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Short Communication

Mitochondrial gene cytochrome c oxidase I (CO1) used for molecular identification of *Bactrocera zonata* in Pakistan

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Abstract: *Bactrocera zonata* is fruit pest mostly attacked on peach and cause heavy destruction in production of peach fruits by sucking their juice. For their management, we start to detect them on basis of their molecular characterization. As mitochondrial genome encodes a gene COI used as biomarker for identification of eukaryotes including insects. In present study, we amplified COI gene and cloned into pTZ57R/T vector (Fermentas). Cloned gene was confirmed through restriction analysis and sequenced through its entirety on both strands from Macrogen (South Korea) by Sanger sequencing method. Different computational tools were utilized for comparative analysis of sequence with other related sequences retrieved from databases. Related species were identified through phylogenetic analysis using Mega 7 tool. Pairwise sequence alignment showed the sequence identity about 96% with *Bactrocera zonata*. By identifying the pests with more authentic molecular biomarker may help the research to control them more effectively in future.

Key words: Fruit fly; COI; DNA barcoding; Phylogenetic analysis; *Bactrocera zonata.*

Introduction

Bactrocera zonata was originated from South and South-East Asia which spread towards Arabian Gulf and in 1990, first reported in Egypt (Africa), in Iran and Lebanon (1) and also in Iraq (2). *B. zonata* causes heavy damage to fruits in Asia (3) and is a serious pest of peach (*Prunus persica*) . Females of *B. zonata* lays their eggs on the fruit while maggots demolish the pulp. Subsequently the secondary infection with bacterial and fungal disease are frequent and infested fruits drop down (4). Mitochondrial genomic studies show that their tRNAs have typically clover-leaf structure with an exception for trnS$^{(AGN)}$. Gene evolutionary rate analysis revealed that *cox1* and *atp6* exhibits lowest and highest gene substitution rates, respectively than other genes (5).

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. Identification of insect parasitoids is often difficult, and they are suspected to contain many cryptic species (6). It was Herbert with his team (7) who first time give idea of DNA barcoding by amplifying the 680bp region of cytochrome c oxidase I (CO1) gene using mouse mitochondrial genome as a reference. The two main ambitions of DNA barcoding are to (i) assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible morphology (7).

DNA barcodes could aid in the routine identification of insects in applied settings by enabling the recognition of morphologically cryptic species, by associating immature forms with adults (pest management), and by identifying eggs (phytosanitary applications) and fragmentary remains (food quality, ecological analyses) (8). Barcoding not only discriminates among host-specific morphospecies, but it also raises the species count by revealing that each of the generalist species are arrays of highly host-specific cryptic species(6). Memon et al. (9) confirmed the usefulness of variation in CO1 sequences in circumscribing a new hemipteran species, but found broad overlap in intraspecific and interspecific distances among sequences of 373 species of Hemiptera downloaded from databases. However, most of the latter data derive from studies specifically directed towards elucidating relationships within taxonomically problematic groups. Thus, the available data are biased towards situations in which recent speciation reduces the observed level of inter-species sequence divergence, and may underestimate the utility of DNA barcoding as an identification tool among Heteroptera in genera.

Our group previously identified phylogenetic relationship of cotton dusky bug with other insects (10). With aiming to identifying more insects that attack on crops and lower their productivity, we have studied peach fruit fly on molecular basis to understand their

genetic marker and relationships with other species, so might be able to devise a sustainable control strategy against attack.

Materials and Methods

Sample collection and genomic extraction

Samples of peach fruit fly (PFF) had been collected from fields of Ayyub Agriculture Research Institute Faisalabad, Pakistan Genomic DNA was extracted from samples by Cetyl trimethyl ammonium bromide (CTAB) method as already reported in (10). For a short time, insects were placed in nitrogen and then grinded with pestle mortar to make fine powder. 700µl of solution containing CTAB buffer and 2% β-marceptoethanol put into powder, proceeding to incubating on 65°C for proper disruption of cell membrane. At that moment, 700µl of chloroform and isoamyl alcohol mixture (24:1) was added and centrifuged to distinct DNA in aqueous phase from extra cell debris. Then isopropanol (0.6: 1 of isopropanol with aqueous phase) was used to precipitate DNA followed by centrifugation to pellet down of genome. Then pellet was washed with 70% ethanol twice to purify DNA from salts and air dried. The purification DNA was analyzed using nanodrop.

Amplification of barcoding signature

CO1 gene was amplified by using forward primer (C1J2195: 5'-TTGATTYTTTGGTCATCCAGAAGT-3') and reverse primer (TL2N3014: 5'-TCCAATGCACTAATCTGCCATATTA-3') (11). For reaction, PCR protocol followed by initial denaturation temperature 95°C for 5 minutes, denaturation temperature 95°C for 1 minute, annealing temperature 45°C for 1 minute and elongation/extension temperature 72°C for 1 minutes. Gel electrophoresis was used for confirmation of COI gene amplification.

Cloning of CO1 gene

Amplified product was ligated into pTZ57R/T plasmid vector (Fermentas/ThemoFisher Scientific, Massachusetts, USA) and then ligation product was transformed into *E.coli* top10 strain by heat shock method.

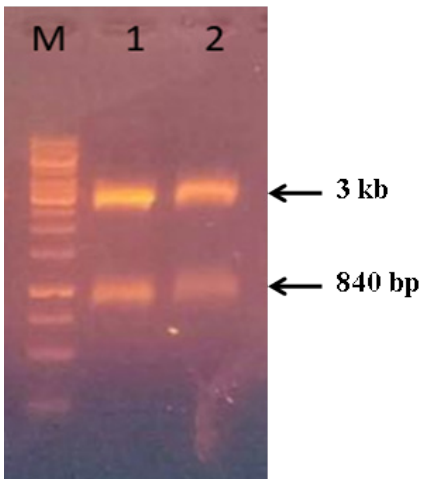

Figure 1. Clone confirmation by restricting the plasmid (pTZ57R/T vector+COI gene) using BamH1 and EcoR1. Two samples (1,2) run at the same time along with 1kb DNA ladder (Fermentas)(M). Upper band confirm the presence of pTZ57R/T plasmid vector of 3 kb size while lower band was COI gene of 840bp length.

Clones were confirmed by restriction analysis using *EcoR1* and *BamH1* restriction enzymes (Figure 1). 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

Lasergene package (DNASTAR, Madison, Wisconsin) was used to analyze the sequencing data. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find out closely related sequences from databases. Closely related sequences retrieved from databases in FASTA format; MEGA7.0 software was then used for multiple sequence alignment and phylogenetic analysis while tree display and manipulation was performed through Tree View Software. MegAlign application used to pairwise distance analysis for evolutionary divergence between sequences (12).

Results and Discussion

Agarose gel electrophoresis was used to confirm PCR amplification of 840 base pairs fragment. When fragment ligate with vector, it was transformed into *E.coli* which was confirmed by restriction analysis (*BamH1* and *EcoR1* enzymes used) (Figure 1). Two bands had been obtained: one for vector and second for COI gene. Then clone of COI gene was sequenced through its entirety in both directions. The sequence was submitted into GenBank database (accession no: MG770092).

Closely related sequences of COI genes were downloaded from databases in FASTA format and used in MEGA 7.0 software package to analyze phylogenetic analysis by neighbor-joining algorithm. The tree was constructed with default values while bootstrap value for this tree was 1000 times replicates. Results in Figure 2 showed that sample is closely grouped together with

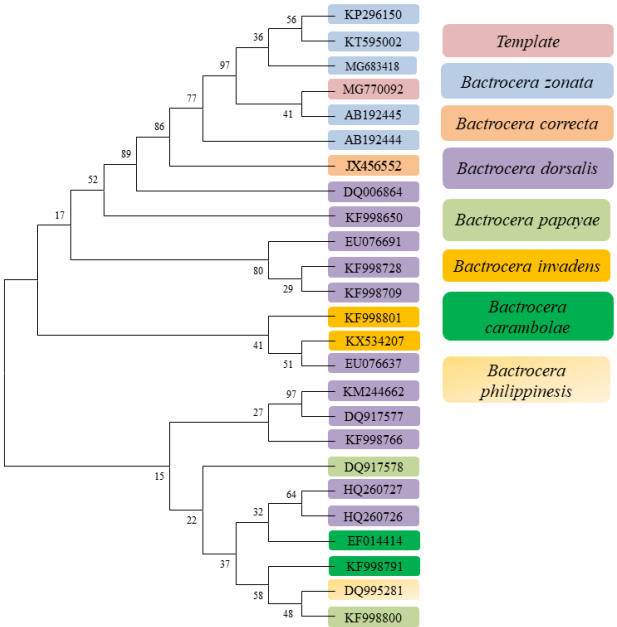

Figure 2. Phylogenetic tree was constructed in MEGA 7.0 software using neighbor-joining algorithm. The analysis shows relationship of COI gene sequence of peach fruit fly (template) with that of other closely related insect species.

Table 1. Pairwise nucleotide sequence identities of COI gene of Peach fruit fly (Template MG 770092) with different species of genus Bacterocera obtained from database.

	AB192444	AB192445	DQ006864	DQ917577	DQ917578	DQ995281	EF014414	EU076637	EU076691	HQ260726	HQ260727	JX456552	KF998650	KF998709	KF998728	KF998766	KF998791	KF998800	KF998801	KM244662	KP296150	KT595002	KX534207	MG683418	MG770092
AB192444	***																								
AB192445	95.6	***																							
DQ006864	82.5	83.7	***																						
DQ917577	82.2	82.5	92.6	***																					
DQ917578	82.3	82.6	92.8	99.2	***																				
DQ995281	11	2.8	5.8	4.6	4.6	***																			
EF014414	81.9	81.9	92.2	98.6	98.8	5.1	***																		
EU076637	82.2	82.5	92.7	99.1	99.4	4.5	98.7	***																	
EU076691	83.5	83.9	92.6	98	98.5	4.5	97.6	98.5	***																
HQ260726	82.2	82.5	92.7	98.6	99.1	4.5	98.7	98.7	97.8	***															
HQ260727	89.8	89.5	86.3	91.2	91.5	4.5	91.3	91.4	92.4	92.1	***														
JX456552	83.7	83.4	90.1	92.3	92.6	6.5	91.8	92.2	91.3	92.1	84.9	***													
KF998650	82.7	83	93.2	99	99.5	4.5	98.8	99.1	98.2	98.8	91.2	92.6	***												
KF998709	82.3	82.8	93.1	99	99.5	4.5	98.6	99.4	98.7	98.8	91.3	92.3	99.2	***											
KF998728	82.3	82.8	93.1	99	99.5	4.5	98.6	99.4	98.7	98.8	91.3	92.3	99.2	100	***										
KF998766	82.3	82.6	92.7	99.1	99.4	4.5	98.5	99	98.1	98.7	91	91.9	99.1	99.1	99.1	***									
KF998791	82.3	82.6	93	99.1	99.6	2.7	98.7	99.2	98.3	99	91.3	92.2	99.4	99.4	99.4	99.2	***								
KF998800	82.5	82.7	92.8	99	99.5	2.7	98.6	99.1	98.2	98.8	91.2	92.3	99.2	99.2	99.2	99.1	99.9	***							
KF998801	82.5	82.7	92.8	99.2	99.2	4.5	98.8	99.6	98.2	98.6	91.2	92.3	99.2	99.2	99.2	99.1	99.1	99	***						
KM244662	82.2	82.5	92.6	100	99.2	4.5	98.6	99.1	98	98.6	91.2	92.3	99	99	99	99.1	99.1	99	99.2	***					
KP296150	60.3	63	68.4	68.8	68.9	2.8	68.2	68.8	68.1	68.9	61.3	68.8	69.1	69.1	69.1	69	69	69.1	68.8	68.8	***				
KT595002	88	90.9	90.7	90.3	90.3	2.8	89.5	90.3	91.7	90.3	87.1	90.8	90.5	90.7	90.7	90.4	90.4	90.5	90.3	90.3	71.3	***			
KX534207	82.2	82.5	92.8	99.2	99.2	4.5	98.6	99.6	98.2	98.6	91.2	92.6	99	99.2	99.2	98.8	99.1	99	99.5	99.2	68.8	90.3	***		
MG683418	92.1	95.6	86.4	85.7	85.7	2.8	84.9	85.7	87.1	85.7	91.4	86.6	85.9	86	86	85.5	85.8	85.9	85.7	85.7	66.2	94.2	85.7	***	
MG770092	85.4	88.7	92.1	92.1	92.1	2.8	91.5	91.9	91.3	92.1	84.8	92.6	92.6	92.4	92.4	92.4	92.2	92.3	92.3	92.1	72.6	96.7	92.1	91.3	***

different isolates of *Bactrocera zonata* with accessing no AB192445 which was sampled from Cairo, Egypt, another *B. zonata* belongs to India (KP296150), Chiang Mai (KT595002) and Pachong (AB192444) Thailand.

Inter species distance shows the similarity from template and between species. The result obtained by MegAlign shows that template (MG770092) has highest sequence identity value with *Bactrocera zonata* (KT595002) with value of 96.7%. While *Bactrocera dorsalis* (KF998650) and *Bactrocera correcta* showed 92.6% similarity, the 2nd highest among the sequences (Table 1).

Genetic identification of local insect's populations which attacks on crops which are economically important and cash crops so would indirectly also boost and help the control strategy of these insect pests by the farmers. Genetic basis studies of insects are limited and mostly farmer use chemicals extensively for insect control which also pollute land and cause economic loss to the farmers. Sustainable control measures of well identified insect pest will directly enhance the productivity of crops. This authentic identification of insect pest will ultimately boost the economy of agriculture sector and economy of the country. Concluding the finding of this study is that authentic identification of insect pests by DNA barcoding or on genetic basis, will help in targeting the insect pests with more specified strategy which directly help to enhance the production of crops.

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