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Short Communication

Molecular Identification and Sequence Analysis of Dusky Cotton Bug, *Oxycarenus hyalinipennis* (Hemiptera:Lygaiedae) Infesting Cotton Field in Pakistan

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Authors' Contributions
JNA and SJNA presented the ideas and did experimentation. JNA collected insects from cotton fields and MJ extracted DNA for PCR. MWJ, SJNA and SM reviewed the literature. JNA, SJNA edited the manuscript.

Key words
***Oxycarenus hyalinipennis*, Molecular identification, PCR, Phylogeny.**

ABSTRACT

The identification of dusky cotton bug, *Oxycarenus hyalinipennis* (Hemiptera:Lygaeidae) is difficult because of its close resemblance to other bugs of genus Oxycarenus. Molecular identification and characterization of dusky cotton bug (DCB), *Oxycarenus hyalinipennis* (Oxycarenidae: Hemiptera) was done through Polymerase chain reaction and sequencing. In PCR, mitochondrial cytochrome oxidase I (COI) gene based primers were used for identification of this pest collected from various cotton fields in Punjab. Phylogenetic analysis was also complemented to differentiate DCB identified from other countries of the world. The PCR bands obtained in gel electrophoresis amplified PCR fragments from all DCB species at 710 bp. The sequencing results and phylogenetic analysis with sequences submitted at NCBI database revealed that DCB have 99-100% similarity with *Oxycarenus hyalinipennis* OH1 (JQ342987.1), *Oxycarenus hyalinipennis* OH2 (JQ342988.1) reported from USA. This is the first report of molecular identification and DNA barcoding of *Oxycarenus hyalinipennis* infesting cotton from Pakistan. DCB is becoming alarming pest, so, this study will be highly helpful to observe DCB correct identification for management.

Cotton, *Gossypium hirsutum* is the most important industrial cash crop of Pakistan. Dusky cotton bug (*Oxycarenus laetus* Kirby) (Hemiptera:Lygaeidae) is a pest of cotton lowering the market value of cotton by staining lint and other end products when the adults and nymphs are crushed at the time of ginning (Sweet, 2000). Both adults and nymph suck the cell sap gregariously from reproductive parts of plants and immature seeds and deteriorates the seed quality which remains light in weight (Vennila *et al*., 2007). Besides damaging the seeds and the reproductive parts it also deteriorates the lint quality resulting in poor ginning of cotton fibers (Awan, 2013). Dusky cotton bugs (*Oxycarenus* spp.) on cotton reduce seed weight 32%, oil content 6 %, and overall yield loss up to 6.8% (Sewify *et al*., 1993). Dusky cotton bug has various host plants and after the introduction of Bt in 2000 from Pakistan, it is becoming a threat to early and late cotton crop (Shah *et al*., 2016). The bugs transmit viruses and are known to cause severe damage to plants. There are 24 small genera and one large genus, *Oxycarenus* in the family of Oxycarenidae. The genus *Oxycarenus* contains about 55 species, of which at least 6 species are listed as pests, especially on cotton and hibiscus plants. The precise, timely and correct identification of bug species is important to monitor and control in fields. Morphological based identification is time consuming and problematic because of non-availability of authenticated specimens, old literature and requires an expert taxonomist. Similarly, immature stages and damaged specimen cannot be easily identified to a particular species (Tembe *et al*., 2009). Molecular identification is latest technique aiming at identification of new specimens by assessing their DNA sequence similarity. The amplification of mitochondrial mitochondrial cytochrome oxidase I (COI) gene fragment using universal primers of Folmer *et al*. (1994) is generally

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considered as a reliable, cost-effective and easy molecular identification tool for a wide applicability across metazoan taxa (Hebert *et al*., 2004, 2004a, 2005; Smith *et al*., 2008; Hajibabaei *et al*., 2006). In this study we analyzed, DNA barcoding (molecular determination) of DCB for molecular identification using mitochondrial COI gene primers.

Materials and methods

Hand picking of dusky cotton bug (DCB) was done during 2017-2018 in cotton at Post Graduate Agriculture Research Centre, Faisalabad, Department of Horticulture, University of Agriculture Faisalabad as well as AARI Faisalabad and CCRI Multan. Adult specimens of were collected from infested opened bolls of cotton. Before proceeding molecular identification, morphological study of the species was carried out under laboratory using light microscope. Some of the collected specimens were preserved in 96% alcohol or either stored at −20 °C in a freezer or prior to analysis.

For molecular study, total genomic DNA from whole body of insect or parts of adult body (legs) of DCB was extracted using CTAB method with slight modifications to increase DNA yield (Ahmad *et al*., 2017; 2018). Mitochondrial cytochrome oxidase I (mtCOI) based primers were used for PCR. PCR amplifications were performed in PCR machine (Peqstar, Germany) for primers (LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) conditions as described by Folmer *et al*. (1994). Simply, an initial denaturation at 95 °C for 5 min, 40 cycles at 94 °C for 40 sec, 47 °C for 40 sec, extension at 72 °C for 45 min and final extension at 72 °C for 15 min. The amplified PCR products were tested for the confirmation of genomic DNA presence using gel electrophoresis on 2.0% agarose gel. The required size of DNA fragment from fruit fly samples was estimated by comparing with 1 kb DNA ladder markers (GeneMark). The amplified band corresponding to the target PCR product was documented using SYNGENE Gel documentation system under UV light. The amplified PCR products (710 bp) were sequenced directly in both directions using services of M/S Macrogen (Korea) with primers (LCO-1490/HCO-2198) after purification with a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The obtained sequences were analyzed using Lasergene v. 7.1 software package (DNASTAR, USA) and were further aligned using CLUSTAL W method of Bio-Edit software. Comparison of obtained sequences with sequences available in GenBank was accomplished using BLAST (Basic Local Alignment Search Tool)service available at http://www.ncbi.nlm.nih.gov:80/BLAST. Pairwise alignment tree for our samples sequence query (dendrogram) was constructed with NCBI data base available. The studies were performed with software BLASTN 2.8.0+ employing a methodology for pairwise alignment for the construction of phylogenetic tree. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Results and discussion

The nucleotide sequencing of DNA fragments were analyzed and aligned through BLASTN with sequence data of DCB previously reported in NCBI site (Fig. 1). The reference accession numbers of sequences used for alignment were DCB (HQ908084.1, JQ342988.1), (KM022344.1, KM023038.1, KJ541660.1, HQ105989, KM021657.1, KR918399.1 and KX053389.1. In base pair sequence alignment, homology of DCB spp. was compared with previously reported NCBI database through BLAST option. Pairwise alignment of dendrogram tree indicated that our dusky cotton bug Seq (>180220-034-O01-8-DCB-HCO-2198.ab1) or Seq (>180220-034-O01-8-DCB-LCO-1490.ab1) share same cluster with *Oxycarenus*

>180220-034_O01_8-DCB_H_C_O_-_2_1_9_8.ab1 605 bp

TGATGTATTGAAATTTCGGTCTGTTAATAATATAGTAATGGCTCCAGCTAATACTGGTAATGATAATAA
TAATAATAATGCAGTAATTCCTACTGATCAAACAAAGAGGGGGATTCGTTCAAGGGTTATACCAGCTG
GTCGTATATTAATAATAGTTGAGATAAAGTTGATTGCTCCTAAAATTGATGATACACCTGCTAAATGTA
GGGAAAAAATTGCTAAATCTACAGATGCTCCTCTATGGAATAATCTATTAGATAAAGGTGGATAAACT
GTTCATCCTGTTCCTGCTCCTATTTCTACTAAGCTACTTGATAACAGGAGTGTTAATGATGGGGGTAAT
AGTCAGAATCTTATATTATTTATTCGGGGGAAGGCTATATCTGGGGCTCCAATTATTAATGGGACTAAT
CAATTTCCAAATCCTCCAATTATAATAGGTATAACTATAAAGAAAATTATAATAAATGCATGTGCTGT
GACTACAACATTATAAATTTGATCATCACCAATAAAAGACCCTGGTTGACCTAATTCAATACGGATAA
TTCATCTTAATGATGATCCAACTATACGGATCATATACCAAATAAAAAGT

Fig. 1. Nucleotide sequence of the PCR amplified mitochondrial cytochrome C oxidase subunit I (COI) of dusky cotton bug (*Oxycarenus hyalinipennis*).

hyalinipennis OH-1 (JQ342987.1), *Oxycarenus hyalinipennis* OH2 (JQ342988.1) isolates *and Oxycarenus laetus* (HQ908084.1) while other species (*Oxycarenus lavaterae* and *Oxycarenus modestus, Oxycarenus pallens*) of same genus have separate clusters (Figs. 2, 3). In homology sequence study, DCB showed 100% similarity with themselves and 100% and 99% similarity with NCBI GenBank database, *Oxycarenus hyalinipennis* OH-1 (JQ342987.1), *Oxycarenus hyalinipennis* OH2 (JQ342988.1), respectively with 97-99 % good query coverage while 99 % similarity was observed for *Oxycarenus laetus* (HQ908084.1). The pairwise alignment also showed 87 to 94 % homology with other species of same genus (Accession numbers: KM022344.1, KM023038.1, KJ541660.1, HQ105989, KM021657.1) and 86% similarity for other Hemiprerans (KR918399.1, KX053389.1) (Table I).

The pairwise alignment of nucleotides indicated a very high and significant match confirming our sequence to be a part of Cytochrome C Oxidase subunit1 family with good query coverage (Fig. 1). The amplified sequenced segment of COI was closely related to insect species from the Order Hemiptera by using BLAST search (Figs. 2, 3). The pairwise phylolenetic tree (Fig. 2) indicated that all the species belonging to *Oxycarenus* being clustered together with high query coverage score 97-99. As we expected, our entry *Oxycarenus hyalinipennis* (605bp) was found to be clustered with the same species of members of the genus *Oxycarenus* showing 99-100% sequence similarity with

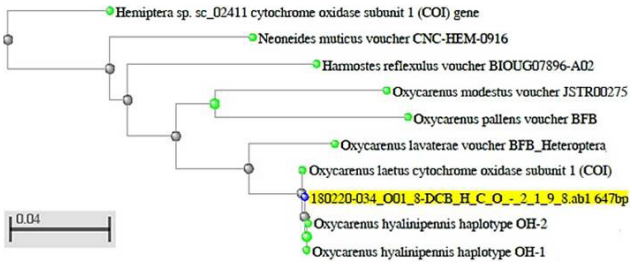

Fig. 2. Pairwise alignment of constructed tree based on cytochrome oxidase I (COI) gene sequence of dusky cotton bug (*Oxycarenus hyalinipennis*) from Pakistan.

Table I.- Comparison and identity of fragment of the cytochrome oxidase I (COI) gene of *Oxycarenus hyalinipennis* of major cotton growing districts of Punjab with that of referred gene bank available in NCBI.

Query	Subject	Max score	Total score	Query cover	E. value	Identity	Accessions
>180220-034_E01_3-FF-C_H_C_O_-_2_1_9_8.ab1	*Oxycarenus hyalinipennis* OH-1	942	942	97%	0.0	100%	JQ342987.1
	Oxycarenus hyalinipennis OH-2	937	937	97%	0.0	99%	JQ342988.1
	Oxycarenus laetus cytochrome oxidas	1067	1067	99%	0.0	99%	HQ908084.1
	Oxycarenus lavaterae BFB	928	928	100%	0.0	94%	KM023038.1
	Oxycarenus lavaterae BFB	893	893	95%	0.0	94%	KM022344.1
	Oxycarenus modestus JSTR00275	735	735	99%	0.0	87%	KJ541660.1
	Neoneides muticus voucher CNC-HEM-0916	726	726	99%	0.0	87%	HQ105989.1
	Oxycarenus pallens BFB_Heteroptera	717	717	99%	0.0	87%	KM021657.1
	Harmostes reflexulus BIOUG07896-A02	706	706	96%	0.0	87%	KR918399.1
	Hemiptera sp. sc-02411	695	695	98%	0.0	86%	KX053389.1

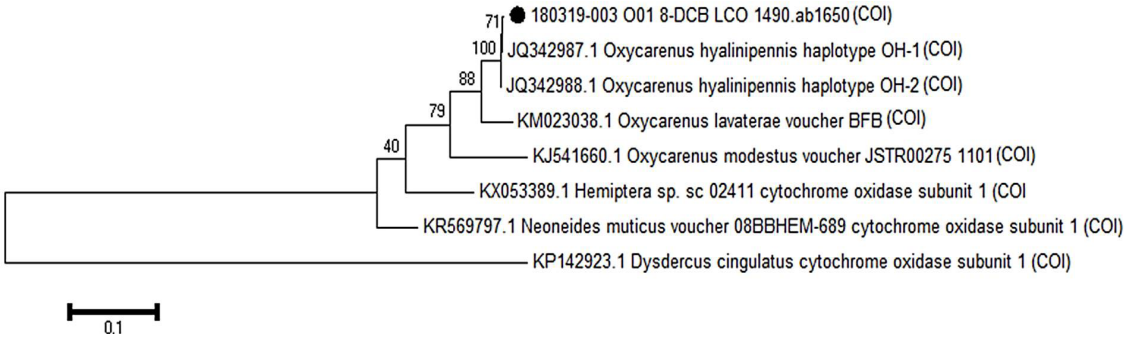

Fig. 3. Multiple alignment of constructed tree based on cytochrome oxidase I (COI) gene sequence of dusky cotton bug (*Oxycarenus hyalinipennis*) collected from Pakistan with high scored NCBI gene bank data available.

Oxycarenus hyalinipennis reported from USA (Nagoshi *et al*., 2012). *Oxycarenus laetus* has been reported and identified from India using (COI) based primers showing 99% similarity (Habeeb and Sanjayan, 2011). Currently morphological based identification methods for DCB complex are time consuming and technically difficult for closely related species. Further, for most of the adult and immature stages, diagnostic morphological characters are not completely suitable (White and Elson-Harris, 1992).

Conclusion

Our sequence will serve as an exclusive DNA barcode for this species. This is probably the first report of molecular identification of this insect from Pakistan.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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