Genetic variation in coconut black headed caterpillar (*Opisina arenosella* Walker) population in India

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Abstract
Opisina arenosella Walker or leaf eating caterpillar or the black headed caterpillar, a serious defoliator of coconut palm, is an outbreak pest in Andhra Pradesh. In order to characterize the genetic relationship among the O. arenosella collected from various districts of Andhra Pradesh, 625 bp of mitochondrial cytochrome oxidase subunit I (COXI) gene was sequenced for 18 larval samples collected from five districts of Andhra Pradesh. The sequence analysis data revealed that out of 625 sites, 14 sites (2.24 %) were variable (polymorphic) without any insertion or deletion and remaining 611 sites were invariable (monomorphic). Phylogenetic analysis revealed the existence of high genetic diversity among all samples collected from different districts. Eleven haplotypes could be observed among the 18 individuals. The high haplotype diversity and low nucleotide diversity support the high level of genetic diversity among the O. arenosella populations.

Keywords: *Opisina arenosella*, mitochondrial DNA, cytochrome oxidase, genetic diversity, haplotypes.

Introduction

In India, coconut crop is mainly confined to the four southern states accounting for about 90% of the area under coconut, among which Andhra Pradesh shares about 1.04 lakhs ha area. In Andhra Pradesh, East Godavari (50167 ha), West Godavari (20394 ha), Srikakulam (14645 ha) and Visakhapatnam (6928 ha) are important coconut growing coastal districts[6]. There is a tremendous potential to increase the area under coconut cultivation to about 5 lakhs ha in Andhra Pradesh.

The coconut palm, with its luxuriant green foliage, offers an abundant supply of food to a large number of caterpillar pests. One of the major factors that contributes to the loss of production and productivity in coconut is the damage due to the insect pests, especially the leaf eating caterpillar or the black headed caterpillar *Opisina arenosella* Walker[4,21]. It is an outbreak pest and in severe cases the whole plantation presents a burnt up appearance due to drying of the leaves, the attacked leaves droop, bunches buckle and immature nuts shed heavily[18]. The caterpillar is a serious defoliator of coconut palm in India[25], Sri Lanka[12], Burma[9] and Bangladesh[1]. For a long time, the pest was known as *Nephantis serinopa*[16] and included under the order Lepidoptera and family Cryptophasidae.

Becker[4] found that *Nephantis* and *Opisina* are monotypic genera and the type species of the both in British museum were conspecific. Hence, the coconut caterpillar was renamed as *Opisina arenosella* Walker and placed under family Oecophoridae.

The pest, in its larval stage, causes serious damage to the plant while the adult moth, having a short life span, is harmless. Normally the damage of the pest is restricted to outer whorls of leaves. The caterpillars construct galleries out of silken webs reinforced with the excreta and the scraps of leaf bits. Hiding in these galleries, the caterpillars feed on the chlorophyll containing parenchymatous tissues. Only the upper epidermis is left intact. These portions get dried presenting a scorched up appearance to the foliage. The infested palms restore to the normal yield potential in the fourth year after pest attack[7].

Mitochondrial DNA (mtDNA) has been used as a powerful tool for evolutionary and molecular diversity studies of insects and mtDNA analyses have provided insights into population structure and gene flow, hybridization, biogeography and phylogenetic relationships. Mitochondrial cytochrome C oxidase *(COX)* is one of the common stretches of DNA used in bar-coding. It is considered as an ideal marker due to its uniparental inheritance, haploidy, resistance to degradation and relatively high evolutionary rate[2,10,17]. It is one of the extensively used molecular markers for studying intra-specific and inter species variation in insects[13,14,23]. The study of intra-specific mtDNA variation can expose information about the interconnectivity of populations and past demographic measures such as population expansions[2]. The size of the *COX* gene is approximately 1548 bp which has 70.2 % total T content[27]. *COXI, COXII* and *COXIII* are tree different subunits of cytochrome C oxidase; among these, *COXI* is largest one and the most conserved between them[3].Versatile primers that are designed for *COX1* enable simple and conventional amplification[5].

O. arenosella breeds all year round on coconut palms without undergoing diapause. Although the species infests coconut groves almost throughout peninsular India, it has never been found to occur as a large contiguous population in coconut-growing areas. Infested areas are always interspersed with un-infested ones, suggesting the existence of spatially segregated populations[24]. Therefore, a study

was conducted to know about the relativity of these spatially segregated populations of *O. arenosella* in five districts of Andhra Pradesh.

Material and Methods

Sample collection: Larval population of similar age group (third to fourth instar) were collected from lower whorl leaves of coconut palm from coconut gardens during roving surveys from Bidimi village, Mandasa mandal (Srikakulam district), S. Rayavaram village and mandal (Visakhapatnam district), Matlapalem village, Tallarevu mandal (East Godavari district), Poduru village and mandal (West Godavari district) and Vizianagaram district. The districts are major coconut growing areas and adjacent coconut plantations are not contiguous and the samples collected are far and distant from each other. The larval samples were collected from the multi-aged stand (15 to 40 years) gardens of these districts which had a history of harbouring *O. arenosella* population for one month before the roving survey was carried out. The number of samples collected from each districts and number of samples are shown in table 1.

Genomic DNA extraction: The genomic DNA was extracted from 18 larval samples using HiPurA™ Insect DNA Purification Kit (Hi-Media). The extracted DNA was eluted in 200 µl elution buffer. The quality and quantity of extracted DNA was verified by using spectrophotometer and agarose gel electrophoresis. DNA samples were stored at -20°C.

Gene-specific primer designing: In order to design gene specific primer, the *COX1* gene nucleotide sequence of *Opisina* was retrieved from NCBI. A pair of primer was designed using the software tool FASTPCR (http://primerdigital.com/fastpcr.html) [Forward primer (5'-AAATTGGGTCACCTCCTCCT-3') and reverse primer (5'-TTCGGAAGATGAGCAGGAAT-3')]

PCR amplification: Amplification was carried out in a 20 µl reaction volume which contained 2 µl of 10X Buffer, 0.8 µl dNTPs (0.25 mM each), 3 µl primer (2µM of each forward and reverse primer), 0.3 µl of 3U Taq DNA polymerase and 3 µl DNA. The PCR cycling condition consisting of an initial denaturation (2 min at 94ºC) was followed at 35 cycles of denaturation (30 sec at 94ºC), annealing (1 min at 50 ºC) and extension (1 min at 72 ºC), with a final extension (10 min at 72 ºC). After amplification, the PCR products were separated on 1.2% agarose gel in 1X TBE buffer by electrophoresis stained with ethidium bromide. The gel visualized in a gel documentation system.

Cloning and sequencing: The PCR products were purified using NucleoSpin Gel and PCR Clean-up kit. The purified PCR fragments were ligated into pTZ57R/T PCR cloning vector. The recombinant plasmids were transformed into *Escherichia coli* strain (*DH5α*). The positive clones confirmed by colony PCR were sequenced. Each sample was subjected to three sets of sequencing reactions using the forward and reverse primers for high accuracy and to eliminate sequencing errors.

Sequence analysis: Both forward and reverse raw nucleotide sequence of *COX1* obtained after sequencing were trimmed to remove the vector contamination using VECSCREEN (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). These sequences were then assembled in BIOEDIT (bioedit.software.informer.com/7.1/) sequence assembler. Each nucleotide sequence obtained was compared with its related species using BLASTn tool (http://blast.ncbi.nlm.nih.gov/). The final 625 bp *COX1* sequences all the individuals were aligned by Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

The phylogenetic analysis was carried out based on the maximum likelihood (ML) method and the sequence divergence among the nucleotide sequence of *O. arenosella* population estimated by computing the rate of nonsynonymous versus synonymous substitutions (Ka/Ks) were performed using MEGA6 software (www.megasoftware.net/mega.php). A median joining network was constructed to illustrate relationships among haplotypes using the program Network *ver*. 4.6.1.2 (http://www.fluxusengineering.com/sharenet.html). Further analyses of nucleotide sequences were carried out using the software DnaSP ver 5.10 (http://www.ub.edu/dnasp).

Results and Discussion

Fragments of sizes between 600-700 bp of were obtained from the amplification of the 18 larval samples using the designed primers to amplify the *COX1* gene. BLAST results of these sequences confirmed the sequence to be *COXI*. The sequences were deposited in GenBank DNA database (accession numbers KP995715 - KP995732). The average base composition of these 18 sequences was T=30.0 %, C=15.2 %, A=38.6 %, and G=16.2 %. Median joining analysis revealed haplotype network of *COXI* in which more frequent haplotype was surrounded by less frequent haplotype (fig. 1). A total of 11 haplotypes could be observed among 18 individuals of five populations. Out of the 18 individuals, nine individuals shared a single haplotype. Two haplotypes were shared by more than two individuals. Among these, one haplotype was shared by four individual's viz., Visakhapatnam 1, Visakhapatnam 2, East Godavari2 and West Godavari 1. Similarly another single haplotype could be observed for five individuals (Srikakulam 1, Srikakulam 3, Visakhapatnam 3, West Godavari 4 and Vizianagaram 2).

The sequence alignment data of 18 individuals analyzed using DnaSP[26] software showed that out of 625 sites, 14 sites (2.24 %) were variable (polymorphic) without any insertion or deletion and remaining 611 sites were invariable (monomorphic). Among 14 variable sites, 10 sites were singleton variable and four sites were parsimony

informative site. Total number of mutation, Eta was found to be 14. Pairwise genetic distance (Table 2) among the 18 individuals ranged from 0.00-0.0113 for *COXI* gene. Analysis of genetic distance, based on nucleotide differences showed slight genetic difference.

Diversity estimates were analyzed for individuals collected from different districts. All the individual sequences showed high haplotype (gene) diversity and low nucleotide diversity. East Godavari, West Godavari and Vizianagaram were the most variable populations with all the individuals from these districts possessing distinct haplotype (Hd = 1.00) but not all the haplotype were private. The number of haplotypes varied from two to four. The haplotype diversity was high in all the populations ranging from 0.667- 1.00 whereas nucleotide diversity was comparatively low for the populations studied (Table 3).

By analyzing all the populations the haplotype diversity, Hd, was found to be 0.895 and variance of haplotype diversity was found to be 0.00297. Nucleotide diversity, Pi, for the 18 *COXI* sequences, was found to be 0.0046. These differences suggested that all individual showed high level of genetic diversity. Tajima's *D* values (-1.06799) and the Fu's *Fs* values (-4.614) were negative. These patterns of diversity are indicative of recent population growth and expansion.

Phylogenetic analysis based on maximum likelihood tree (fig. 2) showed that *O. arenosella* collected from different districts formed two separate clusters. *O. arenosella* individuals collected from East Godavari district were aligned in a first cluster; similarly those from Srikakulam districts were aligned in a second cluster separately. While samples collected from other three districts, *viz*., Visakhapatnam, West Godavari and Vizianagaram were not aligned in a single clade separately indicating that overlap exists among these samples with respect to *COXI* nucleotide sequences.

To examine the evolutionary rates among *O. arenosella*, pair wise divergence at nonsynonymous and synonymous sites were calculated across each population based on Nei-Gojobori method[20]. The ratio of nonsynonymous (Ka) to synonymous nucleotide substitutions (Ks) was calculated for all populations. When Ka exceeds Ks (Ka/Ks ratio >1), it indicates a positive or diversifying selection, implying the increase of advantageous mutations during the course of evolution. Whereas, a Ka/Ks ratio <1 (Ka is less than Ks) indicates a purifying or negative selection, implying the elimination of most of the nonsynonymous substitutions or the amino acid changes is reduced[11, 19]. In this study the Ka/Ks ratio were generally higher in Vizianagaram and Visakhapatnam population due to decreased synonymous divergence compared to other population. This value, which is greater than one, suggests positive selection. West Godavari population shows negative selection because the Ka/Ks value was less than one. In order to make the comparison fair, we eliminated populations, when any non-applicable or infinity values was observed in Ks (Table 4).
DNA based tool such as mtDNA markers are very useful for phylogenetic analysis, diversity studies and also taxonomic identification. Mitochondrial *COX1* is a highly conserved electron transport protein coded by multiple genes containing regions that evolved at different rates [15]. *COXI* gene has been successfully applied in diversity studies of many lepidopteron species [22]. In the present study, we have utilized *COXI* gene for determining the genetic diversity of *O. arenosella* collected from different districts of Andhra Pradesh viz. Srikakulam, Visakhapatnam, East Godavari, West Godavari and Vizianagaram. Phylogenetic analysis based on the maximum likelihood (ML) method revealed that the samples from Srikakulam and East Godavari showed low degree of genetic variation whereas the samples from Visakhapatnam, West Godavari and Vizianagaram displayed significantly high genetic variation.

The diversity of *O. arenosella* collected from different regions may be because of their movement between these locations due to environmental influences, mainly winds. This movement of individuals to one location would contribute to the increase in population and attaining genetic similarity among them. Tajima's *D* test[28] is based on the allele frequency distribution of segregating nucleotide sites. A positive value indicates a bias towards intermediate frequency alleles, while a negative value indicates a bias towards rare alleles, so the negative value is a mark of recent population expansion. Similarly, Fu's *FS* test[8] is based on the distribution of alleles or haplotypes and in this case also, negative values can indicate recent population growth. In this study high haplotype diversity, low nucleotide diversity and significant negative Tajima's *D* and Fu's *Fs* values across the population supports the recent rapid population growth and expansion. *Fs* value compares the number of polymorphic site to the total number of nucleotide differences to detect the population growth.

In conclusion, this study exposes the high genetic diversity in the *O. arenosella* from Andhra Pradesh, which may be explained by the dispersal of insects between localities. The result of this study also confirms that Mt DNA markers are highly informative and effective tool for estimating the genetic diversity among *O. arenosella* populations.

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Table 1
Number of individuals of *O. arenosella* collected from different districts of Andhra Pradesh

S. N.	Districts	No. of individuals collected
1.	Srikakulam (SRL)	3
2.	Visakhapatnam (VSP)	5
3.	East Godavari (E.GD)	2
4.	West Godavari (W.GD)	4
5.	Vizianagaram (VZN)	4

Table 2
Pair wise genetic distance of 18 individuals of *O. arenosella*

	SRL1	SRL2	SRL3	VSP1	VSP2	VSP3	VSP4	VSP5	E.GD1	E.GD2	W.GD1	W.GD2	W.GD3	W.GD4	VZN1	VZN2	VZN3	VZN4
SRL1																		
SRL2	0.0016																	
SRL3	0.0000	0.0016																
VSP1	0.0048	0.0064	0.0048															
VSP2	0.0048	0.0064	0.0048	0.0000														
VSP3	0.0000	0.0016	0.0000	0.0048	0.0048													
VSP4	0.0064	0.0081	0.0064	0.0016	0.0016	0.0064												
VSP5	0.0016	0.0032	0.0016	0.0064	0.0064	0.0016	0.0081											
E.GD1	0.0064	0.0080	0.0064	0.0016	0.0016	0.0064	0.0032	0.0080										
E.GD2	0.0048	0.0064	0.0048	0.0000	0.0000	0.0048	0.0016	0.0064	0.0016									
W.GD1	0.0048	0.0064	0.0048	0.0000	0.0000	0.0048	0.0016	0.0064	0.0016	0.0000								
W.GD2	0.0064	0.0080	0.0064	0.0016	0.0016	0.0064	0.0032	0.0080	0.0032	0.0016	0.0016							
W.GD3	0.0097	0.0113	0.0097	0.0048	0.0048	0.0097	0.0064	0.0113	0.0064	0.0048	0.0048	0.0064						
W.GD4	0.0000	0.0016	0.0000	0.0048	0.0048	0.0000	0.0064	0.0016	0.0064	0.0048	0.0048	0.0064	0.0097					
VZN1	0.0064	0.0081	0.0064	0.0016	0.0016	0.0064	0.0032	0.0081	0.0032	0.0016	0.0016	0.0032	0.0032	0.0064				
VZN2	0.0000	0.0016	0.0000	0.0048	0.0048	0.0000	0.0064	0.0016	0.0064	0.0048	0.0048	0.0064	0.0097	0.0000	0.0064			
VZN3	0.0097	0.0113	0.0097	0.0048	0.0048	0.0097	0.0064	0.0113	0.0064	0.0048	0.0048	0.0064	0.0064	0.0097	0.0032	0.0097		
VZN4	0.0064	0.0081	0.0064	0.0016	0.0016	0.0064	0.0032	0.0081	0.0032	0.0016	0.0016	0.0032	0.0064	0.0064	0.0032	0.0064	0.0064	

Table 3
Summary statistics for *COX1* sequences of *O. arenosella*

Population	Polymorphic sites, S	No. of Haplotypes, h	Haplotype diversity, Hd	Nucleotide diversity, Pi	Tajima's D value	Fu's Fs value
Srikakulam	1	2	0.667	0.00107	_	_
Visakhapatnam	5	4	0.900	0.00416	0.56199	-0.567
East Godavari	1	2	1.00	0.00160	_	_
West Godavari	7	4	1.00	0.00560	-0.81734	-1.012
Vizianagaram	7	4	1.00	0.00587	-0.38921	-0.946

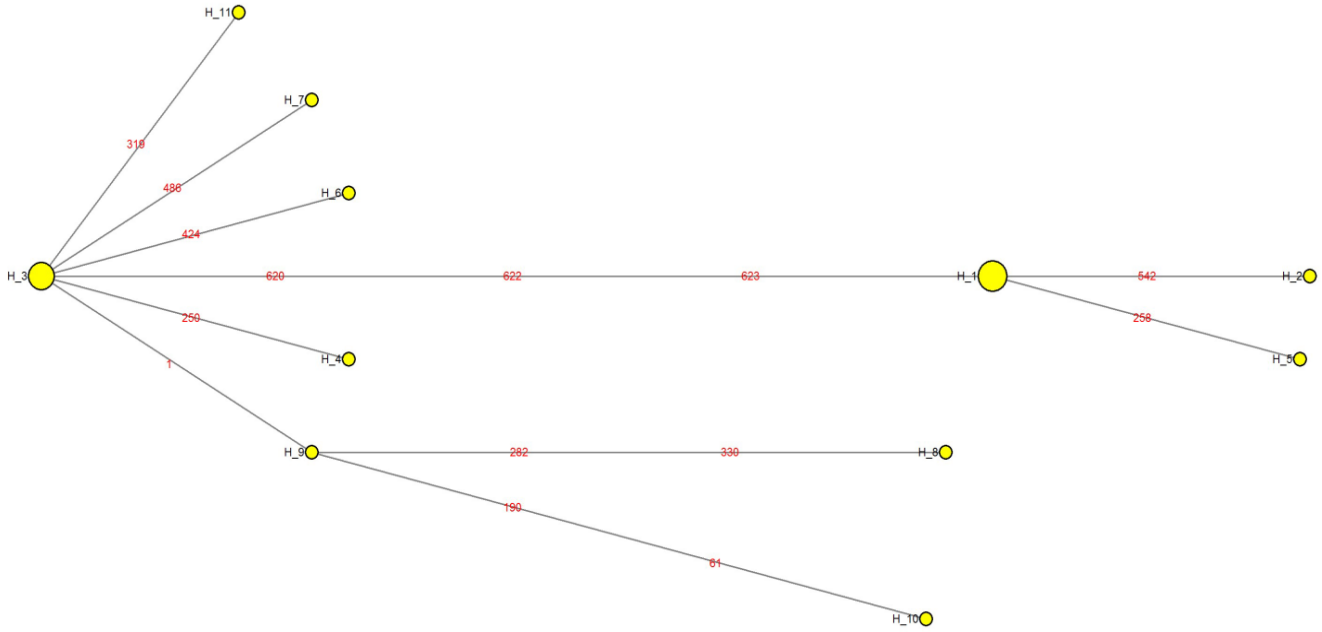

Figure 1: Most Parsimonious median joining (MJ) network for *O. arenosella COX1* haplotypes. Red marking region represent variable sites. These mutations present along the lines connecting the each haplotype (single nucleotide change).

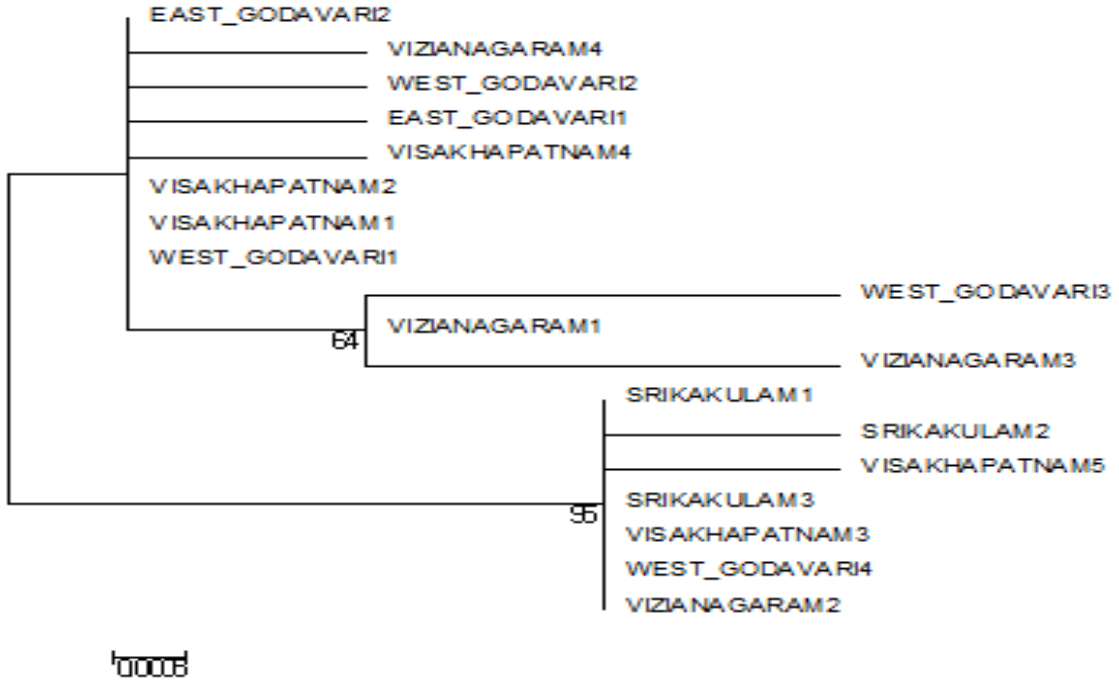

Figure 2: Maximum likelihood tree using 1000 bootstrap replicate

Table 4
Summary of non-synonymous (Ka) and synonymous (Ks) value and their ratio of different *O.arenosella*

Population	Ka	Ks	Ka/Ks
Srikakulam	0.0015	0	∞
Visakhapatnam	0.0040	0.0033	1.21
East Godavari	0.0022	0	∞
West Godavari	0.0034	0.0083	0.41
Vizianagaram	0.0056	0.0041	1.37

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