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LIFE STAGE INDEPENDENT IDENTIFICATION OF FRUIT FLIES (DIPTERA: TEPHRITIDAE) USING 28S RDNA SEQUENCES

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ABSTRACT
With well over 500 species, *Bactrocera* is one of the most species-rich clades within the dipteran family Tephritidae, the true fruit flies. In quarantine and plant protection activities, their immature stages are metwith and diagnosis of these is important. For this purpose molecular diagnosis becomes handy, because it is independent of sex, color morphs and life stages. Alignment of the sequences of 28S rDNA from various life stages of the four species of *Bactrocera* shows that the molecular identification is independent of life stages and polymorphism of the target species. This study evaluated the utility of 28S rDNA for the quick and accurate species diagnosis of eggs, larvae, pupae and adults of all the *Bactrocera* spp. studied. The phylogram for the *Bactrocera* spp. suggests that *B. tau* is phylogenetically distant from the rest of the three species viz. *B. dorsalis*, *B. zonata* and *B. correcta*, which was supported by 100% bootstrap value. Moreover *B. dorsalis*, *B. zonata* and *B. correcta* had maximum sequence identity (98%) with very few variable sites in the 28S rDNA sequences. Even though, 28S rDNA region will have high reliability for species identification in these species studied and it was never reported before. This study indubitably proved the utility of 28S rDNA for the quick and accurate species diagnosis of egg, larva, pupa and adult of *B. zonata* Saunders, *B. tau* Walker *and B. dorsalis* Hendel.

INTRODUCTION

Bactrocera (Tephritidae: Diptera) is among the largest genera within Tephritidae with about 500 described species (Drew and Hancock, 2000) and are the world's worst pests of fruits causing enormous economic loss every year (Armstrong and Jang, 1997). The genus *Bactrocera* is of particular concern throughout much of Asia, where they constitute a significant threat to agricultural resources (Nagappan *et al.*, 1971). Globalization has increased the threat of introduction of invasive species like *Bactrocera*. Therefore establishing accurate and timely identification of species of fruit flies at the port of entry is of paramount importance. In this regard, morphological identification always requires presence of adults, whereas immature stages are often encountered at the port of entry. At this juncture, molecular identification comes handy. In this regard, identification of *Bactrocera* using conventional taxonomy has certain limitations owing to homoplasmy on most morphological characters and difficulty in identifying in its immature stages. On the other hand, molecular identification augments conventional taxonomy, in which species identification is not limited by polymorphism, sex and stage of development of the target species. In the present study, we conducted analyses using 28r DNA sequence data in order to identify the *Bactrocera* spp. viz. *B. zonata, B. tau*, *B. correcta* and *B. dorsalis* in their various life stages.

MATERIALS AND METHODS

Stock culture maintenance

Adults of *B. dorsalis, B. correcta, B. tau* and *B. zonata* were obtained from Delhi using bait traps and net sweeps. These insects were mass-reared on pumpkin *Cucurbita maxima*, kept in plastic cages (24.5cm × 20.5cm × 20.5cm) and provided with water, yeast powder bacto applied on sugar cubes and a piece of pumpkin as a food source and site of oviposition. Individual pairs of newly emerged adults were separated from this stock and maintained separately. These were observed for oviposition, and as soon as oviposition started pieces of pumpkin were removed to separate plastic jars (14.0cm × 10.5cm) each provided with a layer of sand (10-15 cm) to facilitate pupation; the mouths of these plastic jars were covered with muslin cloth and secured by rubber bands. Observations were made on the larvae and puparia daily, and puparia were removed to separate containers before adult emergence to record the sex ratio and other details. Morphological identification of all 4 species of *Bactrocera,* viz., *B. tau, B. zonata, B. correcta* and *B. dorsalis,* was carried out according to White and Elson-Harris (1992) prior to molecular studies.

DNA isolation and Polymerase Chain Reaction

Total DNA was extracted from individual fruit fly adults using the DNA easy Tissue Kit (Qiagen) according to manufacturer's protocol. Two micro litres were used as the template for Polymerase Chain Reaction (PCR). Polymerase Chain Reaction was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells) with the following cycles; 94ºC for 4 minutes as initial denaturation followed by 35 cycles of 94ºC 40 seconds, 47ºC for 45 seconds 72ºC for 45 seconds and 72ºC for 20 minutes as final extension. Primers specific to specific

to 28S rDNA, (28SS- 5'- GAC CCG TCT TGA AMC AMG GA -3'; 28SA- 5'- TCG GAR GGA ACC AGC TAC TA -3', Chen *et al*., 2006), resulting in the amplification of approximately 330 bp fragment. PCR was performed in 25-μL total reaction volume containing 20 picomoles of each primer, 10mM Tris HCl (pH-8.3), 50mM KCl, 2.5mM $MgCl_2$, 0.25mM of each dNTP and 0.5U of Taq DNA polymerase (Fermentas Life Sciences). The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10μg/mL) and visualized in a gel documentation system (UVP).

Molecular cloning and Sequencing

The PCR amplified fragments were eluted using Nucleospin® Extract II according to the manufacturer's protocol (Macherey-Nagel, Germany) and ligated into the general purpose-cloning vector, InsT/Aclone (Fermentas GmBH, Germany) according to the manufacturer's protocol. Blue/white selection was carried out and all the white colonies were maintained on LBA containing ampicillin (100 mg/mL), incubated at 37ºC overnight and stored at 4ºC until further use. Plasmids were prepared from the overnight culture of the positive colonies cultured in LB broth (enzymatic casein- 10g, yeast extract-5g, NaCl-5g in 1000mL of water, pH-7.0) using GeneJET™ Plasmid Miniprep Kit (Fermentas GmBH, Germany) according to manufacturer's protocol, from the overnight cultures of the five randomly selected clones grown in LB broth. Sequencing was carried out in an automated sequencer (ABI Prism® 3730 XL DNA Analyzer; Applied Biosystems, USA) using M13 universal primers both in forward and reverse directions.

RESULTS AND DISCUSSION

The PCR amplicon size of different length (*B. correcta*-329bp, *B. tau*- 336bp, *B. dorsalis*- 327bp, *B. zonata*- 327bp) was amplified from all the four species of *Bactrocera* in their different life stages viz. adult, pupa, larva and egg. Alignment of the sequences from various life stages does not showed any differences in the 28S rDNA sequences for all the four species of *Bactrocera* (Fig. 1), which proved that the molecular identification is independent of life stages and polymorphism of the target species. Comparison of the 28S rDNA sequences of all four species of *Bactrocera* viz. *B. correcta*, *B. zonata*, *B. dorsalis*, *B. tau* showed very less variations in sequences (Fig. 1).

Table 1: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution of *Bactrocera* species

	A	T	C	G
A	-	*2.32*	*1.25*	29.6
T	*2.45*	-	3.79	*1.64*
C	*2.45*	7.07	-	*1.64*
G	*44.23*	*2.32*	*1.25*	-

The Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for *Bactrocera* spp. sequences was performed using MEGA 4.0 (Tamura *et al*., 2007). The reliability of the clustering pattern in the trees was determined by the bootstrap test, with 1000 replication. Each entry showed the probability of substitution from one base (row) to another base (column) instantaneously. Rates of different transitional substitutions were indicated in bold and those of transversional substitutions are shown in italics (Table. 1). The *Bactrocera* spp. nucleotide frequencies were 0.32 (A), 0.303 (T), 0.163 (C) and 0.214 (G). The base composition of the 28S rDNA gene fragment was biased toward Adenine (A) and Thymine (T), which together constituted 62.3% of the total. The overall transition (ti)/ transversion (tv) bias of *Bactrocera spp* nucleotide sequence was R= 4.293, where $R = [A*G*k_1 + T*C*k_2] / [(A+G)*(T+C)]$. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated from the datasets (Complete deletion option). All calculations were conducted in MEGA

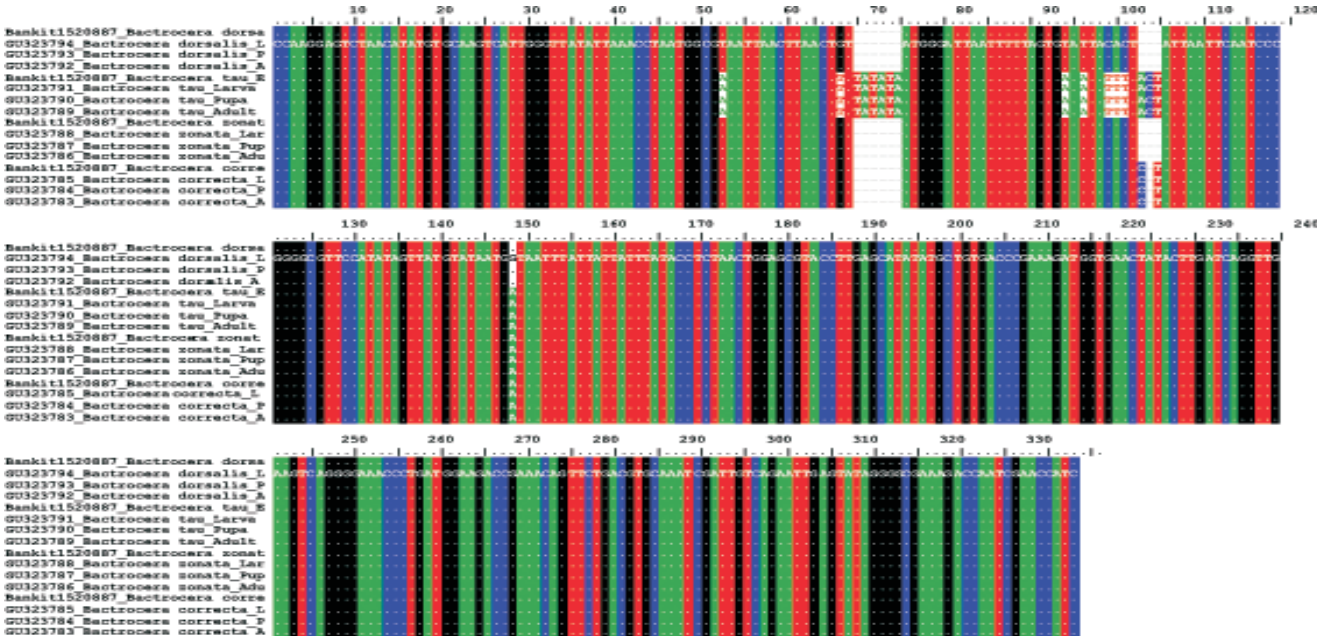

Figure 1: Consensus sequence of 336 bp from the Ribosomal 28SrDNA gene for the four *Bactrocera* species in their different life stages viz. egg, larva, pupa and adult

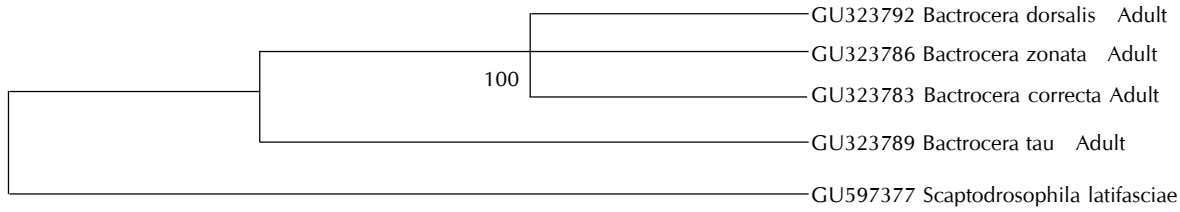

Figure 2: Neighbour-joining tree with bootstrap support (1000 replicates) showing clustering of *Bactrocera* species using 28SrDNA sequences. *Scaptodrosophila latifasciae* was used as an outgroup

4.0 (Tamura *et al.,* 2007). Summary statistics for the different substitutional changes are shown in Table 1. The phylogram for the *Bactrocera* spp. suggests that *B. tau* is phylogenetically distant from the rest of the three species viz. *B. dorsalis*, *B. zonata* and *B. correcta*, which was supported by 100% bootstrap value. Moreover *B. dorsalis*, *B. zonata* and *B. correcta* had maximum sequence identity (98%) with very few variable sites in the 28S rDNA sequences. The above results clearly showed that molecular identification is not limited by life stages and polymorphism of the insects. The above results of phylogenetic analysis based on the 28S rDNA were never suggested before.

White and Elson-Harris (1992) have investigated about 250 fruit fly pest species known to damage various fruits, vegetable plants and host plant of these pestiferous species. In India, larvae of *Bactrocera* spp. cause serious damage to a variety of horticultural crops viz. Mango, Melons, Pumpkins, Bitter guards and Tomato etc. cultivated in almost every part including high altitude area from throughout the year. Most of the *Bactrocera* species are native of oriental and are potential agricultural pest and also as consequence of the introduction of exotic invasive species, which cause a serious economic problem especially due to quarantine interception in the form of egg or other immature stages. In this regard, identification of *Bactrocera* spp using conventional taxonomy having certain limitations owing to homoplasy on almost morphological characters and difficult to identify in its immature life stages. Some of the species can take upto 40 days to reach adulthood (Kim, 1999). Accurate and timely identification of quarantine pests' viz. *Bactrocera* spp. is of high economic importance in countries like India and it is necessary to identify the species at the port of entry. In this regard, various molecular markers have been employed by researchers for identification of insects, viz. Cytochrome b (Raboudi *et al.,* 2005), 16S rRNA (von Dohlen and Moran, 2000), 18S rRNA, 28S rRNA, 5.8S rRNA (Ji *et al.,* 2003), internal transcribed spacers, elongation factor-1α (Djernaes and Damgaard, 2006; Ji *et al.,* 2003), mitochondrial cytochrome oxidase (mtCOI) (von Dohlen *et al.,* 2006), etc., in various insects. Molecular identification is a very useful tool when there is a polymorphism in the target species exists as in the case of *Ceratitis capitata* (Wiedmann) and *Anastrepha fraterculus* (Wiedmann) (Sonvico *et al.,* 1996). 28S rDNA is a nuclear locus that has been provided good results for thrips phylogeny (Toshiro and Tamito, 2007). Nuclear genes are slowly evolving hence used in many groups of insects. In the present study, the third domain (D3) of the ribosomal 28S locus (28S rDNA) sequences was proved to be useful in identification of *Bactrocera* species. Asokan *et al.,* (2011) used COX-1 as a molecular marker for differentiating *Bactrocera* spp. and is a better molecular marker than 28S rDNA for one most important reason that cox-1 has enough species level variations than 28S rDNA. This is because 28S rDNA is under stringent selection where genetic variations are discouraged. On the other hand COX-1 can have synonymous mutations giving enough genetic variation but not sufficient for the protein to become non-functional. Even though, we strongly believe that 28S rDNA region will have high reliability for species identification in these four species of fruit flies and it was never reported before. Moreover, it will help in timely, accurate and life stages independent identification of this quarantine pest species' at the port of entry.

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