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Genetic Analysis of *Bactrocera dorsalis* (Diptera; Tephritidae) Populations Collected from Fruit Growing Areas in Western Tamil Nadu, India

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ABSTRACT

Fruit flies under Tephritidae are one of the diverse and agriculturally important insects. It needs reliable taxonomic procedures to resolve species complexes and genetic structure analysis to effectively control this pest. Little attention has been given to the analysis of genetic diversity in South Indian populations. By using mitochondrial cytochrome oxidase I and II (*cox1* and *cox2*), the genetic analysis of *B. dorsalis* from eight locations of major fruit growing areas in western parts of Tamil Nadu were investigated. Genetic diversity indices such as a number of haplotypes (H), haplotype diversity (Hd), nucleotide diversity (π), and the average number of nucleotide differences between sequences (k) of the populations revealed that *Bactrocera dorsalis* has a high level of genetic diversity with distinct genetic structure. Future studies should concentrate on extensive sampling from varied geographical locations of south India along with complete species delimitation, and also microsatellite marker analysis which would enable us to understand the complete genetic and population structure of this pest. This information will be useful to devise an area-wide management programme for this destructive pest.

Keywords

Bactrocera dorsalis, *cox1*, *cox2,* Genetic diversity, Western Tamil Nadu

Article Info

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Introduction

Fruit flies under Tephritidae, are one of the most species-rich families in Diptera with distinguishing body colour and wing patterns (Aluja and Norrbom 1999, White and Elson-Harris 1992). But Dacini is one of the tribes in Tephritidae is having very few key observable features which lead to blurred species resolution for taxonomy and agricultural diagnostics. The tribe Dacini comprises totally 10 percentpest species on commercial fruits and vegetable crops (Vargas *et al.,* 2015). For instance, the *Bactrocera dorsalis* complex has five sibling fruit fly species; *B. dorsalis B. papayae* (Drew and Hancock), *B.philippinensis* (Drew and Hancock), *B. carambolae*(Drew and Hancock), *and B. invadens* (Drew) due to overlapping morphological characters except for minor differences such as in colour patterns (Drew and Hancock 1994), genital characters (Iwahashi 2001) including length of aedeagus (Drew *et al.,* 2008), and variations in wing

shape (Schutze *et al.,* 2012). But now, all the sibling fruit flies were synonymized as *B. dorsalis* (San Jose *et al.,* 2018). In addition, generic complex assignment of the Dacini tribe by molecular data resulted in four genera, including important pestiferous genera such as *Bactrocera, Zeugodacus, Dacus.* However, few scientists (Drew and Hancock, 2016) considered *Zeugodacus* as subgenus of the genus *Bactrocera.* Due to inconclusiveness in morphological and molecular studies, there is incongruence between both aspects which necessitates a reliable taxonomic starting point. As ecological studies that focus on the behavior of species, host selection, distributional patterns or pollination associations are hindered when species are inaccurately identified (San Jose *et al.,* 2018).

In India, fruit flies have been recognized among the ten most serious pests of agricultural crops causing annual monetary losses to the tune of rupees 7000 corers. Furthermore, as the larvae feed and develop from inside the fruit, there is an increasing threat of migration of fruit flies through the export of fruits and vegetables.

For instance, among the Dacini flies, *B. dorsalis* poses the most important quarantine pest status, because of its extreme invasiveness and high survivability to the newer ecological niches. Hence, the global horticultural industry including India is facing significant threat due to the detrimental effect of this pest. To address the impact of important invasive fruit flies, the International Plant Protection Convention (IPPC) has created diverse International Standards for Phytosanitary Measures (ISPM). The standards cover all aspects related to invasive risk assessments, monitoring, management, and control (Jiang *et al.,* 2018).

Therefore to devise an effective pest management strategies, for instance, Area-Wide Integrated Pest Management (AWIPM), Sterile Insect Technique (SIT) programme and timely application of quarantine treatments will rely upon the key issues such as genetic diversity, genetic and population structure and gene flow of the concerned species (Qin *et al.,* 2016, Choudhary *et al.,* 2016, Jiang *et al.,* 2018).

An informative and neutral molecular marker can be obtained from invertebrate Mitochondrial DNA (mtDNA) which may be useful in phylogeographic structure analysis (Wan *et al.,* 2011)and for determining intra and inter-specific relationships.

Since, it has features such as high copy numbers, haploid mode of inheritance with no recombination, lack of introns makes molecular evolution as constant with moderate rate, and a lower effective population size (Mun *et al.,* 2003; Nardi *et al.,* 2005; Prabhakar *et al.,* 2013; Prabhakar *et al.,* 2012; Wan *et al.,* 2012; Simon *et al.,* 1994; Roderick 1996). Hence, we analyzed two mitochondrial genes (*cox1* and *cox2*) of adult fruit flies species of *B. dorsalis,* collected from the Western agroclimatic zone of Tamil Nadu to infer about the genetic diversity of this mango fruitfly.

Materials and Methods

Field sampling

B. dorsalis adults were collected from various fruit growing regions in the Western agroclimatic zone of Tamil Nadu (*i.e.* Krishnagiri, Dharmapuri, Coimbatore districts) from 2017 to 2018. The collection sites encompass locations that varied in latitude and longitude as furnished in (Table 1). Fruit flies were trapped using methyl eugenol traps and preserved in 70 percent ethanol and further stored at -20˚C until DNA extraction.

DNA extraction, amplification, and genotyping

Genomic DNA was isolated from the legs of a single insect of *B. dorsalis* from each geographical locations by using the HotSHOT method (Montero- Pau *et al.,* 2008). Two buffers were utilized *viz.,* alkaline lysis buffer (pH 12) which contained 25mM NaOH, 0.2mM Na_2EDTA, and neutralizing solution contained 40mM Tris-HCl. Six legs of individual fruit fly sample were removed and homogenized with 100µl alkaline lysis buffer (pH 12) with micropestle and incubated at 95˚C in a hot water bath for 30 minutes.

After incubation, the samples were removed from the hot water bath and were allowed to cool at 4˚C in a refrigerator for 5-10 mins. Then, 100µl of the neutralizing solution was added to each tube, vortexed and spun to settle down the debris. The extracted DNA samples were stored at -20˚C.

The two fragments of mitochondrial gene (Cytochrome oxidase I and II) were amplified using the primer pairs *cox1*, LCO 1490 (GGTCAACAAATCATAAAGATATTGG) HCO 2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Vrijenhoek 1994) and Tephriditae specific *cox2* of the mitochondrial gene (Cytochrome oxidase II) using the primer pairsTephFdeg (GACAACATGAGCHGSHYTHGGBCT) and TephR (GCTCCACAAATTTCTGAACATTG) (Jiang *et al.,* 2018). Polymerase chain reactions (PCR) were carried out in 25µl of cocktail mixtures containing, 11.5µl of nuclease-free water, 2.5 µl of reaction buffer (10X with 25Mm $MgCl_2$), 2.5 µldNTPs, 1.5µl of forward primer, 1.5 µl of reverse primer, 1µl of 25Mm $MgCl_2$ and 1µl of DMSO (Dimethyl sulfoxide) (as PCR additives)and 5 of µl of template DNA. The PCR amplification of *cox1* was accomplished by initial denaturation for 2min at 94˚C, 35 cycles of denaturation at 94˚C for 1min, primer annealing for 1min at 52˚C and an extension for 1min at 72˚C and final extension step at 72˚C for 5 min and the storage and cooling temperature was held at 4˚C.

The PCR amplification of *cox2* was accomplished by initial denaturation for 3min at 94˚C, 35 cycles of denaturation at 94˚C for 15sec, primer annealing for 1min at 52˚C and an extension for 1min at 60˚C and final extension step at 60˚C for 5 min and the storage and cooling temperature was held at 4˚C.Amplified products of *cox1* and *cox2* genes were separated using agarose gel electrophoresis [1.5% (*w/v*)]. Amplified PCR products (20 µl) were sequenced at Agrigenome labs Pvt. Ltd., Cochin, Kerala.

Sequence analysis

Mitochondrial DNA gene *cox1*and *cox2*partial sequences were aligned by using Geneious v.11.1.3 software. After manual correction and assembly, unique sequences were deposited in gene bank under accession numbers MN016984 to MN016996 for *cox1*, and MN017012 to MN017024 for *cox2* gene sequences. Pairwise differences between sequences (k) were calculated in MEGA v6.06 (Tamura *et al.,* 2013).

Descriptive statistics number of haplotypes (H), Haplotype diversity, nucleotide diversity (π), and the average number of nucleotide differences between sequences (k) were calculated in DnaSP v5.0 software (Librado and Rozas 2009). Evidence of genetic structuring was assessed by analysis of molecular variance (AMOVA) and population comparisons and differentiation using the Tamura and Nei+γ model in ARLEQUIN version 3.5.2.2 (Excoffier and Lischer, 2010) with the resulting phi statistics assessed using 10,000 permutations at 0.05 significance level.

Results and Discussion

In the current study, we used the sequences of *B.dorsalis* obtained from eight locations (accession number of *cox1*: MN016984 to MN016996; *cox2*: MN017012 to MN017024) of Western Agroclimatic zone of Tamil Nadu. The aligned mtDNA *cox1*and *cox2* genes partial sequences used for genetic diversity analysis of *B.dorsalis* in different fruit growing areas of Krishnagiri, Dharmapuri, Coimbatore districts. A final sequences length of 634 and 598 bp for *cox1* and *cox2* genes respectively, were used for analysis. Overall 81 variable sites, including 51 parsimony informative sites and 30 singleton sites in the *cox1* gene and 52 variable sites including 15 parsimony informative sites and 37 singleton sites in *cox2* gene were observed in the nucleotide sequence alignment.

Analysis of sequences revealed that 12 haplotypesin the *cox1* gene and14 haplotypes in the *cox2* gene were detected from all the populations of *B. dorsalis* (Table 2). Among 12 haplotypes detected in the *cox1* gene, only four were shared by at least two populations and remaining 12 haplotypes were exclusive haplotypes unshared by any other populations. Likewise, only two out of 14 haplotypes detected in the *cox2* gene, were shared haplotypes. Results obtained from descriptive genetic diversity analysis such as Haplotype diversity, the average number of nucleotide differences (k), nucleotide diversity (p) of eight populations based on both gene sequences are summarized in (Table 3 and 4). Results of genetic diversity indices indicated that almost all the populations retain higher levels of genetic diversity. The analysis of molecular variance (AMOVA) showed that most of the genetic variation was present among the populations (51.62 from *cox1* and 66.11% from *cox2* gene sequences, $P<0.001$) than within the populations (48.38 % from *cox1* and 33.89 % from *cox2* gene sequences, $P<0.001$) (Table 5). The overall fixation index (Fst) calculated for all populations with both genes were statistically significant (0.51 at $P<0.01$ and 0.66 at $P<0.01$ levels)

Table.1 Locations of *B. dorsalis* sample collected from the western agroclimatic zone of Tamil Nadu

S. No	Code	District	Locations	Latitude	Longitude
1.	Cbe	Coimbatore	Coimbatore	11.0120° N,	76.9378° E
2.	Dpi-Km	Dharmapuri	Karimangalam	12.3060° N,	78.2045° E
3.	Dpi-Mo		Morapur	12.1281° N,	78.3913° E
4.	Dpi-Pe		Pennagaram	12.1334° N,	77.8967° E
5.	Kki-Ho	Krishnagiri	Hosur	12.7409° N,	77.8253° E
6.	Kki-Kp		Kaveripattinam	12.4215° N,	78.2174° E
7.	Kki-Ve		Veppanapalli	12.7020° N,	78.1942° E
8.	Kki-Py		Paiyur	12.3696° N,	78.2191° E

Table.2 Descriptive statistics of Genetic diversity of *B. dorsalis* populations based on*cox1* and *cox2* partial sequences

Species	Marker	Number of polymorphic sites	Haplotype (H)	Haplotype diversity (Hd±SD)	Average number of nucleotide differences (K±SD)	Nucleotide diversity (π±SD)
B. dorsalis	*cox1*	62	12	0.917±0.031	9.133±1.452	0.0145±0.00663
	cox2	51	14	0.983±0.028	12.108±2.049	0.0204±0.00643

Table.3 Descriptive statistics of Genetic diversity of *B. dorsalis* populations based on *cox1* sequences

S. No	Code	Location	Polymorphic site	Haplotype (H)	Haplotype diversity (Hd±SD)	Average number of nucleotide differences (K±SD)	Nucleotide diversity (π±SD)
1.	Cbe	Coimbatore	0	1	0	0	0
2.	Ho	Hosur	4	2	1.000±0.500	4.000±2.707	0.0057±0.0028
3.	Mo	Morapur	7	2	1.000±0.500	7.000±4.583	0.0105±0.0052
4.	Km	Karimangalam	4	2	1.000±0.500	4.000±2.707	0.0060±0.0030
5.	Kp	Kaveripattinam	4	2	1.000±0.500	4.000±2.707	0.0060±0.0040
6.	Py	Paiyur	4	2	1.000±0.500	4.000±2.707	0.0058±0.0028
7.	Pe	Pennagaram	2	2	1.000±0.500	2.000±1.452	0.0031±0.0015
8.	Ve	Veppanapalli	7	2	1.000±0.500	7.000±4.583	0.0100±0.0049

Table.4 Descriptive statistics of Genetic diversity of *B. dorsalis* populations based on *cox2* sequences

S. No	Code	Location	Polymorphic site	Haplotype (H)	Haplotype diversity (Hd±SD)	Average number of nucleotide differences (K±SD)	Nucleotide diversity (π±SD)
1.	Cbe	Coimbatore	2	2	1.000±0.500	2.000±1.450	0.0033±0.0016
2.	Ho	Hosur	5	2	1.000±0.500	5.000±3.333	0.0083 ±0.0041
3.	Mo	Morapur	6	2	1.000±0.500	6.000±3.959	0.0099 ±0.0050
4.	Km	Karimangalam	5	2	1.000±0.500	5.000±3.333	0.0083 ±0.0041
5.	Kp	Kaveripattinam	4	2	1.000±0.500	4.000±2.707	0.0067 ±0.0033
6.	Py	Paiyur	6	2	1.000±0.500	6.000±3.959	0.0099 ±0.0049
7.	Pe	Pennagaram	2	2	1.000±0.500	2.000±1.453	0.0033±0.0016
8.	Ve	Veppanapalli	8	2	1.000±0.500	8.000±5.205	0.0132±0.0066

Table.5 Summary of AMOVA analysis of different populations of *B. dorsalis*

Gene analyzed	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index
Cox1	Among populations	7	112.44	5.469Va	51.62	F_{st}0.5 1*
	Within populations	8	41.000	5.125 Vb	48.38	
	Total	15	153.44	10.594		
Cox2	Among populations	7	75.062	4.268Va	66.11	F_{st}0.66*
	Within populations	8	17.500	2.188 Vb	33.89	
	Total	15	92.562			

* significant at P < 0.01 level.

Analysis of partial mtDNA sequences proved the significant level of genetic diversity was found to exist within and among populations, as exemplified by high values of haplotype diversity, the average number of nucleotide differences, and nucleotide diversity from both *cox1* and *cox2* genes. Haplotype diversity (Hd>0.5) and nucleotide diversity (π>0.005) except for Coimbatore and Pennagaram locations were high.

The observed high value of genetic diversity indicated that the analyzed populations were stable with a very long evolutionary history. The possible justification for Coimbatore (π=0.0 <0.005 for *cox1*and π=0.0033<0.005 for *cox2)* and Pennagaram (π=0.030 <0.005 for *cox1*and π=0.0031<0.005 for *cox2)* populations showed that the moderate genetic diversity might be due to collection of samples from a smaller portion of populations. Krishnagiri and Dharmapuri districts being the main horticultural hub of Tamil Nadu which might have paved way for the pest to reside continuously throughout the year in fruit fly orchards. Hence, the number of generations per year are being constant may be the reason behind the high level of genetic diversity. These results are in accordance with (Aketarawong *et al.,* 2007) who predicted that the origin of Indian populations.

The molecular variance results obtained from both *cox1* and *cox2* genes demonstrated that the same as above that the observed genetic differentiation was higher because of significant high fixation index (F_{st}) value for both *cox1,*(F_{st}0.51> 0.25@ P < 0.01) and *cox2,*(F_{st}0.66 >0.25@ P < 0.01) (Table 5). It was evident that the existence of population differentiation among populations because overall genetic differentiation between the populations having highly significant values (51.62, 66.11 as percent variance for *cox1, cox2,* respectively), as compared to differentiation within the populations (48.38, 33.89 as percent variance for *cox1, cox2,* respectively).The results are in contrast to the other Indian populations (Choudhary *et al.,* 2016).Although Coimbatore location had a small proportion of populations having significant pairwise differences (varied from 0.929 to 1.000 for *cox1*and 0.892 to 930 for *cox2*) (Table 5) indicating little gene flow between Coimbatore and all other populations in Krishnagiri and Dharmapuri districts. The high genetic differentiation with limited gene flow is likely to be attributed to the presence of cryptic species within each location. Because of having high value in the average pairwise difference across sequence for both *cox1* (9.133±1.452 bp), and *cox2* (12.108±2.049 bp) and with the range pairwise difference between sequences being 0% - 7.2% (Table 5).

Hence, the species delimitation process is very important which can be solved by using an alternate identification system such as PCR-RFLP analyses for this *B. dorsalis* complex(Chua *et al.,* 2010). Moreover, the existing genetic pattern may be explained by *wolbachia* infection mediated hybrid inviability between populations. As *wolbachia* is one of the speciation agents, that increases speciation rates between sibling species of the tephritid fruit flies (Jamnongluk *et al.,* 2003).

Therefore determination of infection status of *wolbachia* in various populations can be explored for this destructive pest. Also, the current pattern of gene flow can be confirmed by fast-evolving microsatellite marker analysis with extensive sampling from varied biogeographical locations of south India. Concurrently, *B. dorsalis* mtDNA genes (*cox1* and *cox2*) partial sequences (26 in total)can serve as a representative sequence for the Tamil Nadu locations which will enrich the database of this pest.

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