS, Singh S (2005). Chemosensory Processing in the Fruit Fly Drosophila Melanogaster: Generalization of a Feeding Response Reveals Overlapping Odor Representations. *J. Biosci.* **30**: 679-688.

Crozier RH, Crozier YC (1993). The mitochondrial genome of the honeybee *Apis mellifera*: Complete sequence and genome organization. *Genetics.* **133**: 97-117.

Chandra BCS, Geetha L, Abraham VA, Karanth P, Thomas K, Srinivasan MV, Gadagkar R (1998). Uniform Discrimination of Pattern Recognition by Honeybees. *Animal Behavior* **56**, 1391-1398.

Damus, M.S. and Otis, G.W. (1997). A morphometric analysis of *Apis cerana* F. and *Apis nigrocincta* Smith populations from southeast Asia, *Apidologie* **28**, 309-323.

De Salle, R. and Templeton, A.R. (1992). The mtDNA genealogy of closely related *Drosophila silvestris*. *J.Hered.,* **83**: 211-216.
Deowanish S., Nakamura J., Matsuka M., Kimura K. (1996). mtDNA variation among subspecies of *Apiscerana* using restriction fragment length polymorphism, *Apidologie,* **27**, 407–413.
Gray MW, Burger G, Lang BF (1999). Mitochondrial evolution. *Science*. **283**: 1476-1481.
Gray MW, Burger G, Lang BF (2001). The origin and early evolution of mitochondria. *Genome Biol*. **2**: 1-5.
Ruttner F. (1988). Biogeography and Taxonomy of Honeybees, Springer Verlang, Berlin.
Ruttner F. (1992). Naturgesichte der Honigbiene, Munchen, Ehrenwirth.
Rueppell O, Chandra BCS, Pankiw T, Fondrk MK, Beye M, Hunt J, Page RE (2006). The Genetic Architecture of Sucrose Responsiveness in the Honey Bee (*Apis mellifera* L.). *Genetics* **172**: 243-251.
Smith, D.R. and Hagen, R.H. (1996).The biogeography of *Apis cerana* as revealed by mitochondrial DNA sequence data. *J. Kansas Entomol. Soc.* **69**: 294-310.
Saccone, C., Gissi, C., Lanave, C., Larissa, A., Pesole, G.R. (2000). Evolution of the mitochondrial genetic system: an overview. *Gene,* **261**: 153-59.