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A STUDY ON THE MITOCHONDRIAL COI DNA SEQUENCE AND PHYLOGENETIC STATUS OF *ANASTATUS BANGALORENSIS* MANI & KURIAN AND *ANASTATUS ACHERONTIAE* NARAYANAN, SUBBA RAO & RAMACHANDRA RAO (HYMENOPTERA: EUPELMIDAE)

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ABSTRACT

Anastatus (Hymenoptera: Eupelmidae) species are end parasitoids of a wide variety of insect eggs of Hemiptera, Lepidoptera and Orthoptera. It included the family Eupelmidae of Hymenoptera and they are an important egg parasitoid of several major insect pests. Here we report the partial sequence of cytochrome oxidase sub unit I gene (COI) of *Anatatus bangalorensis* (KU052674) *and Anastatus acherontiae* (KU052673) and its phylogenetic relationship. The COI gene sequence of *A. bangalorensis and A. acherontiae* are showed considerable variation with other related species. The mitochondrial COI DNA barcode developed in this study can be used for the accurate identification. The COI partial coding sequence of *A. bangalorensis* (KU052674) are showed 1.6% difference to *A. acherontiae* (KU052673), *A. bangalorensis and A. acherontiae* demonstrates the efficiency of the barcoding gene in discriminating global phylogeographical variants among the related species complex. *A. bangalorensis and A. acherontiae* are in a same clade on the phylogenetic tree.

KEYWORDS: *A. Acherontiae*, *A. Bangalorensis*, Cytochrome Oxidase, DNA Bar-coding, Molecular Phylogeny

INTRODUCTION

Species of *Anastatus* (Hymenoptera: Eupelmidae) are included in the egg parasitoids of Lepidoptera, Orthoptera, Hemiptera and Phasmidae. The common host of *A. acherontiae* is *Acherontia styx* which is a Sphingid moth commonly found in Asia. They are also known to be a pest of *Citrus junos* in South Korea, using their tongue to pierce and damage the fruit. It is also found in other eggs of lepidopterans, *A. bangalorensis* lays eggs on *Halymorpha marmorea* (Heteroptera: Pentatomidae), a serious pest of arecanut plant causing tender nut fall. Head of *A. bangalorensis* and *A. acherontiae* is dark with metallic green refringence. Antennal pedicel brown or black, scape pale yellow or pale brownish yellow, mesosoma dark brown with metallic green, legs brown with mid tibial spur and tarsus pale yellow (Narendran, 2009). The identification of these two species is difficult in morphologically due to their similarities. Molecular phylogenetic analysis were extensively carried out using COI gene sequences in various group of insects like dipterans (Bindu and Sebastian, 2014; Priya and Sebastian, 2014), lepidopterans (Akhilesh and Sebastian, 2014; Pavana and Sebastian, 2014), heteropterans (Sreejith and Sebastian, 2014), odonates (Jisha and Sebastian, 2015), and hymenopterans (Rukhsana and Sebastian, 2014).

MATERIALS AND METHODS

A. bangalorensis and *A. acherontiae* used in the present study was collected from Wayanad district in Kerala, India. Mitochondrial genomic DNA was extracted from the experimental insects, *A. bangalorensis* and *A. acherontiae*. The tissue was homogenized using a glass pestle and mortar. The genomic DNA in the homogenate was extracted using a GeNei Ultrapure Mammalian Genomic DNA Prep Kit in accordance to the manufacturer's instructions. About 2 ng of genomic DNA was amplified for mitochondrial cytochrome oxidase subunit I (COI) gene using the forward primer with DNA sequence 5'- GGTCAACAAATCATAAAGATATTGG -3' and reverse primer with DNA sequence 5'- TAAACTTCAGGGTGACCAAAAAATCA -3'. The PCR reaction mixture consisted of 2 ng of genomic DNA, 1µl each forward and reverse primers at a concentration of 2.5 µM, 2.5 µl of dNTPs (2mM), 2.5 µl of 10X reaction buffer, 0.20µl of Taq polymerase (3U/µl) and 11.8 µl H_2O. The PCR profile consisted of an initial denaturation step of 2 minutes at 95^0C, followed by 30 cycles of 5s at 95^0C, 45s at 50^0C and 45s at 72^0 C and ending with a final phase of 72^0C for 3 minutes. The PCR products were resolved on a 1% TAE-agarose gel, stained with Ethidium Bromide and photographed using a gel documentation system. After ascertaining the PCR amplification of the corresponding COI fragment, the remaining portion of the PCR products were column purified using Mo Bio Ultraclean PCR Clean-up Kit (Mo Bio Laboratories, Inc. California) as per the manufacturer's instructions. The purified PCR products were sequenced from both ends using the forward and reverse primers used for the PCR using Sanger's sequencing method (Sanger, 1975). The forward and reverse sequences obtained were trimmed for the primer sequences, assembled by using ClustalW and the consensus was taken for the analysis. The nucleotide sequence and peptide sequence were searched for its similarity using BLAST programme of NCBI (www.ncbi.nlm.nih.gov/) and Inter and intra specific genetic diversity were calculated using Kimura 2-parameter model with the pair wise deletion option and the difference in the nucleotide in codon usage partial COI sequence of *A. bangalorensis* and *A. acherontiae* was analyzed using MEGA6 software.

RESULTS AND DISCUSSION

The PCR of the COI gene fragment of *A. bangalorensis* (KU052674) *and A. acherontiae* (KU052673) yielded products of 652 bp and 663 bp respectively. The BLAST search using the sequences revealed that the sequences obtained in this study was novel. The average divergence in inter specific comparisons is 1.6%. Partial COI DNA sequence of *A. bangalorensis* showed 1.6% difference to that of *A. acherontiae* sequenced.

The average nucleotide composition across the species *A. bangalorensis* was T=45.3%; A=30.5%; C=10.6%; G=13.5% and *A. acherontiae* was T=43.2%; A=29.3%; C=11.7%; G=15.8% (Table 1). This results show that analysis based on mitochondrial gene can be useful for unraveling phylogenetic relationships in these species. The percentage of A+T was higher than that of G+C which reflected further in the codon usage in these two species. The probability of substitution (r) from one base to another base also calculated for 14 nucleotide sequences (Tamura *et al*, 2004) shows in Table 2. The nucleotide frequencies are 31.62% (A), 43.49% (T/U), 11.82% (C), and 13.08% (G). The transition/transversion rate ratios are k1 = 1.394 (purines) and K2 = 0.628 (pyrimidines). The overall transition/transversion bias is R = 0.364, where R = [AxGxk1 + TxCxk2] / [(A+G) x (T+C)] (Tamura *et al*., 2013).

The evolutionary history was inferred using the Neighbor-joining method using COI partial sequence. The evolutionary history of *A. bangalorensis and A. acherontiae* were inferred using the Neighbor- joining method (Figure 1). The amino acids arginine and glutamic acid are not found in these partial nucleotide sequences (Table 3). DNA sequence

based identification technique has revealed the morphological and ecological traits of many species during larval stages (Foltan, 2005; Smith, 2006; Hayashi and Sota, 2010). Gurney *et al.* (2000) reported that closely related species have 90% similarity in the standardized DNA sequence and distantly related species have less than 90% similarity in the same genes sequence. Here we can clearly classify the two species, *A. bangalorensis and A. acherontiae* by comparing the nucleotide sequences.

Table 1: The Evolutionary Nucleotide Divergence of *A. Bangalorensis and A. Acherontiae* with Various Hymenopterans Species

Accession No. and Organism	% of Divergence
KU052674 *Anastatus bangalorensis*	0%
KU052673 *Anastatus acherontiae*	1.60%
HQ599571 *Aphelinus varipes*	13.15%
KP072609 *Perilampus tristis*	13.81%
KF573404 *Trichogramma pretiosum*	13.59%
JX442923 *Trichogramma brassicae*	13.37%
JQ268913 *Aphytis hispanicus*	14.49%
JQ756596 *Megastigmus aculeatus*	15.23%
KJ208871 *Eupelmidae* sp.	13.18%
JQ756561 *Philocaenus medius*	15.75%
JQ756575 *Philocaenus liodontus*	15.94%
FM210164 *Metaphycus flavus*	14.06%
GQ374677 *Megastigmus transvaalensis*	15.64%
KJ083443 *Eulophidae* sp.	15.00%

Table 2: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution. Each Entry Shows the Probability of Substitution (r) from One Base (Row) to Another Base (Column)

	A	T	C	G
A	-	14.64	3.98	6.14
T	10.64	-	2.5	4.4
C	10.64	9.2	-	4.4
G	14.84	14.64	3.98	-

Table 3: the Percentage of Various Amino Acids found in the Partial COI Sequences of *A. Bangalorensis* and *A. Acherontiae*

Amino Acid	% in A. Bangalorensis	% in A. Acherontiae
Ala	**0**	**0.6**
Cys	5.1	7.9
Asp	1.7	1.7
Glu	0	0
Phe	18.9	15.2
Gly	1.1	3.4
His	0.6	0
Ile	14.3	15.2
Lys	6.9	5.6
Leu	4	2.8
Met	0	1.7
Asn	12	11.8
Pro	0.6	1.7

Gln	0	0.6
Arg	0	0
Ser	13.7	13.5
Thr	3.4	2.2
Val	1.7	2.2
Trp	6.9	5.6
Tyr	9.1	8.4

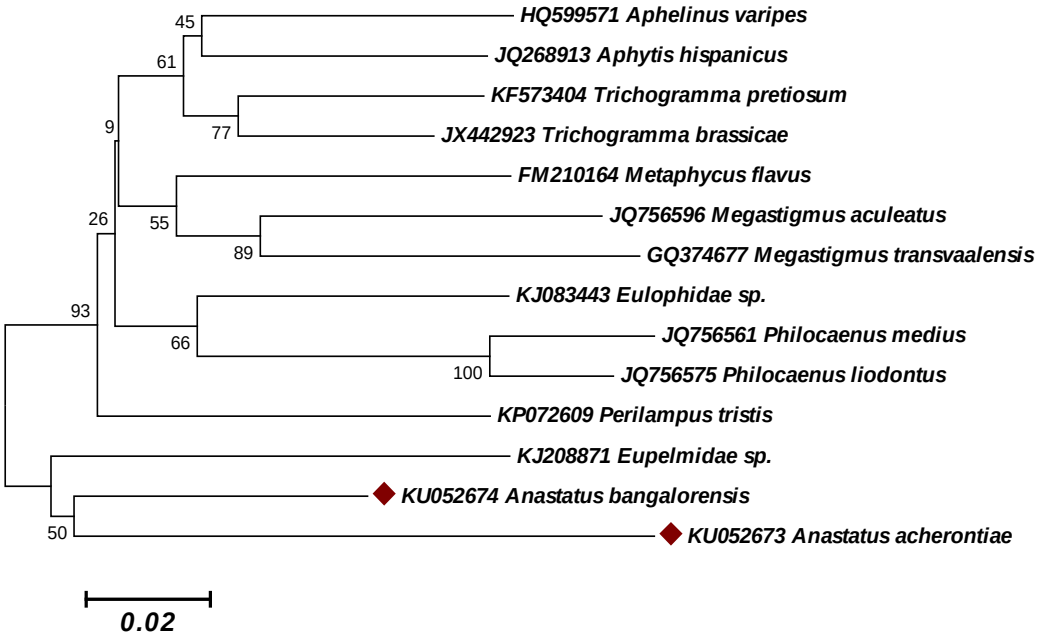

Figure 1: Phylogenetic Status of *A. Bangalorensis* and *A. Acherontiae* Compared with other Hymenopterans Species

CONCLUSIONS

Variation in the nucleotide is fundamental property of all living organisms which can be used for their identification and phylogenetic status. The COI sequence obtained in this study showed nucleotide variation between *A. bangalorensis* and *A. acherontiae* are 1.6%. Phylogeny analysis using NJ tree revealed the sharing of common ancestor to these two species and both species in a same clade. The branch length of *A. bangalorensis* was less compared to the *A. acherontiae* indicating the diversity. The phylogenetically close species of these two are a Eupelmidae species (KJ208871). Inter specific divergence of partial coding fragment of COI gene is very efficient for species identification (Hebert *et al*., 2003).

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