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Genetic Diversity of *Apis mellifera* (Hymenoptera: Insecta) - A Review

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ABSTRACT

The Honey bee is an important model animal for behavioral study as it is a colonial insect with complex social behavior. Hymenopterans are a diverse group of organisms and due to their richness at species level, DNA based identification is useful from genetic point of view. In this review different molecular tools used for identification of this diverse group is made here in.

Key words: Insects, Honey bee, *Apis mellifera*, Diversity.

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. Among the insecta, the Hymenoptera is the 2nd largest order comprising of bees, wasps, sawflies, ants, horntails, chalcids. The name refers to the heavy wings of the insects, and is derived from the ancient Greek ὑμήν (hymen): membrane and πτερόν (pteron): wing, which means insects with membranous wings. The hind wings are connected to the forewings by a series of hooks called hamuli.

The order Hymenoptera is composed of about 3, 00,000 species and 95 families worldwide though only 1, 30,000 species have been named so far. In India 60,600 species have been reported. All the species of order Hymenoptera are distributed all over the world and thus habitat also varies. Many adults are found on flowers. Females typically have a special ovipositor for inserting eggs into hosts or otherwise inaccessible places. The ovipositor is often modified into a stinger. The Hymenopterans show complete metamorphosis, the life cycle consists of larva, pupa and adult. So they are also known as Holometabolous insects (except sawflies which are caterpillar like).The order Hymenoptera is futher classified into two main suborders:

Symphyta: The suborder Symphyta includes the sawflies, horntails, and parasitic wood wasps.

Apocrita: The suborder Apocrita includes wasps, bees, and ants.

Hymenoptera originated in the Triassic, the oldest fossils belonging to the family Xyelidae. Social hymenopterans appeared during the Cretaceous. Among most or all hymenopterans, sex is determined by the number of chromosomes an individual possesses. Fertilized eggs get two sets of chromosomes (one from each parent's respective gametes), and so develop into diploid females, while unfertilized eggs only contain one set (from the mother), and so develop into haploid males. This phenomenon is called haplodiploidy. However, that the actual genetic mechanisms of haplodiploid sex determination may be more complex than simple

chromosome number. In many Hymenoptera, sex is actually determined by a single gene locus with many alleles. In these species, haploids are male and diploids heterozygous at the sex locus are female, but occasionally a diploid will be homozygous at the sex locus and develop as a male instead. This is especially likely to occur in an individual whose parents were siblings or other close relatives. Diploid males are known to be produced by inbreeding in many ant, bee and wasp species. One consequence of haplodiploidy is that females on average actually have more genes in common with their sisters than they do with their own daughters.

Honey bees (or honeybees) are a subset of bees in the genus *Apis*, primarily distinguished by the production and storage of honey and the construction of perennial, colonial nests out of wax. Honey bees are the only extant members of the tribe Apini, all in the genus *Apis*. Currently, there are only seven recognised species of honey bee with a total of 44 subspecies, though historically, anywhere from six to eleven species have been recognised. Honey bees represent only a small fraction of the approximately 20,000 known species of bees. Some other types of related bees produce and store honey, but only members of the genus *Apis* are true honey bees.

Geographic Range

Honey bees as a group appear to have their centre of origin in South and South East Asia (including the Philippines), as all but one of the extant species are native to that region, notably the most plesiomorphic living species (*Apis florea* and *Apis andreniformis*). The first *Apis* bees appear in the fossil record at the Eocene–Oligocene boundary, in European deposits. The origin of these prehistoric honey bees does not necessarily indicate that Europe is where the genus originated, only that it occurred there at that time.

As a group of economically important insects, it constitutes the major dominants among pollinators of world. About 15% of crops are pollinated by bees out of which 80% by honey bees. Most of honeybee species have historically been cultured or at least exploited for honey and bee wax by human indigenous to their native ranges. Worker bees of a certain age will secrete beeswax from a series of glands on their abdomens. Propolis or bee glue is created from resins, balsams and tree saps. Propolis is consumed by humans as a health supplement in various ways and also used in some cosmetics.

Only two of these species have been domesticated. These are European honeybee- *Apis mellifera* and Asian honeybee- *Apis cerana*. Honeybees represent only a fraction of bee diversity of approximately 25000 known species of bees, there are only seven presently recognized species with a total of 44 sub species. These bees are the only living members of tribe apini. Biodiversity in honeybees includes all species and subspecies of the genus *Apis* (family-apidae) and ecosystem and ecological process of which they are part. Biodiversity is generally considered at two different levels taxonomic and systematic. Due to lack of published literature and higher level of variations within species and recent divergent lines between taxa, the systematics of small and the useful group is not clearly understood. Taxonomic questions on the status of species within *Apis* remain controversial. Subsequent workers tended to ignore Maa's (1953) classification and recognized only 4 species in the genera: *Apis mellifera, Apis cerana, Apis florae, Apis dorsata.* Recent studies on the diversity of Asian honeybees led to the rediscovery of two of Maa's species: *A.aderniformis* (Wu and Kuang 1987) and *A.koschevnikovi* (Koeniger *et al.,* 1988). As far the species diversity of honeybees in India is concerned the Asian subcontinent is supposed to be richest but as far our state is concerned 4 major species of honeybees are found namely: *Apis mellifera, Apis cerana, Apis florae and Apis dorsata.*

Genetic Identification

Hymenopterans are a diverse group of organisms and due to their richness at species level, DNA based identification is useful from genetic point of view. In earlier days morphometric methods were the important tools and being used to resolve systematic issues. The characterisation based on morphmetric characters is not well suited for phylogeographical studies because they can be sensitive to environmental selection pressures, need a lot of time and experience, and sometimes are unsuitable for identifying some hybrids. But now-a-days in the modern hitech world, the field of molecular biology has been exploited greately (Hawksworth 1994; Hoy 1994; Crampton *et al*., 1996, Roderick 1996, Karp *et al*., 1998, Kumar and Negi 2004).DNA analyses are techniques which are lately being used to understand and confirm insect systematic (Mestriner 1969; Burns and Johnsan 1971; Sylvester 1982; Berlocher 1984; Sheppard and Berlocher 1989). Misidentifications at the level of species and genera have therefore, created serious problems for researchers in the field of ecology, physiology and genetics for a comparison and evolution of their results. Thus molecular studies have therefore proved to be extremely useful for this purpose. So, besides behavioral, morphological and cytogenetic evidence, molecular data provide strong support for phylogenetic relationships among insects.

RAPD analysis

Molecular methods have opened up a wide range of new approaches to invertebrate research, particularly with regard to molecular phylogenetic

and taxonomic studies. The molecular characterization included the study of genetic diversity, genetic relatedness and phylogenetic analysis at species level. 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. Molecular markers are widely used in biology to address questions related to ecology, genetics and evolution. The recent characterization of genomes, completely or partially, and knowledge of the molecular basis of genetic variation have been very important sources for the development of markers and establishment of evolutionary models at the inter and intra-specific levels (Li 1997). In bees, molecular studies addressing those issues have focused on *Apis* and *Apis mellifera*.

RAPD stands for Randomly Amplified of Polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction. Randomly amplified polymorphic DNA markers (RAPD) are an important technique for genetic polymorphism and relatedness). No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species. RAPD is a preliminary study for genetic polymorphism and largely been carried out, but now a days is less popular due to its poor reproducibility faint or fuzzy products and difficulty in scoring bands, which lead to inappropriate and non-authenticated inferences.

One of the most basic techniques of molecular biology is expression cloning which is used to study the function of protein. In this technique, DNA coding for a protein of interest is cloned (using PCR and/or restriction enzymes) into a plasmid (known as an expression vector). This plasmid may have special promoter elements to drive production of the protein of interest, and may also have antibiotic resistance markers to help follow the plasmid. This technique is very useful in DNA-sequencing. The advent of DNA sequencing has significantly accelerated biological research and discovery. In order to amplify any DNA sequence *in vivo* and *in vitro*, the sequence in question must be linked to primary sequence elements capable of directing the replication and propagation of themselves and the linked sequence in the desired target host. The required sequence elements differ according to host, but invariably include an origin of replication, and a selectable marker. In practice, however, a number of other features are desired and a variety of specialized cloning vectors exist that allow protein expression, tagging, single stranded RNA and DNA production and a host of other manipulations that are useful in downstream applications. The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of many animal, plant, and microbial genomes. The complete genome of *Apis mellifera* has been sequenced and consists of 10,000 genes with approximately 236 million base pairs. The size of the genome is a tenth of the human genome . Besides the discovery of new genes for the use of pollen and nectar, researchers found that, in comparison with other insects, *Apis mellifera* has fewer genes for immunity, detoxification and the development of the cuticula .

Mitochondrial DNA analysis

Mitochondrial DNA was discovered in the 1960s by Nass and Nass (1963) by electron microscopy as DNase-sensitive thread inside mitochondria, and by Haslbrunner *et al.,* (1964) by biochemical assays on highly purified mitochondrial fractions. Animal mtDNA is a circular molecule (there are few exceptions) composed of about 37 genes coding for 13 proteins, 22 tRNAs, and two rRNAs. Also there is a non-coding region, the control region (or 'A+T' rich region), that is responsible for transcription and replication (Wolstenholme 1992). Although, the genome content has been described as very conservative, the order in which the genes are organized in the mtDNA molecule is more variable than was initially predicted, especially for tRNA genes (Wolstenholme 1992; Dowling *et al.*, 1996). The mitochondrial genome is maternally inherited in most animals without recombination, so the whole set of genes is inherited as one unit (Avise 1994). The mutation rate of animal mtDNA is higher than that of nuclear DNA, so, mtDNA is a powerful tool for tracking ancestry through females (matrilineage) and has been used in this role to track the ancestry of many species back hundreds

of generations. The high mutation rate makes mtDNA most useful for comparisons of individuals within species and for comparisons of species that are closely or moderately-closely related, among which the number of sequence differences can be easily counted. As the species become more distantly related, the number of sequence differences becomes very large thus changes begin to accumulate and an accurate count becomes impossible. However within the molecule there are genes or regions with higher and lower base substitution rates (Vawter and Brown 1986; Brown 1983). Therefore it is possible to select an appropriate region for analysis according to the taxonomic level under study. *Apis mellifera* was the first member of the Apidae to have its mtDNA completely sequenced (Crozier and Crozier 1993) and most microsatellite loci known within Apidae have been described from this species (Estoup *et al.,* 1993).

Analysis of mitochondrial DNA variations in honeybees permits the identification of major racial lineages similar to those based on morphology (Badino *et al.,* 1988a; Cornuet 1982; Garnery *et al.,* 1992; Moritz *et al.,* 1986; Sheppard and Huettel 1988; Smith and Brown 1990). Most of the studies focused on mitochondrial DNA to improve the understanding of the phylogenetic relationship between the various insect groups (Garey *et al.,* 1996, 1999; Moon and Kim 1996; Aguinaldo *et al.,* 1997; Giribet and Ribera 2000; Giribet *et al.,* 2000; Giribet and Wheeler 2001).Initial data on honeybees' mtDNA have confirmed the presence of three lineages in Africa, Western Europe and South-Eastern Europe. Recently the existence of a fourth mitochondrial lineage in the Middle East has also been confirmed. The molecular clock does not tick at a rate in all taxa but maybe influenced by species characteristics. Eusocial species (those with reproductive division of labour) have been predicted to have faster rates of molecular evolution than their non-social relatives because of greatly reduced effective population size; if most individuals in a population are non-reproductive and only one or few queens produce all the offspring, then eusocial animals could have much lower effective population sizes than their solitary relatives, which should increase the rate of substitution of ''nearly neutral'' mutations. An earlier study reported faster rates in eusocial honeybees and vespid wasps but failed to correct for phylogenetic non independence or to distinguish between potential causes of rate variation. Because sociality has evolved independently in many different lineages, it is possible to conduct a more wide-ranging study to test the generality of the relationship.

16S- rRNA alaysis

The 16S-rRNA gene is used for phylogenetic studies as it is highly conserved. In addition to these, mitochondrial and chloroplastic rRNA is also amplified. Sequence analysis of the 16S rRNA sequences is done with the help of several primers, called "universal primers." These primers target the conserved region of 16S rRNA gene and amplify the target in parts. Finally the several amplified parts could be assembled together to have the entire sequence of amino acids. In recent decades, we have witnessed significant progress in reconstructing phylogenies based on molecular data (nucleotide or amino acid sequences). Good examples are the analysis of small-subunit ribosomal RNA of 2,551 species (Van de Peer et al., 2000), as well as the analysis of over 500 proteins of six genomes (Wolf *et al.,* 2004). However, the results of these studies often conflict with respect to phylogeny. Therefore, it is necessary to improve the phylogenetic analysis using additional molecular markers and tighter species sampling. Beyond gene sequences, such markers include singular character states as transposable element insertions, gene order changes, code variants, and intron positions (Rokas and Holland 2000). Sequences coding for the 16S rRNA gene have been used for estimating phylogenies over a notable range of taxonomic levels. The existence of sites that are changing at widely differing rates within this single gene (Simon *et al.,* 1994) suggests that 16S sequences contain historical information that is useful at more than one level of phylogenetic divergence. In a study, Simon *et al.,* (1994) suggested that data from 16S might be useful primarily for phylogenetic estimation at higher levels, because few sites were variable at lower levels among closely related species, and even some of those quickly saturated. In contrast, Engel and Schultz (1997), in a reanalysis of Cameron's (1991, 1993) data for estimating relationships among the corbiculate bees, suggested that *Apis* (honey bee) species relationships recovered from 16S sequences (Cameron *et al.,* 1992) were strongly congruent with those inferred from morphological data.

During the present investigations, *Apis sp.* of Family: Apidae viz. *Apis mellifera, Apis cerana* and *Apis dorsata* have been studied for their molecular analysis. Molecular genetical studies revealed the various numbers of variations in the *Apis. sps.* as reported by earlier workers. These changes might have occurred due to environmental mutations of different geographical ranges.

The era of molecular genetics began with the discovery of DNA structure in 1953. In the late 1970s, yet another milestone in genetics was reached when researchers found that they could manipulate DNA molecules in a test tube (*in vitro*), essentially "cloning" the first gene. This discovery led to further molecular genetic advances

including recombinant DNA technology and study of mitochondrial DNA. Mitochondrial DNA was extensively used for studying the genetic variation, evolutionary relationships and phylogeny of individuals within species and among closely or moderately-closely related species; this due to their small size, high copy number, relatively infrequent gene rearrangements, high rate of mutation, their low effective population size (Brown *et al*., 1997; Behura 2006; Singh 2008; Lee *et al*., 2009). Also, due to the maternally inheritance mtDNA is a powerful tool for tracking ancestry through females (matrilineage) back hundreds of generations (Brown *et al*., 1997). Mitochondrial DNA was believed to be better suited to identification of older skeletal remains than nuclear DNA because the greater number of copies of mtDNA per cell increases the chance of obtaining a useful sample, and because a match with a living relative is possible even if numerous maternal generations separate the two (Stone *et al*., 2001). At the technical level there are two advantages of using mtDNA as molecular markers such as: universal primers are available for insect species which their sequences are not known (Simon *et al*., 1994) and the circular nature of mtDNA facilitates the sequencing of the total mitochondrial genome by amplifying previously sequenced genes (Cameron *et al*., 2006). Animal mitochondrial genomes are potential models for molecular evolution and markers for phylogenetic and population studies. DNA markers have made a significant contribution to molecular studies of insects (Behura 2006). Different molecular markers satisfactorily resolved the phylogenetic relationships among organisms, however, none of which have proven to be the best tool (Cameron *et al*., 2006). Different regions of the ribosomal DNA (rDNA) gene have been used successfully to investigate the genetic variation in different insect species (Black 1993; Campbell *et al*., 1993; Paskewitz *et al*., 1993; Birch *et al*., 1994; Fenton *et al*., 1994; Schmitz & Moritz 1998; Townson & Onapa 1994; Pfeifer *et al*., 1995; Loxdale & Lushai 1998; Haine *et al*., 2006). Honeybee mtDNA sequences have been used to infer geographic origin, genetic relatedness, phylogeny, and population structure. Previous research has shown interesting features in hymenopteran mitochondrial genomes. A perusal of literature reveals that the mtDNA has attracted the attention of many molecular biologists and geneticists. This review focuses on the molecular genetic aspects as the present study deals with the molecular genetic variation of honeybee species.

Schmitz and Moritz (1990) studied mtDNA variation in social wasps and constructed the distance trees that support the hypothesis of monophyly of genera *Vespula* and *Dolichovespula*. Sheppard *et al.,* (1991) examined restriction enzyme analysis and indicated that mitochondrial DNA in some neotropical populations is almost entirely of African origin, and these data had been cited as evidence for asymmetrical gene flow between African and European-derived populations. In the Neotropics, introduced European honey bees (*Apis mellifera* L.) have been largely supported by bees descended from an African race, *A. m. scutellata*, which were introduced into Brazil in the 1950s. Evaluation of the nature of hybridization in the Neotropics is, however, confounded by possible population size advantages for the African-derived group. As an alternative approach, genetic interactions studied in transition areas between zones ecologically and climatically adaptive for both racial groups and described the results of a survey transecting regions populated by African- and European-derived honey bees in Argentina. Mitochondrial DNA, morphological and isoenzyme analysis showed that substantial hybridization occurs between the two racial groups.

De Salle and Templeton (1992) predicted a sequential pattern of colonization going from north to south *Drosophila* flies on east side of the island of Hawaii and examined their prediction using mtDNA restriction site with four base cutters and DNA sequencing.

Cameron (1993) made the phylogenetic analyses of DNA sequence information from the mitochondrial genome (large-subunit ribosomal RNA gene) of representative *Apis* bees. It suggested that advanced eusocial behaviour evolved twice independently within this assemblage. These results depart from previous hypotheses of *Apis* relationships by indicating a close phylogenetic relationship between the primitively eusocial bumble bees and the stingless bees. The remarkably high level of colony organization found in the honey bees and stingless bees (family Apidae) is extremely rare among animals. Yet there is controversy over whether these two groups independently evolved advanced eusocial behaviour or inherited it from a common ancestor.

Landry *et al.,* (1993) developed a system based on DNA markers to rapidly characterize individuals of five species of micro hymenoptera from genus *Anaphes* and *Trichogramma*. The main components of their system was a rapid and simple DNA micro-extraction method and fast DNA polymorphism analysis based on randomly amplified polymorphic DNA markers. Arias and Sheppard (1996) amplified, cloned and sequenced the mitochondrial DNA region encompassing part of the NADH dehydrogenase subunit 2 and isoleucine transfer RNA genes for 14 morphometrically identified *Apis mellifera* subspecies and the New World "Africanized" honeybee. Twenty different haplotypes were detected and phylogenetic analyses supported the existence of 3 or 4 major subspecies groups similar to those based on morphometric measurements.

However, some discrepancies reported concerning the subspecies composition of each group. Based on the sequence divergence of *Drosophila* (2% per Myr) found that the four lineages may have diverged around 0.67 Myr. The variability found in this region enables us to infer phylogenetic relationships and test hypotheses concerning subspecies origin, dispersion, and biogeography.

Smith and Hagen (1997) sequenced the non-coding intergenic region in bees from 110 colonies of *A. cerana* collected over most of the species' range and found two major forms of non-coding sequence: a western form, occurring in bees from India, Sri Lanka and the Andaman Islands; and an eastern form, occurring in bees from Nepal, Thailand, Malaysia, Indonesia, the Philippines, Hong Kong, Korea, Japan, and India. The non-coding intergenic region of the *Apis cerana* mitochondrial genome provides a rapidly evolving source of characters for study in intra-specific biogeography. Thus the eastern and western haplotypes co-occur in India. Within the eastern form, phylogenetic analysis of sequence variation indicated two well supported groups of haplotypes: a "Sundaland group," which was found in bees from peninsular Malaysia, Borneo, Java, Bali, Lombok, Timor, and Flores; and a "Philippine group" which was found in bees from Luzon, Mindanao, and Sangihe. Haplotypes from both the Sundaland group and the Philippine group were found on the island of Sulawesi, suggesting that this island was colonized independently by two groups of *A. cerana*. In addition, the bees of Taiwan and a third group of Sulawesi bees had mitochondrial haplotypes characterized by absence of most of the non-coding sequence. Variation in the sequence of the remaining non-coding region, as well as comparison of coding sequences with other populations of *A. cerana*, indicated that these are independent deletions of the non-coding region.

Francisco *et al.,* (2001) characterized the mitochondrial DNA (mtDNA) of five species of *Plebeia* (*P. droryana*, *P. emerina*, *P. remota*, *P. saiqui* and *P.* sp.) and generated a data set to be used in further population, phylogenetic, and biogeographic studies. The mtDNA of each species was analyzed using 17 restriction enzymes and restriction maps were built. A high level of interspecific variability was found. The total size of the mtDNA was estimated to be 18500 bp. Through a combination of PCR and examination of restriction fragment length polymorphism, the locations of 14 of the main mitochondrial genes were located on restriction maps. They verified a gene order identical to *Apis mellifera*.

Weinlich *et al.,* (2004) characterized the mitochondrial genome of Meliponini. They described the restriction and partial genomic map of seven *Melipona* species (*M.bicolor*, *M.compressipes*, *M.marginata*, *M.melanoventer*, *M. quadrifasciata*, *M.rufiventris* and *M.subnitida*). The maps were obtained through RFLP and PCR-RFLP using 15 restriction enzymes. The total number of sites mapped ranged from 12 to 19, indicating a high level of genetic diversity among those species. MtDNA total size was estimated to be 18500 bp. Brito and Arias (2005) characterized the mitochondrial DNA of two stingless bee species of the genus *Partamona*. Partial restriction maps were obtained based on digestion of PCR amplified fragments with 8 restriction enzymes. Using *Melipona bicolor* mtDNA sequence as a model, we were able to amplify 12120 bp of *P.mulata* and 10300 bp of *P. helleri*, about 65.5% and 55.7% of their mitochondrial genome, respectively. The digestion assays showed 16 restriction sites for *P. mulata* and 20 for *P. helleri*, some of which were exclusive to the genus and others shared with other Meliponini species. The main mitochondrial genes could be mapped and through sequencing analysis verified that the intergenic region that occurs between the genes CO I and CO II in *Apis* was absent in *Partamona.*

Shi *et al.,* (2005) examined the phylogenetic relationships among the Braconidae using homologous 16S rDNA, 28S rDNA D2 region, and 18S rDNA gene sequences and morphological data using both PAUP* 4.0 and MRBAYES 3.0B4 from 88 in-group taxa representing 35 subfamilies. The monophyletic nature of almost all subfamilies, of which multiple representatives presented in this study, well-supported except for two subfamilies, Cenocoelinae and Neoneurinae that should probably be treated as tribal rank taxa in the subfamily Euphorinae. The topology of the trees generated in the present study supported the existence of three large generally accepted lineage or groupings of subfamilies: two main entirely endoparasitic lineages of this family, referred to as the "helconoid complex" and the "microgastroid complex," and the third "the cyclostome." The Aphidiinae was recovered as a member of the non-cyclostomes, probably a sister group of Euphorinae or Euphorinae-complex. The basal position of the microgastroid complex among the non-cyclostomes has been found in all our analyses. The cyclostomes were resolved as a monophyletic group in all analyses if two putatively misplaced groups (*Mesostoa* and *Aspilodemon*) were excluded from them. Certain well-supported relationships evident in this family from the previous analyses were recovered, such as sister-group relationships of Alysiinae + Opiinae, of Braconinae + Doryctinae, and a close relationship between Macrocentrinae, Xiphozelinae, Homolobinae, and Charmontinae.

Collet *et al.,* (2006) described three 16S mtDNA PCR-RFLP patterns, each one completely associated with a previously determined A, M, or C Dra I restriction pattern of the COI-COII region. These results indicated that the COI-COII and the 16S genes had a very closely linked evolutionary

history. Although distinct patterns were obtained with *Eco* RI, *Alu* I, *Hinc* II and *Taq* I, the best differentiation among the three patterns was observed with *Dra* I and *Vsp* I enzymes. Nucleotide sequence analysis of the16S gene fragment displayed 10 sites of base substitution (1.35%) among the three patterns and two insertions in the *A. m. scutellata* pattern. Phylogeographic and morphometric evidence can be used to cluster *Apis mellifera* subspecies into evolutionary lineages or branches. Mitochondrial DNA sequence and restriction site analyses have shown similar clustering of subspecies groups. Thus, mtDNA variation can be used to infer honey bee evolutionary relationships.

Magnacca and Danforth (2007) worked on the Hawaiian bees based on mitochondrial DNA and morphology, appeared to support a recent origin for the group, but support for the resulting tree was weak. Four nuclear genes with varying evolutionary rates-arginine kinase, EF-1, opsin, and wingless—were sequenced for a reduced taxon set in an attempt to Wnd one or more data set that would provide better support. All showed very low variation (<2%) in the ingroup. Comparison among genes revealed a much higher than expected rate of evolution in mtDNA, especially at ist and second positions. While the data from the nuclear genes showed insufficient variation for phylogenetic analysis, the strong sequence similarity among the Hawaiian species supports the previous hypothesis of a recent origin for the group.

Quezada-Euan *et al.,* (2007) analysed the genetic structure of populations of the stingless bee *M. beecheii* from two extremes of its geographic range. The results showed that populations from Costa Rica and Yucatan exhibit substantial phenotypic and molecular differentiation. Bees from Yucatan were smaller and paler than those from Costa Rica. The value of multilocus *FST* = 0.280 ($P < 0.001$) confirmed that there were significant molecular genetic differences between the two populations. Populations showed significant deviation from Hardy Weinberg equilibrium and the values of *FIS* (the inbreeding coefficient) were positive for Costa Rica = 0.416 and the Yucatan Peninsula = 0.193, indicating a lack of heterozygotes in both populations possibly due to inbreeding. The DNA sequence of 678 bp of the mitochondrial gene COI differed between populations by 1.2%.

Tan *et al.,* (2007) investigated DNA sequence diversity in a non-coding portion of the mitochondrial genome in samples of *Apis cerana* from 47 locations in China. Nine haplotypes (mitochondrial genotypes) were found: Japan1, Japan2, Korea4, and Cambodia2, which were previously reported from other populations, and China1-5, which are new. All nine sequences belong to the Mainland mitochondrial lineage, and none differs from the Japan1 haplotype by more than a single base substitution and/or a single insertion/deletion. Japan1 is the most common haplotype, making up 39 of 49 sequences. Haplotype diversity was 0.4 (s.d. 0.089) and nucleotide diversity was 0.00569 (s.d. 0.00154). By both measures the Chinese samples were more diverse than those from Japan and Thailand, similar to populations from Pakistan, Burma and Korea, and less diverse than samples from Indochina (Laos-Cambodia-Vietnam).

Tavares *et al.,* (2007) and Quezada-Euan *et al.,* (2007) found high levels of genetic differentiation between several geographic populations of the endangered stingless bee *Melipona rufiventris*, indicating the presence of a cryptic species which should be considered separately for conservation purposes. Similarly, surveyed genetic diversity at several microsatellite markers and 678 bp of the mitochondrial gene COI in the endangered stingless bee *Melipona beecheii* and found high levels of genetic differentiation between populations in the Yucatan peninsula and Costa Rica. They recommended that movement of colonies between\ southern Mexico and Central America should be reconsidered given that the two regions may harbor two cryptic species.

Thummajitsakul *et al.,* (2008) collected the samples of the stingless bee *Trigona pagdeni* Schwarz from north, northeast, central and peninsular Thailand and studied genetic variation and population structure were investigated using a DNA fingerprinting technique, TE-AFLP, and Analysis of Molecular Variance (AMOVA). They found high levels of genetic variation among individuals in all populations, but mean expected heterozygosity was highest in the Northeast. AMOVA calculations revealed significant genetic differentiation among the four populations (φPT = 0.18, P = 0.01). They also detected differentiation (φPT = 0.13, P = 0.001) between samples collected north and south of the Kra ecotone, a biogeographical zone of transition betweenseasonal evergreen and mixed moist deciduous forests. However the greatest differentiation was detected between samples from the northeast and the other locations combined (φPT = 0.21, P = 0.001). This method can be applied to the study of population structure in *T. pagdeni* and other stingless bees, and may provided a useful tool for management and conservation of this species.

Kawakita *et al.,* (2008) discussed possible future approaches for resolving the frustratingly persistent corbiculate bee controversy. The corbiculate bees comprise four tribes, the advanced eusocial Apini and Meliponini, the primitively eusocial Bombini, and the solitary or communal Euglossini. Recovering a robust phylogeny forthe four tribes is of considerable importance for understanding the evolution of eusociality, yet previous morphological and molecular studies reached strikingly different conclusions. An

expanded dataset consisting of 12 nuclear genes to explore lines of supported for the molecular hypothesis. Results corroborate previous molecular studies; support increases as more genes are added. Across genes, support for the molecular hypothesis is positively correlated with the number of informative sites and the relative substitution rate. Phylogenetic signals supporting the molecular tree rest almost entirely upon synonymous changes at the first and third codon positions.

Rasmussen and Camargo (2008) tested the Stingless bees which exhibit extraordinary variation in nest architecture within and among species and analyzed the phylogenetic association of behavioral traits for species of the Neotropical stingless bee genus *Trigona*. A phylogenetic hypothesis was generated by combining sequence data of 24 taxa from one mitochondrial (16S rRNA) and four nuclear gene fragments (long-wavelength rhodopsin copy 1 (opsin), elongation factor-1α copy F2, arginine kinase, and 28S rRNA). Fifteen characteristics of the nest architecture were coded and tested for phylogenetic association. Several characters have significant phylogenetic signal, including type of nesting substrate, nest construction material, and hemipterophily, the tending of hemipteroid insects in exchange for sugar excretions. Phylogenetic independent habits encountered in *Trigona.* include coprophily and necrophagy. Arias *et al.,* (2008) described a set of 24 oligonucleotides that allow PCR amplification of the entire mitochondrial genome of the honey bee *A. mellifera* in 12 amplicons. These fragments have important applications for the study of mitochondrial genes in different subspecies of *A. mellifera* and as heterospecific. They further concluded that Mitochondrial DNA markers have been widely used to address population and evolutionary questions in the honey bee *Apis mellifera*. Most of the polymorphic markers restricted to few mitochondrial regions.

Krauss *et al.,* (2008) analysed the evolutionary positions of bees and wasps (Hymenoptera) and beetles (Coleoptera) in relation to moths (Lepidoptera) and dipterans (Diptera) using recently completed genome project data to test the suitability of such near intron pairs (NIPs) as a marker class for phylogenetic analysis by scanning 758 putatively orthologous gene structures of *Apis mellifera* (Hymenoptera) and Tribolium castaneum (Coleoptera) and identified 189 pairs of introns, one from each species, which are located less than 50 nt from each other. The reconstruction of the organismal evolutionary tree based mainly on molecular sequence data and resulted that the sequence data are sometimes insufficient to reliably resolve in particular deep branches. Thus, it is highly desirable to find novel, more reliable types of phylogenetic markers that can be derived from the wealth of genomic data. It was suggested that 31 and 12 intron positions apomorphic for *A. mellifera* and *T. castaneum*, respectively, which seem to represent changes inside these branches. Another 12 intron pairs indicate parallel intron gain or extraordinarily small exons. It showed that the analysis of phylogenetically nested, nearby intron pairs is suitable to identify evolutionarily younger intron positions and to determine their relative age, which should be of equal importance for the understanding of intron evolution and the reconstruction of the eukaryotic tree.

Zayed (2009) reviewed how haplodiploidy and complementary sex determination affect genetic parameters pertinent to the viability and future evolutionary potential of bee populations and how genetic tools can improve the conservation management of bees. He found that bees are especially prone to extinction for genetic reasons, and that genetics can provide invaluable tools for managing bee populations to circumvent pollinator decline. The emerging threat of pollinator decline has motivated research on bee conservation biology in order to both understand the causes of declines and to develop appropriate conservation strategies. The application of genetics to the conservation of diploid animals has proven to be important for both overcoming genetic threats to population viability and for providing tools to guide conservation programs. However, the haplodiploid bees have several unusual genetic properties of relevance to their conservation, which warrant special attention.
Lopes *et al.,* (2010) compared the number of polymorphic loci and alleles per locus and observed the heterozygosity in *Melipona rufiventris* and *M. mondury* populations, using specific and heterologous primers to their high degree of polymorphism.The microsatellites are considered useful tools for studying population genetics. Nevertheless, studies of genetic diversity in stingless bees by means of these primers revealed a low level of polymorphism, possibly the consequence of the heterologous primers used, since in most cases these were not specifically designed for the species under consideration. The use of specific primers placed in evidence the greater frequency of polymorphic loci and alleles per locus, besides an expressive increase in observed heterozygosity in *M. rufiventris* and *M. mondury*, thereby reinforcing the idea that populational studies should be undertaken by preferably using species-specific microsatellite primers.

Martimianakis *et al.,* (2011) studied *Apis mellifera* L. populations from various areas of Albania, Bulgaria, Cyprus, Greece, Italy, Slovenia and Turkey, together with a genetically improved commercial strain exported all over the world analyzed the sequences of two mitochondrial regions, the ND5 and the COI gene segments. Their study examined the phylogenetic relationships

among these honey bee populations and investigated the existence of gene flow as a result of migratory beekeeping and commercial breeding. Seven and eight different haplotypes were revealed for the COI and ND5 gene segments respectively, while the combined data set consisted of twelve different haplotypes. Among the two DNA segments studied, the highest genetic divergence values were observed in COI. In both genes the highest divergence value was among *A. m. ligustica* colonies and all others. All the phylogenetic trees constructed by Maximum Parsimony, Neighbour-Joining and Bayesian Inference analyses exhibited exactly the same topology for COI and ND5 separately, as well as for the concatenated data set; *A. m. ligustica* forms the most distinct clade. The study presented the first comprehensive sequencing analysis of *A. mellifera* subspecies occurring in Greece and it is the first time that sequencing data from ND5 mtDNA gene segment have been obtained at the population level.

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