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Genetic barcoding and phylogenetic analysis of dusky cotton bug (*Oxycarenus hyalinipennis*) using mitochondrial cytochrome *c* oxidase I gene

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Abstract: Cotton dusky bug (*Oxycarenus spp.*) mostly attack on cash crops such as *Gossypium*, *Cola* and *Hibiscus* which affect the national economy therefore sustainable pest management is needed. Cytochrome c oxidase I (COI) gene is utilized as marker gene for DNA barcoding, genetic and ecological study of insects. In present study insect (cotton dusky bug) samples were collected from cotton fields in Faisalabad. COI gene was amplified from genomic DNA of bug and cloned into pTZ57R/T vector (Fermentas). The clone was sent to Macrogen (South Korea) for Sanger sequencing. The phylogenetic analysis and pairwise multiple sequence alignment showed that our cotton dusky bug grouped with two species of *Oxycarenus* genus and highest sequence identity was 91.1% with *Oxycarenus hylinipennis*. This is the first report of genetic barcode of *Oxycarenus hylinipennis* from cotton from Pakistan.

Key words: Cotton dusky bug; COI; DNA barcoding; Phylogenetic analysis; *Oxycarenus hyalinipennis.*

Introduction

Oxycarenus hyalinipennis also known as dusky cotton bug is a plant bug species belonging to Lygaeidae (1). It is important polyphagous insect which can attack mostly on plants belongs to family Malvaceae. Malvaceae has an importance due to the presence of cash and economic species such as *Gossypium*, *Cola* and *Hibiscus* etc. (1,2). Cotton can play an important role by contributing 1% GDP and 5.1% contribution to the national economy in agriculture sector (3). *O.hyalinipennis's* adults and nymphs feed on leaves and seed of plants and suck oil contents from seed and reduce their weight by 15%. Seed become useless as it does not have the ability to grow (4) so the quality and yield of cotton also fall down. It has been reported first in Africa and spread towards Caribbean Basin and the Pakistan (5, 6,7). Cultivation of Bt. cotton on large scale has reduced the problem of chewing insects and reduced the numbers of sprays. On the other hand, due to fewer sprays, some minor pests are becoming major pests. In Pakistan, dusky cotton bug problem is increasing and going to get the status of the major pest. For any successful integrated pest management program, it is imperative to identify the pest. The accuracy of delimiting species is fundamental in identification and discovery of species. Over a decade ago, DNA barcoding was proposed as a fast, cost-effective and simple taxonomic method based on the use of a unique, short and standardized gene region for the specimen identification and expediting discovery of putative new species (8). DNA barcoding is elusive to many taxonomists. As barcoding numbers on commercial products, DNA barcoding tries to attach a variety of sample with a portion of its DNA, mostly mitochondrial gene 'Cytochrome Oxidase I' which is also known as COI gene (9). Fragment size of COI has been shown to provide high resolution to identify cryptic species, thereby increasing taxonomy-based biodiversity estimates (10) and its usefulness has been confirmed for orders Coleoptera (11), Diptera (12), Ephemeroptera (13), Hemiptera (14), Hymenoptera (15) and Lepidoptera (16). Other molecular marker like RAPD has also been used to study genetic diversity like in order Neuroptra (17, 18). A crucial premise of DNA barcoding in animals is that genetic variation within species is lower than genetic variation among species (8, 18,19). In other words, there is an existence of 'barcoding gap' which allow unknown specimens to be identified as an existing species or flagged as a putative new species. The presence of global barcoding gap in birds, fish, butterflies (20,21,22) sometimes disregarding the importance of local barcoding gaps (23). The accuracy of species delimitation also depends on the completeness of DNA reference library, the geographic extent of sampling, the intensity of intra-specific sampling and divergence time among closely related species (24,25,26). DNA barcoding has proved to be particularly expedient in the study of taxonomically thought-provoking taxa, where morphology-based identifications are exasperated due to cryptic diversity (27) or phenotypic plasticity (28).

We have studied local bug which is one of the main pest attacking on cash crop such as cotton, *Hibiscus* etc. By interpreting their genetic marker and understanding their genetic relationships with other species, we might be able to devise a sustainable control strategy against pest attack.

Materials and Methods

Sample collection

The sample of insects had been collected from cotton crop in the region of district Faisalabad and samples were stored in -20^{0}C freezer.

DNA extraction from insect tissue

DNA extraction from insect tissues was done using already reported CTAB method(29). Briefly, insects were ground with the help of pestle mortar in liquid nitrogen. 700µl of CTAB buffer along with 2% β-marceptoethanol mixture was used and incubated on 65 ^{0}C to disrupt cell membrane. Then 700µl of chloroform and isoamyl alcohol mixture (24:1) was added and centrifuged to separate DNA in aqueous phase from other cell debris. DNA was then precipitated using isopropanol (0.6 volume of aqueous phase) and centrifuged to pellet down the DNA. The pelleted DNA was washed with 70% ethanol to remove the salts and the pellet was airdried.

COI gene amplification

For amplification of COI gene, primer pairs (CAACATTTATTTTGATTTTTTGG as forward primer, TCCAATGCACTAATCTGCCATA-TTA as reverse primer) was used (30). PCR protocol followed by initial denaturation temperature 95°C for 5 minutes, denaturation temperature 95°C for 1 minute, annealing temperature 45°C for 1munite and elongation/extension temperature 72°C for 1 minutes. Amplification was confirmed through gel electrophoresis.

Cloning of COI gene

The amplified product was ligated into pTZ57R/T plasmid vector (Fermentas/ThermoFisher Scientific, Massachusetts, USA) and cloned into *E. coli* top10 strain. Ligation mixture was transformed into competent cells of *E. coli* top10 strain by heat shock method. Clone of COI gene was confirmed by restriction analysis using EcoRI and BamHI restriction enzymes. Confirmed clone plasmids were purified by Plasmid purification kit (Fermentas/ThermoFisher Scientific,Massachusetts, USA) and were sent to Macrogen, South Korea for DNA sequencing through Sanger sequencing method using universal primers M13 F and M13 R.

Sequencing and phylogenetic tree

COI gene sequences received from Macrogen wereassembled and analyzed with the help of Lasergene package (DNASTAR, Madison, Wisconsin). Closely related sequences were found through online tool BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with threshold of greater than 80% of query coverage and identity. 37 Highest score sequences have been retrieved in FASTA format. Multiple sequence alignments were performed and phylogenetic trees were constructed by Clustal W using neighbor joining algorithm in MEGA6.0 software. Tree was displayed and manipulated using Tree view software. Pairwise distance analysis for evolutionary divergence between sequences were obtained usingMegAlign application (31).

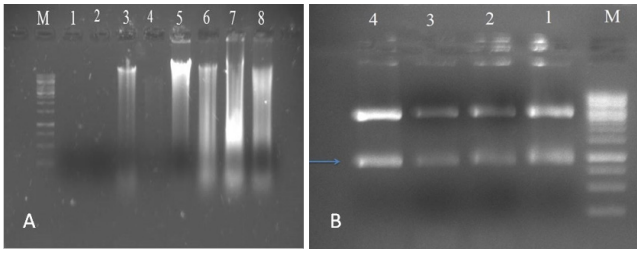

Figure 1. Gel electrophoresis showing genomic DNA isolation of cotton dusky bug (A) and clone confirmation of COI gene in pTZ57R/T vector (Fermentas, USA) using BamH1 and EcoR1 restriction enzymes (B).

Results and discussion

DNA extraction has been confirmed by the presence of band through gel electrophoresis (Figure 1A). PCR product of COI with primers has been confirmed by the presence of a band of size approximately 800 bp and amplified product was cloned and confirmation of clone was done by restriction digestion (Figure1B). The confirmed clone of COI gene was sequenced in its entirety in both orientations. The sequence was submitted into Genbank database (accession no KY213838).

Phylogenetic analysis of sequence showed the relation of dusky cotton bug with bugs while the relationship between our sequence and retrieved sequences from databases remain conserved from Kingdom Animaliato suborder Heteroptera. Divergence originated from infraorder Pentatomomorpha. The bug has most closely related to *Oxycarenus hyalinipennis* and *Oxycarenus lavaterae*. Template COI gene is showing close relationand grouped with members of genus Oxycarenus such as *Oxycarenus lavaterae* (Figure 2). The closely rela-

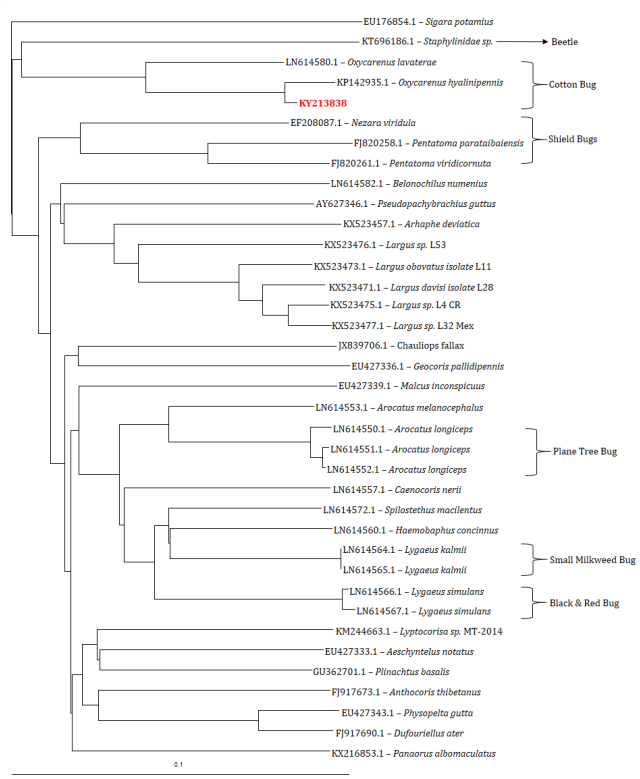

Figure 2. Phylogenetic tree was constructed in MEGA 6.0 software using neighbor-joining algorithm. The analysis shows relationship of COI gene sequence of dusky cotton bug (template) with that of other closely related insect species.

Table 1. Pairwise nucleotide sequence identities of COI gene of dusky cotton bug (KY213838; Font color red) with that other insect species obtained from database.

	AY627346	EF208087	EU176854	EU427333	EU427336	EU427339	EU427343	FJ820258	FJ820261	FJ917673	FJ917690	GU362701	JX839706	KM244663	KP142935	KT696186	KX216853	KX523457	KX523471	KX523473	KX523475	KX523476	KX523477	KY213838	LN614550	LN614551	LN614552	LN614553	LN614557	LN614560	LN614564	LN614565	LN614566	LN614567	LN614572	LN614580	LN614582
AY627346	***																																				
EF208087	80.8	***																																			
EU176854	79.9	75.7	***																																		
EU427333	82.2	84.9	76.1	***																																	
EU427336	81.2	84.9	76.1	83.5	***																																
EU427339	80.7	84.4	75.3	84.3	83.2	***																															
EU427343	81.7	83.6	75.3	85.1	82.6	83.9	***																														
FJ820258	84.3	82.5	79.2	78.3	78.3	78.5	78.3	***																													
FJ820261	83.3	82.8	78.5	79.3	78.5	79.9	78.5	92.1	***																												
FJ917673	81	84.6	77.2	83.6	84.3	84.9	84.6	80	79.9	***																											
FJ917690	81.7	83.8	75.1	85.1	83.8	83.5	95	79.2	79.7	87.5	***																										
GU362701	83.6	85.6	75	87.1	84.7	83.8	86.1	80.7	81.5	85.6	84.7	***																									
JX839706	79.9	83.2	74	84.4	84.3	84.3	83.1	77.6	78.6	84.6	82.4	84.9	***																								
KM244663	81.2	83.8	74.9	86.2	82.8	83.8	86.7	78.3	79	85	85.4	86.7	85	***																							
KP142935	79.2	73.3	80.8	75.4	74.7	72.4	74	77.5	77.2	75.4	75.3	74.9	72.9	74.2	***																						
KT696186	79.4	81.8	75	80.4	80.8	80.7	79.9	76	77.4	81.2	80.6	81.4	80.4	79.7	74.6	***																					
KX216853	81.5	84.4	74.7	84.3	84.2	84.4	83.9	78.1	78.8	84.9	84.4	84.9	83.6	85.3	73.6	80.6	***																				
KX523457	83.1	80	78.8	82.2	80	80.8	82.6	79	79.4	80.3	81.1	82.2	81.5	83.3	76.8	79.6	81.2	***																		
KX523471	85	81.1	77.6	84.3	80.7	83.1	83.2	80.8	81.8	82.5	83.1	83.3	81.9	83.3	75.8	79.9	81.7	86	***																	
KX523473	85.4	82.5	78.3	83.9	81.4	82.5	83.6	82.1	82.6	83.1	84.3	83.6	82.4	82.4	76.5	80.6	82.6	86.8	95.7	***																
KX523475	84.6	81.2	77.8	83.8	81.1	82.1	82.8	81.5	81.4	81.2	81.9	83.1	81.4	81.7	76.5	79.9	82.2	87.5	96	94.9	***															
KX523476	83.6	82.8	78.3	83.1	81.2	81.5	84.7	81.2	80.8	82.8	83.8	83.3	81.5	81.8	76.2	78.5	83.5	87.1	88.5	88.6	88.9	***														
KX523477	84.6	80.8	77.6	83.9	80.8	81.9	82.8	81	81	81.5	81.9	82.6	81.9	82.1	75.8	80	81.5	86.8	95.8	94.2	97.5	88.6	***													
KY213838	79.7	81.5	75.3	83.5	82.9	81.5	82.8	77.8	78.2	83.8	83.2	83.8	81.7	82.8	89.6	81.4	82.8	80.7	80.1	80.8	80.4	79.7	79.4	***												
LN614550	81.7	82.9	74.3	86.4	83.1	85.1	84	77.9	80	84.4	84.4	86.1	85	85	73.2	79.7	84.4	80.6	83.5	84.3	83.6	82.4	83.5	81.8	***											
LN614551	81.2	82.9	74.3	86.9	82.4	85	84	78.3	80	84.7	84.7	85.8	84.6	84.9	73.8	79.9	84.6	81.4	83.3	83.9	83.3	83.2	83.5	82.1	98.8	***										
LN614552	81.5	83.2	74.6	86.9	82.6	85.3	84.3	78.6	80.3	84.7	84.7	86.1	84.9	85.1	73.6	79.6	84.6	80.6	83.8	84.3	83.6	82.9	83.8	82.1	99	99.7	***									
LN614553	81.2	84	74.4	86	83.6	85.4	85.6	77.9	79	84	85	86.2	85.8	85	73.3	81.7	85.1	80.6	83.3	84	83.1	81.7	83.2	81.7	91	90.7	91	***								
LN614557	81.8	84.2	74.3	84.7	83.6	84.9	85.6	78.2	78.6	85.4	84.3	86.7	82.4	84.4	72.8	79.4	85.3	81.7	82.8	82.4	82.1	83.6	82.4	81	87.2	87.4	87.4	88.2	***							
LN614560	81.8	84.6	74.3	84.7	84.2	84.7	85.1	78.2	79	83.5	84.6	85.7	84.6	83.2	72.5	79.7	83.9	80.7	81.5	82.4	81.7	82.1	81.7	81.4	88.3	88.1	88.3	87.6	87.2	***						
LN614564	82.4	84.4	75.6	83.5	82.9	85.3	82.6	79.2	79	83.9	85	84.6	82.9	83.8	73.8	81.7	84.2	79.4	81	81.9	80.7	81.8	81.2	81.4	84.9	85	85	87.8	86	90	***					
LN614565	82.4	84.4	75.6	83.5	82.9	85.3	82.6	79.2	79	83.9	85	84.6	82.9	83.8	73.8	81.7	84.2	79.4	81	81.9	80.7	81.8	81.2	81.4	84.9	85	85	87.8	86	90	100	***				
LN614566	80.1	84.6	73.8	82.4	83.9	83.8	84	78.3	79	83.6	84	86	83.9	84.7	73.3	80.4	83.6	81	81.7	82.2	81.7	80.7	81.2	81.8	86	86	86	88.3	87.6	89.3	88.6	88.6	***			
LN614567	80	84.4	73.8	81.8	84.2	83.8	83.8	78.3	78.9	83.5	83.5	86	83.9	84.4	73.3	80	83.3	80.6	81.4	81.9	81.4	80.3	81	81.9	85.7	85.4	85.7	88.1	87.4	89.6	88.6	88.6	99.4	***		
LN614572	81.7	84.4	73.8	86.4	83.5	85.3	84.7	78.9	79	83.8	84.7	86.2	85.1	85.8	72.6	81.4	84.2	81	82.2	82.5	81.7	82.6	81.7	81.1	87.5	88.2	88.2	89	88.2	90.7	90.1	90.1	89.2	88.6	***	
LN614580	80.8	82.1	76.2	82.5	82.1	81.5	82.5	77.6	79.6	84	83.9	85.3	81	83.8	83.2	83.3	83.1	80.7	80.6	81	80.3	80.7	80.3	91.1	81.5	82.2	81.9	81.9	82.1	81.4	82.8	82.8	81.4	81.2	82.2	***	
LN614582	57.2	60.1	53.6	60.7	59.7	60.6	59.6	56.4	55.7	59.2	59.4	59.2	60.6	60	51.4	58.2	60.6	58.5	59.2	58.9	59.2	59.2	59	58.5	60.8	61.4	61.2	61.1	60.3	61.2	62.4	62.4	59.9	59.4	61.2	58.9	***
	AY627346	EF208087	EU176854	EU427333	EU427336	EU427339	EU427343	FJ820258	FJ820261	FJ917673	FJ917690	GU362701	JX839706	KM244663	KP142935	KT696186	KX216853	KX523457	KX523471	KX523473	KX523475	KX523476	KX523477	KY213838	LN614550	LN614551	LN614552	LN614553	LN614557	LN614560	LN614564	LN614565	LN614566	LN614567	LN614572	LN614580	LN614582

ted bugs are the members of family Pentatomidae like *Nezara virulada*, *Pentatoma parataiba iensis* which are commonly shield bugs. The bugs like plane tree bugs, small milkweed bugs and black & red bugs are grouped in separate clades on the phylogenetic tree (Figure). The members of other infraorder Cimicomorpha and Nepomorpha also belong same order they have different habitat and host range there they are present distantly in phylogenetic tree from our sample sequence. Close relation with bugs such as *Pseudo pachybrachius guttus, Sigara potamius, Pentatoma parataiba iensis, Anthocoristhibetanus, Plinachtus basalis, Lygaeus simulans, Arocatus longiceps* and *Lygaeus kalmii.* This relation showed that all these bugs share common ancestor.

Pairwise distance analysis (Table 1) shows that template is diligently similar to *Oxycarenus hyalinipennis* (89.6%) and then with *Plinachtus basalis* and *Anthocoristhibetanus* (83.8%)while least similarity with *Sigara potamius* (75.3%).

Many insects attack on crops which damage the crop production and affect the individual and national economy. There are fewer study reports on the genetic level identification of *O. hyalinipennis* which cause their morphological identification difficult. So, we propose a unique biological marker for identification of *O. hyalinipennis*. The phylogenetic and pairwise distance analysis shows their similarity with other species belongs to different orders, families and genus.

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