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Amplification, Sequencing and Phylogenetic Analysis of EF-1 α Gene in *Apis* Family (Hymenoptera: Apidae)

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ABSTRACT

In this study, with the help of Elongation Factor (EF-1 α) sequences reported in *Apis* family analyzed the relationship of the same family in Tamilnadu, South India, India, through nucleotide amplification, sequencing and Phylogenetic tree analysis. The worker honey bee specimens were collected from the colonies in different locations of Tamilnadu, South India. Total Genomic DNA was extracted from worker bees thorax region by using slight modification of high salt extraction protocol and the extracted Genomic DNA were subjected to PCR amplification done by using nuclear protein gene EF-1 α primers. Amplified products were analyzed in 1% analytical agarose gel electrophoresis and the molecular weight of the amplified product size of amplicon is approximately ~1100 bp. The amplified product of *Apis cerana indica* (MSS2) were sequenced and submitted in Genbank (GU935342). Phylogenetic analysis done with other four species sequence data of EF-1 α gene collected from Genbank. The phylogenetic tree was constructed by using tree top software program from GeneBee service was started with a set of aligned sequence clustal W. The tree shows clearly that the strain MSS2 is highly cluster with *Apis florea* and *Apis cerana* with 100% bootstrap value. Also strain MSS2 had maximum sequence identity (99%) with *Apis nigrocincta* and *Apis cerana* than *Apis dorsata* (96%) and *Apis florea* (95%). It is assuring that strain MSS2 had a maximum identity 99% and phylogenetically cluster with *Apis cerana indica* alone. EF-1 α analysis of honey bee is an effective tool to classify the honey bee species.

Key words: Honey bee, apiary, genomic DNA, PCR, nuclear protein, amplicon

INTRODUCTION

Insects represent a major life form on earth. So far, nearly 0.9 million insect species are discovered, compromising 75% all the recorded animal species (Jain *et al.*, 2009). In that well known insect is honey bee. The honey bee is an important model organism for behavioral research because it is a colonial insect with complex social behavior (Hunt and Page-Jr, 1994). These members of the insect order Hymenoptera play a key role in the human and natural world. Honey has been traditionally used in various diet preparations, medicines, cosmetics, ointments, candles and house-hold beeswax items, besides Ayurvedic drug preparations. It has long been important for honey production and for the pollination of more than 90 crops (Al-Otaibi, 2008). The honey bee is an excellent model for understanding the evolutionary genomics of eusociality. Appropriate genetic markers can be used to elevate management efficiency of important natural resources

species. The sustainable conservation of these species requires basic knowledge of they are genetic population structure and the use of suitable molecular genetic markers to establish appropriate genetic management programme (Awise, 1994). The honeybee genome has a haploid chromosome number of 16 and a total size of ~180 mega base pairs (Jordan and Brosemer, 1974). The precise identification of insect (honey bees) is a primary importance and so difficulties caused when the body shape and colour of bees are same. Most advanced techniques have been used to split species not to unit them. A number of molecular level approaches have been taken for honey bees. DNA techniques have been applied to study different aspects of the taxonomy, systematic and evolution of honey bees and after a great potential for accurate species identification (Jain *et al.*, 2009).

The development of molecular phylogenetics over the past two decades greatly improved our understanding of eukaryotic phylogeny. Analysis of small-subunit (SSU) and large-subunit (LSU) ribosomal RNA (rRNA) from a wide variety of eukaryotes had done for the classification of species (Roger *et al.*, 1999). This rRNA-based outline of eukaryotic phylogeny has been challenged on several fronts. Cavalier-Smith (1993), O'Kelly (1993) and Patterson (1994) have all developed separate phylogenetic schemes for the early branching order of the eukaryotes based on rRNA trees and ultra structural data. But another one gene called, Elongation factor1- α (EF-1 α) is a conserved nuclear protein coding gene that could provide marker for identifying honey bees. EF-1 α protein involved in the GTP dependent binding of charged transfer (+) RNAs to the acceptor site of the ribosome during translation (Maroni 1993). In *Drosophila* EF-1 α occurs at two copies, EF-1 α F1 and EF-1 α F2 which are expressed at different times during development (Hovemann *et al.*, 1988). EF-1 α genes have been characterized in other animal, including brine shrimp, mice, humans and sand fly (Danforth and Ji, 1998). Amino acid sequence of EF-1 α has recently been used to resolve evolutionary relationships among early eukaryotes (Hasegawa *et al.*, 1993; Kamaishi *et al.*, 1996). However the conserved nature of the amino acid sequence among these desperate organisms, EF-1 α has been identified as a potentially useful gene for studies of higher level phylogenetic relationships, especially in insects. The present aim is to amplify, sequence and phylogenetic analysis of EF-1 α gene in *Apis* family in Tamilnadu, South India, India.

MATERIALS AND METHODS

A total of three species (*Apis florea*, *Apis cerana indica* (MSS2) and *Apis mellifera*) worker bees were collected from two regions in Tamilnadu, South India, India and the work was done between Jul. 2009 to Jan. 2010. The collected fresh and alcohol preserved specimens were used for the DNA extractions (*Apis cerana indica* (MSS2), *Apis mellifera* and *Apis florea*) by slight modification of Bradford *et al.* (1998) and Miller *et al.* (1988) protocol and all gave satisfactory results. The thoracic region of individual specimens grinds presence of homogenizing buffer (100 mM Tris HCl, 100 mM EDTA, 1% SDS). The homogenate were digested with proteinase-K (20 mg mL⁻¹) for 1 h and then incubated at 37°C for 15 min with the addition of RNase and the proteins were precipitated with 4 M ammonium acetate. The DNA was precipitated with equal volume of ice-cold absolute ethanol, washed with 70% ethanol and the pellet was resuspended in 20 μ L TE buffer (pH 8).

The primers used for PCR amplification (Table 1) of EF-1 α F₂ region included the F2 specific forward primer of F₂for 1 and the reverse primer F₂-Rev 1 (Danforth *et al.*, 2004). Synthesized from

Table 1: Forward and reverse primer sequences of EF-1 α

S.No	Primer name	Gene region	Primer sequence
1.	HaF ₂ For1	Ef-1 α F ₂	GGG YAA AGG WTC CTT CAA RTA TGC
2.	F ₂ -Rev1	Ef-1 α F ₂	AAT CAG CAG CAC CTT TAG GTG G

Sigma Aldrich Chemicals Private Limited, Bangalore, India. The extracted genomic DNA was amplified with 30 cycles, initial denaturation of the template DNA at 94°C for 5 min. Each cycle consisted of a denaturation 1 min at 94°C, followed by annealing 55°C for 1 min and primer extension at 72°C for 1 min. The amplified product of *Apis cerana indica* (MSS2) alone partially sequenced. Sequence of 991 bp was deposited in GenBank, accession number is (GU935342). In this study, EF1- α gene of different species (different strains of a species) was obtained haphazardly from GenBank; however 4 species were selected on the basis of high identity (%) for phylogenetic analysis. [*Apis florea* (EU184773), *Apis cerana* (EU184774), *Apis nigrocincta* (AY208279) and *Apis dorsata* (AY208277)]. TreeTop software programme from GeneBee service was started with a set of aligned sequences using Clustal W and searches for phylogenetic trees that are optimal according cluster algorithm.

RESULTS

Species identification based on morphology: In total there are around 22 colonies of log and wild hives in two regions of Tamilnadu, South India, India. Ten worker bees were collected from each colony then the species were identified based on their morphological characters of the head, abdominal segments and wings. This included ten colonies of bees *Apis cerana indica* (MSS2), seven colonies of *Apis florea* and five colonies of *Apis mellifera*.

Genomic DNA extraction and PCR amplification of EF-1 α : The worker honey bee's specimen of *Apis cerana indica*, *Apis mellifera*, *Apis florea* were collected and the genomic DNA was exacted (Fig. 1) from the thorax region of the honey bees by modified high salt extraction method. F₂ copy of EF-1 α gene were amplified by using specific forward and reverse primers (Table 1). The PCR amplification of EF-1 α was successfully achieved for all three species of honey bees that we caught,



Fig. 1: Isolation of genomic DNA from honey bees. L1: *A. florea*, L2: *A. cerana indica* (MSS2), L3: *A. mellifera*

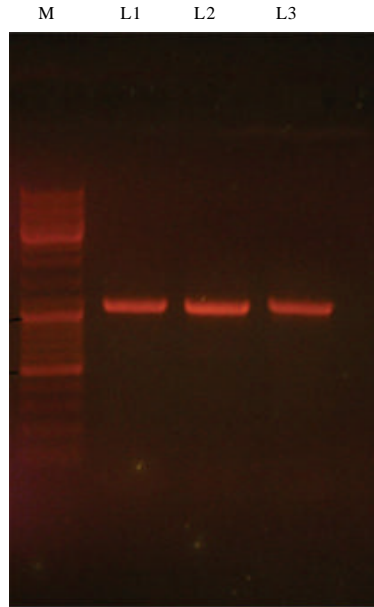


Fig. 2: Agarose gel electrophoresis of EF-1 α Amplified gene product. M: Marker (1kb), L1: Amplified product size ~1100 bp (*A. florea*), L2: Amplified product size ~1100 bp (*A. cerana indica*) (MSS2), L3: Amplified product size ~1100 bp (*A. mellifera*)

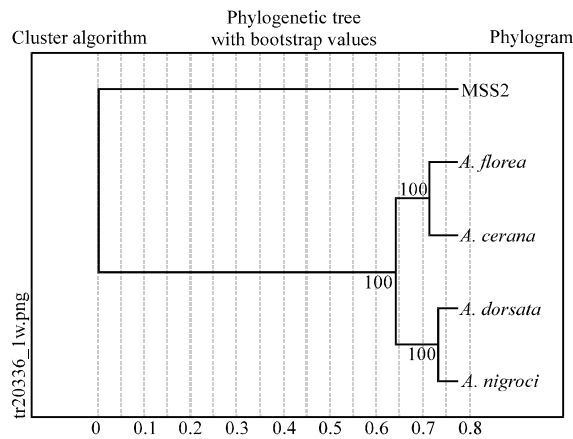


Fig. 3: Phylogenetic tree was constructed based on the nucleotide sequence of EF-1 α gene

but same primer had to be used. The amplified PCR products were visualized in analytical agarose gel with EtBr and confirm the amplification of F₂ copy of EF-1 α gene with DNA marker in all species of *Apis* family (Fig. 2). All amplified PCR products size of Amplicon is ~1,100 bp. The amplified product of *A. cerana indica* (MSS2) alone partially sequenced and has 991 bp [333 A, 210 C, 154 G, 294 T] deposited in GenBank (GU935342). In this study *A. cerana indica* and *Apis cerana indica* (MSS2) both are differentiated clearly by using the cluster algorithm. The EF-1 α gene sequences (F₂ copy) of *A. mellifera*, *A. florea* and *A. cerana indica* were collected from the GenBank are used for the differentiation of *A. cerana indica* (MSS2) by the utilization of EF-1 α gene F₂ copy sequences. Phylogenetic tree was constructed and it reveals that *A. cerana indica* has a maximum identity and phylogenetically cluster with *A. cerana indica* (MSS2) (Fig. 3).

DISCUSSION

Evidence supports that EF-1 α gene present in all major bee families. Regarding the nuclear markers, EF-1 α has two copies in bees, EF-1 α F₁ and EF-1 α F₂, which are expressed at different stages of development. Nuclear loci that have been providing robust result for insect phylogenetic studies. Slowly evolving, nuclear genes are commonly used for phylogenetic analysis in many groups of insects. They have been demonstrated to recover cretaceous age divergences (Danforth *et al.*, 1999, 2004; Wiegmann *et al.*, 2000). Danforth *et al.* (2004) reviewed that phylogenetic utility of EF-1 α , opsin and Wg and commented on the biological functions of their gene product. Among these genes EF-1 α has been most widely used nuclear protein coding gene for phylogenetics.

Degenerate PCR primers initially developed based on comparisons of *Apis* (Walldorf and Hovemann, 1990), *Drosophila* (Hovemann *et al.*, 1988) and Heliothine moths (Cho *et al.*, 1995) nonspecifically amplified two paralogous copies in bees. These primers were used to amplify EF-1 α gene in *Apis mellifera*. Similarly the same primers used in the present study.

They also reported that EF-1 α primer was expected a single 392-bp PCR product correspond to the EF-1 α sequence. However all bees tested, representing most major bee families (including Colletidae, Andrenidae, Halictidae and Apidae). They obtained two bands, even at high annealing temperature (64°C). One band corresponds to the expected 392-bp PCR product, whereas a larger (roughly 600-bp) bands also obtained (Walldorf and Hovemann, 1990). In the present study, we also obtained bright band were visualized at the annealing temperature of (55°C). In the present study also strongly supports the existence of EF-1 α gene in all bees. The EF-1 gene MSS2 (991 base pairs, MSS2) sequence of *Apis cerana indica* sequences were amplified from the genome DNA with forward primer (sequence 5'-GGG YAA AGG WTC CTT CAA RTA TGC-3') and reverse primer (Sequence 5'-AAT CAG CAG CAC CTT TAG GTG G-3') primers, it was submitted to GenBank. In this study, EF-1 α gene of different species (different strains of a species) was obtained haphazardly from GenBank; however four species were selected on the basis of high identity (%) for phylogenetic analysis.

The cumulative results from a limited number of studies of data suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65-83%), with regard to species from 1-14% of the isolates remaining unidentified after testing. The phylogenetic analysis using the *gyrB* gene sequence will be able to classify some bacteria that cannot be classified by their 16S rRNA sequences. Though, *gyrB* sequence method might be more useful for identifying bacteria to the species level (Fukushima *et al.*, 2002). Above the results suggested that the gene sequences enough to identifying the species of pro and eukaryotic organisms. Chouhan and Pardasani (2007) employed the reconstruction of phylogenetic network in honey bees by utilizing the maximum parsimony method. For analyzing this hypothesis and results we have performed the phylogenetic analysis on the basis of an EF-1 α gene. Randomly four different species which are comes under the genus of *Apis* were used for tree construction. The phylogenetic tree showed clearly that our strain MSS2 is highly cluster with *Apis florea* and *Apis cerana indica* with 100% bootstrap value. Moreover strain MSS2 had maximum sequence identity (99%) with *Apis nigrocincta* and *Apis cerana indica* than *Apis dorsata* (96%) and *Apis florea* (95%). It's assuring that strain MSS2 had a maximum identity 99% and phylogenetically cluster with *Apis cerana* alone. It assure that MSS2 is belongs to *Apis cerana indica*. The above the results of a phylogenetic analysis based on the EF-1 α sequence were never suggested before. We believe that the EF-1 α region will have high reliability for identifying honey bees and it was never

reported before. In particular, EF-1 α analysis of honey bee is an effective means to classify closely related species. Further research on EF-1 α sequence analysis will clarify in more detail about the classification of honey bee species.

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