Biochemical Systematics and Ecology 51 (2013) 291–296

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Population genetic structure of the pumpkin fruit fly, *Bactrocera tau* (Walker) (Diptera: Tephritidae) in Himachal Pradesh, India

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ARTICLE INFO

Article history:
Received 4 May 2013
Accepted 21 September 2013
Available online

Keywords:
Bactrocera
tau
Phylogeny
mtCOI gene
Fruit fly
North western Himalaya
SIT

ABSTRACT

The population genetic structure of the pumpkin fruit fly, *Bactrocera tau*, a fruit fly pest that causes significant losses to cucurbit cultivations, has been studied in Himachal Pradesh (India) using mitochondrial cytochrome oxidase I (mtCOI) gene sequences. Levels of differentiation (genetic distances and F_{ST} values) among samples from different locations are minimal, suggesting the local occurrence of a large and geographically undifferentiated population, with the possible exception of population Solan. Nevertheless, overall genetic variability is substantial, with 10 different haplotypes detected in 16 individuals and only one of these – likely the original one as it occupies a central position in the network and is found at a relatively high frequency –shared between multiple populations. The phylogenetic analysis of local *B. tau* samples in the context of the different sibling species that constitute the *B. tau* complex in its South-East Asia region of origin revealed that local *B. tau* is closely related to *B. tau* species *A* from Thailand. This should be taken into account in any intervention aimed at the control of this pest, e.g. area wide integrated pest management (AW IPM). The marked local genetic uniformity and predominance of one single species of the species complex further suggest that the sterile insect technique (SIT) may be a viable option.

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1. Introduction

The pumpkin fruit fly, *Bactrocera tau* (Walker), is widely distributed throughout South Asia (India, Sri Lanka, Bangladesh and Bhutan), South East Asia (Thailand, Malaysia, Vietnam, Philippines and Indonesia) and East Asia (Taiwan and South China) (White and Elson-Harris, 1992; Drew and Roming, 1997; Prabhakar et al., 2012a). Being multivoltine and highly polyphagous, *B. tau* can attack more than 50 cultivated as well as wild plant species from families Anacardiaceae, Cucurbitaceae, Elaeocarpaceae, Moraceae, Myrtaceae, Oxaalidaceae, Rutaceae, Sapotaceae and Solanaceae (Allwood et al., 1999; Huque, 2006). Furthermore, the species infests a wide range of commercially important cucurbit crops such as cucumber, luffa, pumpkin,

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http://dx.doi.org/10.1016/j.bse.2013.09.008

melon, bitter gourd, bottle gourd, ribbed gourd, sponge gourd, ash gourd, snake gourd, sweet gourd and summer squash (Drew and Roming, 1997; Prabhakar et al., 2012a).

B. tau is widely distributed in different regions of India, but its economic damage to cucurbit crops is more severe in the North-Western and North-Eastern hilly regions of the Indian Himalaya (Borah and Dutta, 1996; Prabhakar et al., 2009a, 2012a). Himachal Pradesh, one of the major vegetable growing states of India, suffers significant economic losses that may account for 80% of total cucurbit crops (Prabhakar et al., 2009a; Sood et al., 2010). The majority (96%) of the state is characterized by hills and/or higher mountain ranges, with *B. tau* activity being more significant in areas characterized by low to mid hills and absent in high hill regions (>3200 m amsl).

Based on recent works by Baimai et al. (2000), Jamnongluk et al. (2003) and Thanaphum and Thaenkham (2003), it is now well established that *B. tau* is not to be regarded as a single species, but rather a complex of different sibling species which have been tentatively systematized into eight forms designated as *B. tau* sp *A, B, C, D, E, F, G* and *I* based on specific host-plant preferences, cytological differences, external morphology, allozyme electrophoretic studies, molecular genetic markers and mtCOI gene sequences. This, in turn, led to the recognition that more specific and niche based management programmes are needed for an effective control of this pest (Thanaphum and Thaenkham, 2003).

Morphological differences are often unable to provide adequate resolution to determine the status of species, prompting for the implementation of alternative methods, such as DNA barcoding, to identify and characterize the different species/forms. Due to its pattern of maternal inheritance, absence of recombination and rate of evolution approximately 10 times faster than single-copy nuclear DNA (Brown et al., 1979), the mtCOI is appropriate to characterize taxonomically similar entities and is widely used as a DNA barcode marker (Hebert et al., 2003). Furthermore, mtCOI sequences, and mitochondrial haplotypes in general, have proved to be robust evolutionary markers for determining intra- and inter-specific relationships and phylogeographic structures in various invertebrate taxa, including fruit flies (Armstrong and Ball, 2005; Avise, 2000; Nardi et al., 2005; Prabhakar et al., 2012b).

Aim of the present study is to investigate the genetic variability and population structure in populations of *B. tau* from the Himachal Pradesh (India) and to identify their relationships in the context of the different species that constitute the *B. tau* species complex.

2. Materials and methods

2.1. Collection of infested fruits and rearing of fruit flies

Fruit fly infested cucurbit fruits and flowers were collected during years 2008–2010 from 12 locations in six districts of Himachal Pradesh (Table 1). Samples from each location were kept in separate rearing cages (20 × 15 × 18 cm) under laboratory conditions (Temp. 25±2 °C and RH 75–80%) at Palampur, India (Latitude: 32°6′N, Longitude: 76°3′E, Altitude: 1290 m amsl). Emerging fruit fly adults were identified based on the morphological descriptions given by White and Elson-Harris (1992) and Drew and Raghu (2002). Identified *B. tau* specimens were stored at −20 °C until DNA extraction, and voucher specimens are preserved in the collection of the Department of Entomology, CSK HPKV, Palampur, India.

2.2. DNA extraction

Total genomic DNA was extracted from single *B. tau* specimens following the method of Prabhakar et al. (2009b), with minor modifications. Samples were frozen in liquid nitrogen for 1 min, ground to a fine powder using a micro-pestle,

Table 1
Sampling locations and geographic coordinates of the six *Bactrocera tau* populations from Himachal Pradesh, India.

Sr. no.	Populations	Location(s)	Latitude	Longitude	Elevation m (amsl)	Collection date	Host plant/cue lure	*n*	GenBank accession number
1	Bilaspur	Ghumarwin	31°25′ N	76°43′ E	625	Aug 2009	*Cucumis sativus* Linnaeus	1	HQ378235
2		Chandpur	31°21′ N	76°47′ E	1020	Aug 2009	*Cucumis sativus* Linnaeus	1	HQ378241
3		Nihari	31°25′ N	76°39′ E	681	Sep 2009	Cue lure	1	HQ378243
4	Chamba	Banikhet	32°33′ N	75°57′ E	1538	Aug 2008	*Cucumis sativus* Linnaeus	1	HQ378232
5	Hamirpur	Nadaun	31°46′ N	76°20′ E	460	May 2009	*Lagenaria siceraria* (Molina)	1	HQ378228
6		Nadaun	31°46′ N	76°20′ E	460	May 2009	*Momordica charantia* Linnaeus	1	HQ378229
7		Nadaun	31°46′ N	76°20′ E	460	May 2009	*Cucumis sativus* Linnaeus	1	HQ378233
8	Kangra	Palampur	32°6′ N	76°32′ E	1290	Jun 2009	*Cucumis sativus* Linnaeus	1	HQ378230
9		Palampur	32°6′ N	76°32′ E	1290	Aug 2009	*Momordica charantia* Linnaeus	1	HQ378237
10		Jawalamukhi	31°53′ N	76°17′ E	470	Aug 2009	*Cucumis sativus* Linnaeus	1	HQ378239
11		Jawalamukhi	31°53′ N	76°17′ E	470	Aug 2009	*Momordica charantia* Linnaeus	1	HQ378240
12		Shahpur	32°13′ N	76°11′ E	912	May 2010	*Momordica charantia* Linnaeus	1	HQ378242
13	Mandi	Mandi	31°42′ N	76°55′ E	806	Aug 2009	*Cucumis sativus* Linnaeus	1	HQ378234
14		Barot	32°02′ N	76°50′ E	2690	Aug 2009	*Cucurbita maxima* Duchesne	1	HQ378238
15		Nagwain	31°49′ N	77°10′ E	1116	Aug 2009	*Momordica charantia* Linnaeus	1	HQ378236
16	Solan	Nauni	30°56′ N	77°20′ E	1546	Jul 2009	*Cucurbita pepo* Linnaeus	1	HQ378231

n Number of individuals of *B. tau* sequenced.

Table 2
Average K2P distances between populations (below diagonal) and within populations (diagonal elements) for six populations of *B. tau* from Himachal Pradesh, India.

Populations	Average K2P genetic distances					
	Hamirpur	Kangra	Mandi	Bilaspur	Solan	Chamba
Hamirpur	0.0033					
Kangra	0.003	0.0022				
Mandi	0.004	0.003	0.0033			
Bilaspur	0.004	0.003	0.003	0.0022		
Solan	0.009	0.008	0.009	0.009	n/c[a]	
Chamba	0.004	0.003	0.003	0.003	0.009	n/c

All results are based on the pairwise analysis of 16 sequences.
Mean K2P distances are below diagonal (between Population) and diagonal values are within population K2P distances were obtained by a bootstrap procedure (1000 replicates).
[a] n/c: not calculated.

dissolved in 700 μl of cetyltrimethyl ammonium bromide (CTAB) extraction buffer and incubated at 65 °C for 1 h in a water bath (YORK Scientific Industries, Delhi).

An equal volume (700 μl) of chloroform: isoamyl alcohol (24:1 v/v) was added and tubes were spun at 10,000 g for 12 min at high speed in a refrigerated centrifuge (REMI India) at 4 °C. The aqueous phase was transferred to new microtube and 450 μl of pre-chilled isopropanol was added, incubating samples at −20 °C for 20–30 min, to precipitate the DNA. Tubes were then spun at 10,000 g for 12 min and the supernatant decanted. The DNA pellet was washed three times with 70% ethanol, dried and resuspended in 100 μl of Tris EDTA buffer (10 mM Tris HCl and 1 mM EDTA, pH 8.0). RNAse @ 10 μl/ml (MBI Fermentas) was added and the emulsion incubated for half an hour at 37 °C. The amount of DNA was quantified by recording the absorbance at 260 nm using an UV/VIS spectrophotometer (Bio Rad, SmartSpec 3000) and extractions stored at −20 °C for further use.

2.3. PCR amplification and sequencing

The mtCOI gene was amplified by Polymerase chain reaction (PCR) with forward primer UEA7 5′ TACAGTTGGAATAGACGTTGATAC3′ and reverse primer UEA10 5′ TCCAATGCACTAATCTGCCATATTA3′ developed by Lunt et al. (1996). Amplifications were carried out in a 20 μl reaction volume containing 2 μl of 10X reaction buffer, 2 μl of $MgCl_2$ (25 mM, Life Technologies India, Pvt. Ltd), 1 μl of each primer (20 pmol), 0.5 μl of each dNTP (10 mM, Fermentas), 0.2 μl of *Taq* polymerase (5 units/μl, Life Technologies (India), Pvt. Ltd) and 2 μl (approx. 10 ng) of DNA template. Amplification was performed in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, USA) with an initial denaturation step of 3 min at 94 °C followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final elongation step at 72 °C for 30 min. The amplification products were separated in a 2% (w/v) agarose gel using TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Amplified PCR products, of ~650 bp in length, were freeze dried (CHRIST *ALPHAI-2LD*) and submitted for custom sequencing using same upstream and downstream primers at Xcelris Labs Limited (India) on an ABI PRISM 310™ Genetic Analyzer (Applied Biosystems, USA). The novel *B. tau* sequences obtained in this study have been deposited in GenBank under accession numbers HQ378228–HQ378243.

2.4. Data analysis

All 16 obtained sequences of *B. tau* (16 individual sequences from six populations) were aligned using ClustalW as implemented in MEGA 4.1 (Tamura et al., 2007). Analyses of genetic and phylogenetic relationships between *B. tau* isolates sequenced in this study and different species of *B. tau* (GenBank sequences) were performed in MEGA 4.1 (Tamura et al., 2007), including *Bactrocera dorsalis* (Hendel) (HQ446514) and *Bactrocera zonata* (Saunders) (HQ446513) as out groups. Genetic distances within and between populations of *B. tau* were calculated using the Kimura two-parameter (K2P) method

Table 3
Average number of pairwise differences between populations (above diagonal), within population (bold diagonal elements) and F_{ST} values (below diagonal) for six population of *B. tau* from Himachal Pradesh, India.

Populations	Hamirpur	Kangra	Mandi	Bilaspur	Solan	Chamba
Hamirpur	**2.66667**	1.93333	2.11111	2.33333	6.33333[a]	2.33333
Kangra	0.06327	**1.20000**	1.46667	1.60000	5.60000[a]	1.60000
Mandi	−0.10526	−0.05096	**2.00000**	2.00000	6.00000[a]	2.00000
Bilaspur	0.14286	0.21642	0.16667	**1.33333**	6.00000[a]	2.00000
Solan	0.57895	0.78571	0.66667	0.77778	**0.00000**	6.00000[a]
Chamba	−0.14286	0.25000	0.00000	0.33333	1.00000[a]	**0.00000**

[a] $p < 0.05$.

(Kimura, 1980). The neighbor-joining (Saitou and Nei, 1987) procedure was used for phylogeny reconstruction, assessing confidence levels with 1000 bootstrap replications (Felsenstein, 1985).

A minimum spanning network among *B. tau* haplotypes was constructed under statistical parsimony criteria with TCS 1.21 (Clement et al., 2000). Haplotype frequency and distribution, polymorphic sites and nucleotide diversity were assessed using ARLEQUIN 3.11 (Excoffier et al., 2005). Analysis of molecular variance (AMOVA) was performed to assess the genetic structure and genetic variability within and between the populations of *B. tau*.

3. Results

The final alignment of *B. tau* mtCOI gene sequences consisted of 16 sequences of 585 bp in length, with no indel. Fifteen polymorphic positions were observed (2.56% variable sites), 12 of which were singletons and 3 were parsimony informative sites. Genetic distances (K2P) within population were 0.0022, 0.0022, 0.0033 and 0.0033 in Kangra, Bilaspur, Mandi and Hamirpur populations of *B. tau,* respectively. Mean distances between populations ranged from 0.003 between Kangra and Hamirpur to 0.009 between Solan and Hamirpur, Mandi, Bilaspur and Chamba, respectively (Table 2), with K2P distance between Solan and other populations being larger than in any other pairwise comparison. Among individual sequences, K2P genetic distance ranged from 0.002 to 0.151.

Genetic distances between *B. tau* isolates from Himachal Pradesh (India) and a single isolate from Thailand (*B. tau* sp *A*) were minimal, ranging from 0.002 to 0.014, while genetic distance of Indian isolates with other species of the *B. tau* complex (*B, C, D, E, F, G, I*) of Thailand were higher, ranging from 0.099 to 0.151.

Pairwise F_{ST} estimates (Table 3), in line with the pairwise number of differences between populations, revealed that the Solan population differed significantly from all other populations. In contrast, no significant differentiation appeared between other populations except for Solan.

The phylogenetic analysis (Fig. 1) clustered all the *B. tau* sequences from Himachal Pradesh determined in present study in one clade, whereas Thailand reference samples, corresponding to a wide range of the different species that constitute the *B. tau* species complex, displayed a more pronounced variation. The genetic distance (K2P) between ingroup and outgroup

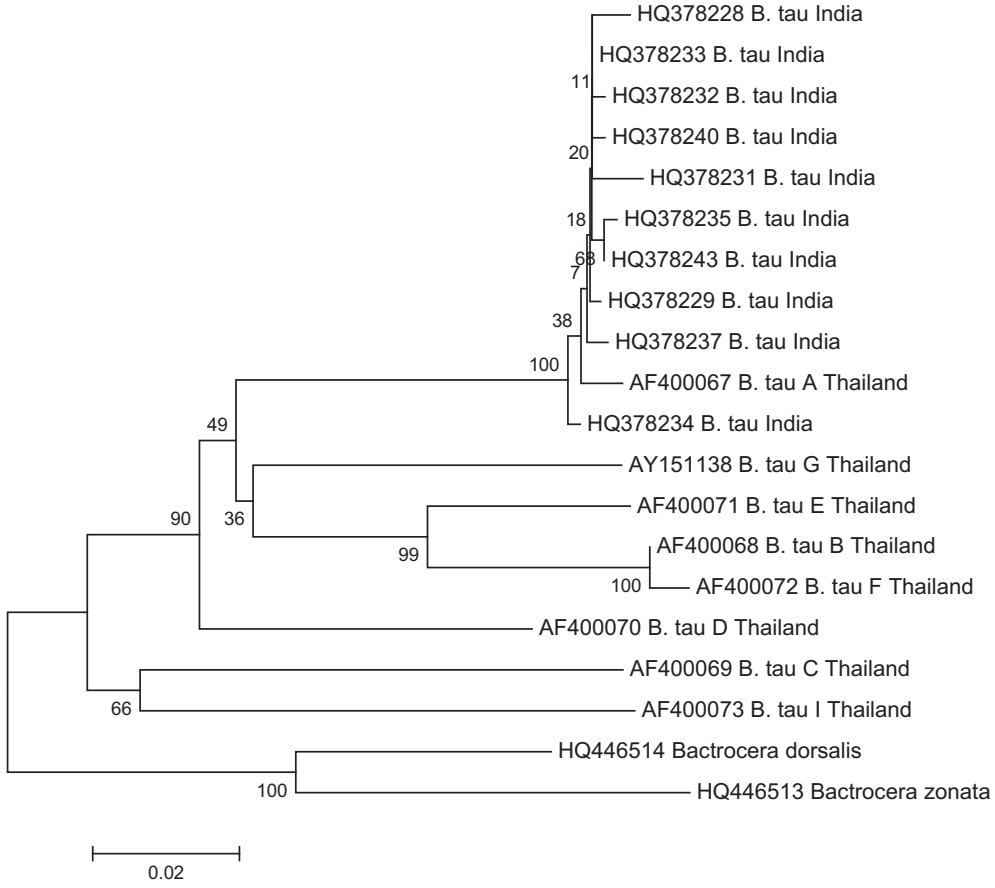

Fig. 1. Phylogenetic tree based on *mtCOI* gene of *B. tau* with *B. dorsalis* and *B. zonata* as out groups using the neighbor-joining method and confidence level calculated with the bootstrap test (1000 replicates).

species varied from 0.147 to 0.171 and 0.155 to 0.189 between *B. tau/B. dorsalis* and *B. tau/B. zonata*, respectively, with *B. dorsalis* being more closely related to *B. tau* on the phylogenetic tree.

Ten different mitochondrial haplotypes were observed in the six *B. tau* populations. Only one haplotype was, however, shared by at least two populations while the remaining nine were unique. The predominant haplotype, here named H1, was observed in a total of seven individuals from Hamirpur, Kangra, Mandi and Bilaspur. The minimum spanning network of haplotypes depicted as Fig. 2 revealed that all haplotypes were found along seven branches that evolved from a central and most frequent haplotype (H1). Four haplotypes (H3, H4, H7 and H10, one from each Hamirpur, Kangra, Bilaspur and Chamba) diverged by single mutation steps, while the most divergent haplotypes (*i.e.* H9, from Solan) was as far as five mutation steps from the central haplotype. There were 8 missing haplotypes in the minimum spanning network.

4. Discussion

The genetic variation in natural populations is the outcome of a balance between evolutionary and demographic processes and provides models and tools for interpreting evolutionary processes and determining the evolutionary potential of a species (Li et al., 2013). Results of mtCOI gene sequence analysis indicated that *B. tau* from Himachal Pradesh (India) is characterized by low levels of genetic divergence, ranging from 0.002 to 0.009 among all populations studied, contrary to Jamnongluk et al. (2003) who reported comparatively higher divergence in the mtCOI gene of *B. tau* from Thailand, in line with the local occurrence of eight different forms (*B. tau* sp *A, B, C, D, E, F, G* & *I*) recognized on morphological, cytological and molecular grounds (Baimai et al., 2000; Jamnongluk et al., 2003).

Although in the context of a limited overall genetic divergence, most (10) mitochondrial haplotypes detected from 16 individuals of *B. tau* from six populations of Himachal Pradesh were unique, with the only exception of haplotype H1 which occupied central position in the minimum spanning network (Fig. 2) and was shared by 7 individuals from four populations, namely Hamirpur, Kangra, Mandi and Bilaspur. The *B. tau* populations in Himachal Pradesh, therefore, seem to retain fairly high level of genetic diversity, as previously documented in *B. dorsalis* (Wan et al., 2012) and *Ceratitis capitata* (Wiedemann) (Meixner et al., 2002).

The high number of different haplotypes observed in the present study was quite unexpected, as the fly was supposed to be secondarily introduced in India from South East Asia, the area of origin of *B. tau*. This observation could be possibly attributed to the occurrence of multiple invasions, with large population numbers of *B. tau* from its native range. Furthermore, the fairly large number (8) of intermediate missing haplotypes in the minimum spanning network suggests a potential genetic drift in *B. tau*. A similar example of introduction from South East Asia has been recently reported by Wan et al. (2012), who studied the route of invasion of *B. dorsalis* in the Asia-Pacific region and suggested, on the basis of haplotype sharing and origin by different populations of *B. dorsalis*, that the fly introduction in the Himachal Pradesh may have originated from South-East China through the nearby region of Myanmar.

Pairwise F_{ST} among 15 population pairs ranged from −0.10526 to 1 (Table 3). Among them, 14 out of 15 showed no statistically significant differentiation ($P > 0.05$), suggesting that most populations in fact constitute one single and uniform genetic group. The higher and statistically significant pairwise F_{ST} value observed for Chamba and Solan populations of *B. tau*, in turn, suggested that population differentiation may indeed take place with more pronounced geographical separation. Nevertheless, the genetic differentiation in most of the populations of *B. tau* studied appears not to be geographically structured and/or associated with their geographical distribution, as evident by neighbor-joining cluster where no sign of geographically associated population structure is evident (Fig.1).

The phylogenetic reconstruction of *B. tau* from the Himachal Pradesh in the context of different species of the *B. tau* complex of Thailand reported by Jamnongluk et al. (2003) revealed that all local *B. tau* sequences clustered with *B. tau* sp *A*.

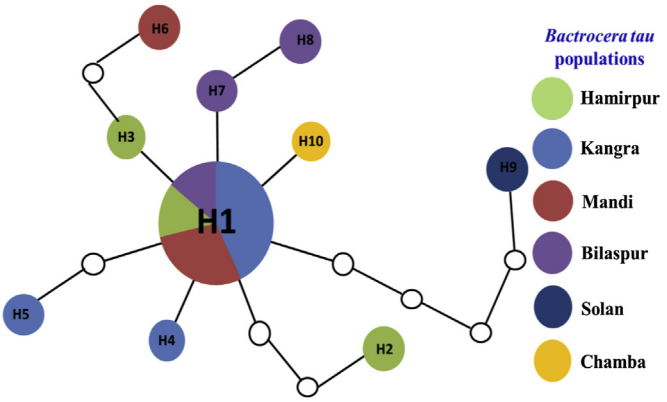

Fig. 2. Minimum spanning network of the 10 mitochondrial haplotypes observed in a set of 16 individuals from 6 populations of *B. tau* collected from Himachal Pradesh (India). Each population is color coded and the empty circles show the missing haplotypes in the network. **H**: Haplotype (H1–H10). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Henceforth, any well-grounded management strategy against this particular species (*B. tau* sp *A*), aimed at minimizing losses to cucurbits cultivation, should take the entire distribution, as well as the possibility of long range introductions of *B. tau* sp *A* into account. An area-wide management program aimed at reducing population numbers and economic damage, possibly based on the sterile insect technique, may also be a logical future strategy for the control of *B. tau* sp *A*.

Acknowledgments

We would like to thank two anonymous reviewers and editor for thoughtful comments on earlier drafts that significantly improved the quality of the manuscript. Authors are grateful to Dr. Francesco Nardi, Department of Evolution Biology, University of Siena, Siena, Italy for critical revision and editing the final draft.

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