Molecular Identification and Phylogeny of Bactrocera Species (Diptera: Tephritidae)

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MOLECULAR IDENTIFICATION AND PHYLOGENY OF *BACTROCERA* SPECIES (DIPTERA: TEPHRITIDAE)

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Abstract

Fruit flies that belong to the genus *Bactrocera* (Diptera: Tephritidae) are major invasive pests of agricultural crops in Asia and Australia. Increased transboundary movement of agricultural produce has resulted in the chance introduction of many invasive species that include *Bactrocera* mainly as immature stages. Therefore quick and accurate species diagnosis is important at the port of entry, where morphological identification has a limited role, as it requires the presence of adult specimens and the availability of a specialist. Unfortunately when only immature stages are present, a lacunae in their taxonomy impedes accurate species diagnosis. At this juncture, molecular species diagnostics based on COX-I have become handy, because diagnosis is not limited by developmental stages. Yet another method of quick and accurate species diagnosis for *Bactrocera* spp. is based on the development of species-specific markers. This study evaluated the utility of COX-I for the quick and accurate species diagnosis of eggs, larvae, pupae and adults of *B. zonata* Saunders, *B. tau* Walker, *and B. dorsalis* Hendel. Furthermore the utility of species-specific markers in differentiating *B. zonata* (500bp) and *B. tau* (220bp) was shown. Phylogenetic relationships among five subgenera, viz., *Austrodacus, Bactrocera, Daculus, Notodacus* and *Zeugodacus* have been resolved employing the 5´ region of COX-I (1490-2198); where COX-I sequences for *B. dorsalis* Hendel, *B. tau* Walker, *B. correcta* Bezzi and *B. zonata* Saunders from India were compared with other NCBI-GenBank accessions. Phylogenetic analysis employing Maximum Parsimony (MP) and Bayesian phylogenetic approach (BP) showed that the subgenus Bactrocera is monophyletic.

Key Words: *Bactrocera*, COX-I, species-specific marker, Bayesian phylogeny, monophyletic

Resumen

Las moscas de la fruta que pertenecen al género *Bactrocera* (Diptera: Tephritidae) son las principales plagas invasoras de los cultivos agricolas en Asia y Australia. El aumento en el movimiento transfronterizo de los productos agricolas ha resultado en la posibilidad de la introducción de muchas especies invasoras que incluyen *Bactrocera* principalmente por los estados inmaduros. Por lo tanto, el diagnóstico de especies con rapidez y precisión es importante en el puerto de entrada, donde la identificación morfológica tiene un papel limitado, ya que requiere la presencia de ejemplares adultos, la disponibilidad de especialistas, y donde la escasez de datos en la taxonomía de los estados inmaduros de las especies impiden el diagnóstico exacto. En esta situación, el diagnóstico molecular de especies basado en el gene COX-I ha llegado a ser util, ya que el diagnóstico de especies no está limitado por el estadio de desarrollo. Sin embargo, otro método de diagnóstico rápido y exacto de especies de *Bactrocera* spp. es basado en el desarrollo de marcadores específicos para las especies. En el presente estudio, se evaluó la utilidad de usar el COX-I para el diagnóstico rápido y preciso de especies usando los huevos, larvas, pupas y adultos de *B. zonata* Saunders, B. *tau* Walker y *B. dorsalis* Hendel. También demostramos la utilidad de usar marcadores específicos de las especies para diferenciar *B. zonata* (500bp) y *B. tau* (220bp). Relaciones filogenéticas entre los cinco subgéneros, es decir, *Austrodacus, Bactrocera, Daculus, Notodacus* y *Zeugodacus* se ha resuelto por primera vez empleando la región 5' de la COX-I (1490 a 2198) donde se compararon las secuencias de COX-I de *B. dorsalis* Hendel *B. tau* Walker, *B. correcta* Bezzi y *B. zonata* Saunders, de la India con las acessiones del NCBI-GenBank. El análisis filogenético utilizando tanto el programa Union de Vecinos (NJ, Neighbor-Joining) y la máxima parsimonia (MP) demostró que los subgéneros del *Bactrocera* son monofiléticos.

Fruit flies are among the world's most serious pests causing enormous yield losses. Tephritid flies of the genus *Bactrocera* are of particular concern throughout Asia and Australia (Kim et al. 1999). There are about 500 described species of *Bactrocera* that are grouped into 28 subgenera (Drew and Hancock 2000). Accidental introduction of alien invasive species, such as *Bactrocera* species, poses a serious and mounting threat to crop production with increasing long distance transportation of agricultural produce. Therefore it is necessary to identify the species quickly and accurately at the port of entry. In this regard identification using morphological characters falls short because it requires adult specimens and the availability of expertise in taxonomy; this is further complicated by the lack of identification keys for identification of immature stages (eggs, larvae and pupae). The problematic immature stages are most often encountered in the import consignments. At this juncture, molecular species diagnostics based on COX-I become handy because they are not limited by developmental stage or sexual gender. Yet another method of resolving identification of closely related species is achievable by the development of species-specific primers that produce a specific amplicons. Such species-specific markers could be used effectively for species diagnosis even by a non-specialist.

In previous studies mitochondrial DNA has been utilized to resolve the phylogeny of *Bactrocera* (Muraji & Nakahara 2001; Smith et al. 2003; Zhang et al. 2010) and host associated genetic differences in thrips (Brunner et al. 2004). In this regard, there are contradicting views on whether the phylogeny of *Bactrocera* is monophyletic or paraphyletic (Zhang et al. 2010). A monophyletic theory for *Bactrocera* was proposed by Drew (1989) based on 5 morphological characters. On the other hand White (2000) proposed the employment of DNA sequencing to resolve the phylogeny of *Bactrocera*. Subsequently Muraji & Nakahara (2001) employed 1.6 kb mitochondrial DNA sequences and concluded that *Bactrocera* is paraphyletic.

In the present study the utility of various developmental stages namely, egg, larva, pupa and adult (male, female) of 4 economically important species of *Bactrocera* (*B. zonata* Saunders, *B. correcta* Bezzi, *B. tau* Walker, *and B. dorsalis* Hendel) were analyzed for accurate species diagnosis by using COX-I sequencing. The utility of species-specific markers in discriminating *B. zonata* from *B. tau* were also evaluated. Phylogenetic analyses carried out employing COX-I (1490-2198) for these species, which were compared to the NCBI-Genbank accessions to understand their phylogeny. Bayesian methods of phylogeny that calculate the posterior probabilities of the clades based nucleotide substitution models were employed to assess the monophyly of the group.

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 in 2008 and 2009. These insects were mass-reared on pumpkin *Cucurbita maxima*, kept in plastic cages (24.5 cm × 20.5 cm × 20.5 cm) 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 (14.0 cm × 10.5 cm) 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 (1992) and Drew (1994) prior to molecular studies. The pure cultures were maintained in insect proof cages (24.5 cm × 20.5 cm × 20.5 cm) at room temperature (30-32 °C) and RH 70-90% for supplying samples of the immature stages from time to time. *B. zonata, B. tau, B. correcta and B. dorsalis* were used for developmental stage non-limiting studies, in which the egg, larva, pupa and adult were drawn from the insect cages from time to time and molecular identifications were carried out.

DNA isolation and Polymerase Chain Reaction

Total DNA was extracted from individual fruit fly adults using the CTAB method (Saghai Maroof et al. 1984). Thoracic tissue was homogenized with liquid nitrogen using a sterile micropestle in 1.5 mL microcentrifuge tube filled with 100 µL STE buffer (100 mM NaCl, 10 mM Tris HCl (pH-8.0), and 1 mM EDTA (pH 8.0)). The homogenate was heated at 65 °C for 60 min before being centrifuged at 6000 rpm for 15 min at room temperature followed by precipitation of DNA and dissolution in molecular biology grade water (DNAase-free and RNAase free) (Eppendorf, Germany). Two µL was used as the template for Polymerase Chain Reaction (PCR).

PCR was carried out in a thermal cycler (AB-Applied Biosystems, Veriti 96 wells) with the following cycles: 94 °C for 4 min as initial denaturation followed by 35 cycles of 94 °C 40 s, 47 °C for 45 s 72 °C for 45 s and 72 °C for 20 min as final extension. Use of primers specific to mitochondrial cytochrome oxidase I (COX-I), (LCO-1490- 5´-

GGT CAA CAA ATC ATA AAG ATA TTG G -3'; HCO-2198- 5´- TAA ACT TCA GGG TGA CCA AAA AAT CA -3´; Hebert et al. 2003) resulted in the amplification of an approximately 700bp fragment. PCR was performed in 25 µL total reaction volume containing 20 pmoles of each primer, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP and 0.5 U of Taq DNA polymerase (Thermo Scientific, Fermentas). The amplified products were resolved in 1.0% agarose gel, stained with ethidium bromide (10 µg/ml) and visualized in a gel documentation system (UVP). For the species-specific primers mentioned in Table 1, PCR mix and PCR cycle parameters were the same except for annealing temperature, viz., 62 ˚C for 45 s for both *B. tau* and *B. zonata*.

Molecular Cloning and Sequencing

The PCR amplified fragments were eluted using Nucleospin® Extract II according to the manufacturer's protocol (Macherey-Nagel [MN], Germany) and ligated into the general purpose cloning vector, InsT/Aclone (Fermentas GmBH, Germany) according to the manufacturer's protocol. Five µL of the ligated vector was cloned into 200 µL of competent *Escherichia coli* (DH5?) cells by heat treatment at 42 °C for 45 s and the whole content was transferred into a tube containing 800 µL of SOC (tryptone - 2% w/v, yeast extract - 0.5% w/v, NaCl - 8.6 mM, KCl - 2.5 mM, MgSO4 - 2.0 mM, glucose - 20 mM in 1000 mL water, pH 7.0) and rotated at 150 rpm, 37 °C for 1 h; 200 µL of the above culture was spread on Luria Bertani agar (LBA) (tryptone - 10 g, yeast extract - 5 g,

TABLE 1. LIST OF TAXA EXAMINED WITH GEOGRAPHIC ORIGIN AND NCBI-GENBANK ACCESSION NUMBERS.

SPECIES	ORIGIN	COI
B (Bactrocera) correcta	India	GU323782
B (Bactrocera) zonata	India	GU323777
B (Bactrocera) verbascifoliae	India	DQ116350
B (Bactrocera) umbrosa	Indonesia	DQ116348
B (Bactrocera) trivialis	Papua New Guinea	DQ116342
B (Bactrocera) trilineola	Vanuatu	DQ116339
B (Bactrocera) tryoni	New Caledonia	DQ116337
B (Bactrocera) redunca	Vanuatu	DQ116330
B (Bactrocera) quadrisetosa	Vanuatu	DQ116328
B (Bactrocera) papayae	Thailand	DQ116326
B (Bactrocera) philippinensis	Philippines	DQ116320
B (Bactrocera) psidii	New Caledonia	DQ116313
B (Bactrocera) occipitalis	Indonesia	DQ116311
B (Bactrocera) melanotus	Cook Islands	DQ116299
B (Bactrocera) latifrons	USA	DQ116297
B (Bactrocera) kandiensis	Sri Lanka	DQ116295
B (Bactrocera) kirki	Tonga	DQ116294
B (Bactrocera) jarvisi	Australia	DQ116288
B (Bactrocera) frauenfeldi	Solomon Islands	DQ116287
B (Bactrocera) facialis	Tonga	DQ116285
B (Bactrocera) endriandrae	Australia	DQ116284
B (Bactrocera) distincta	Tonga	DQ116282
B (Bactrocera) caryeae	India	DQ116266
B (Bactrocera) curvipennis	New Caledonia	DQ116261
B (Bactrocera) carambolae	Malaysia	DQ116259
B (Bactrocera) cognata	Philippines	DQ116252
B (Bactrocera) arecae	Thailand	DQ116236
B (Bactrocera) aquilonis	Australia	DQ116235
B (Bactrocera) cacuminata	Unknown	DQ116241
B (Bactrocera) dorsalis	India	HQ658093
B (Zeugodacus) tau	India	GU323773
B (Zeugodacus) cucurbitae	Philippines	DQ116248
B (Notodacus) xanthodes	Vanuatu	DQ116356
B (Austrodacus) cucumis	Australia	DQ116237
B (Daculus) oleae	Italy	DQ116307
Ceratitis capitata	USA	DQ116371
Anastrepha ludens	Mexico	DQ116207

NaCl - 5 g, agar - 15 g in 1000 mL of water, pH-7.0) containing ampicillin (100 mg/mL), IPTG (4 mg/mL) and X-gal (40 mg/mL) and were incubated at 37 °C for 16 h. Blue/white selection was carried out and all the white colonies (colonies with insert) were maintained on LBA containing ampicillin (100 mg/mL), incubated at 37 °C overnight and stored at 4 °C. Plasmids were prepared from the overnight culture of the positive colonies cultured in LB broth (enzymatic casein - 10 g, yeast extract - 5 g, NaCl - 5 g in 1000 ml of water, pH-7.0) using modified alkali lysis method (Birnboim & Dolly 1979). Plasmids were resolved in 1.0% agarose gel, stained with ethidium bromide (10 µg/mL) and visualized by gel electrophoresis. Clones that had 2.5 kb as compared to control plasmid (1.8 kb) were selected for sequencing. For the purpose of sequencing, plasmids were isolated using GeneJET™ Plasmid Miniprep Kit (Fermentas, Germany) according to manufacturer's protocol from overnight cultures of the 5 randomly selected clones multiplied 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.

Homology searches carried out using BLAST (http://www.ncbi.nlm.nih.gov), and the differences in COX-1 sequences of *B. tau, B. correcta, B. zonata,* and *B. dorsalis* were determined using the sequence alignment editor BioEdit version 7.0.5.3 (Hall 1999). Sequences for *B. tau, B. correcta, B. zonata,* and *B. dorsalis* were deposited with the NCBI database, and the accession numbers were GU323773 - GU323782 and HQ658090 - HQ658093. For the development of species-specific markers for *B. tau* and *B. zonata*, 7 sets of forward and reverse primers were synthesized based on the variable regions in the aligned sequences of GU323774 - *B. tau* and GU323777 - *B. zonata*. The primers thus designed were tested on different identified *Bactrocera* species collected on mango and guava from the experimental farm of Indian Agricultural Research Institute (IARI), New Delhi, India. The PCR amplified fragments resulting from species-specific markers for *B. tau* and *B. zonata* were further cloned, sequenced and analyzed as above. Multiple sequences of all *B. tau, B. zonata, B. correcta,* and *B. dorsalis* from India and *Bactrocera* species from other geographical areas (acquired from GenBank) (Table. 1) were aligned using the multiple alignment programme BioEdit v.7.0.

Phylogenetic Analysis

Phylogeny was assessed using maximum parsimony (MP), and Bayesian phylogeny (BP) using PAUP v4b10 (Swofford 1998) and MrBayes v3.1.2 (Ronquist & Heulsenbeck 2003). MP analysis was performed with 1000 replicates with random sequence addition, TBR (tree bisection reconnection) swapping, and multrees option in effect. Parsimony Bootstrap support (BS) values were calculated based on 10,000 simple stepwise addition replicates with TBR branch swapping and 10 trees saved per replicate. For Bayesian analysis, the best-fit model of nucleotide evolution was evaluated using Modeltest v3.7 (Posada & Crandall 1998) based on the Akaike information criterion (AIC). MrBayes calculates the posterior probability of the phylogenetic tree based on the Bayes theorem and nucleotide substitution models used in maximum likelihood can be imple-

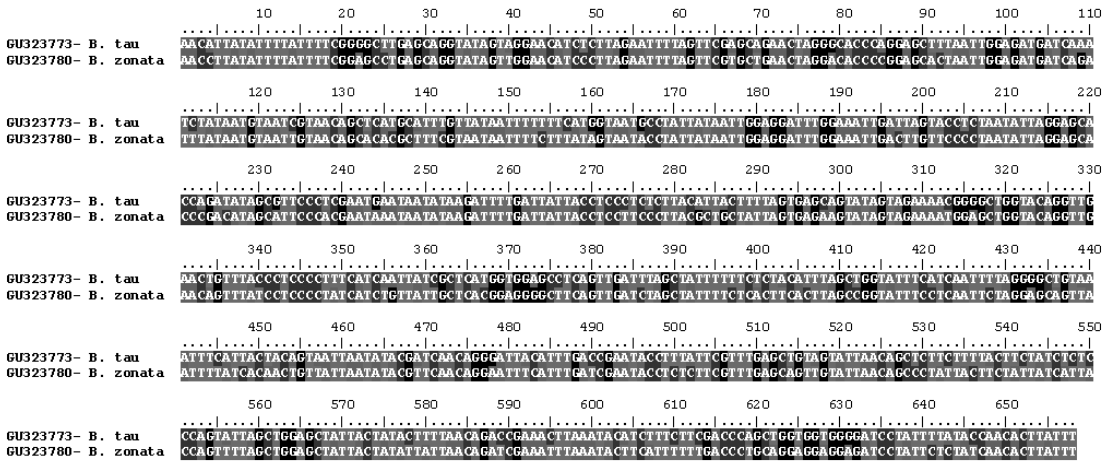

Fig. 1. Sequence comparison for *B. tau* and *B. zonata* from the mitochondrial Cytochrome Oxidase I (COX-I) gene showing differences. Analyses performed using BioEdit v.7.0.

TABLE 2. SPECIES-SPECIFIC MARKERS IDENTIFIED FOR *B. ZONATA* AND *B. TAU*.

Species	Primer	Binding Region (bp)	Product size (bp)
B. zonata	KBR(BZ)-F- 5'- TTT AGT TCG TGC TGA ACT AGG ACA CC -3'	58-83	
	KBR(BZ)-R- 5'- AAC TGG TAA TGA TAA TAG AAG TAA TAG G -3'	529-556	498
B. tau	KBR(BT)-F- 5'- CTC TCT TAC ATT ACT TTT AGT GAG C -3'	274-298	
	KBR(BT)-R- 5'- GAG AGA TAG AAG TAA AAG AAG A -3'	529-550	276

mented. The program uses Monte Carlo Markov Chain (MCMC) approach to approximate the posterior probability of the trees based on the best-fit model for that particular sequence. (Huelsenbeck & Ronquist 2001). The posterior probabilities (PP) of the clades were obtained by 2 independent runs with 4 MCMC each. The run was continued for 5 million generations and trees were sampled every hundredth generation resulting in 50,000 trees. The first 15% were considered as the burn in phase and the remaining 42,000 trees were summarized. The trees obtained were visualized using Figtree v1.3.1 (Rambaut 2009).

RESULTS AND DISCUSSION

The PCR amplicon of the same size (approx. 700bp) was amplified for both *B. tau* and *B. zonata*. Sequencing results showed that the total nucleotide length obtained was 658 bp for both *B. tau* and *B. zonata*. The BLAST search for the eight sequences viz. GU323773 - *B. tau* (egg), GU323774 - *B. tau* (adult), GU323775 - *B. tau* (larva), GU323776 - *B. tau* (pupa), GU323777 - *B. zonata* (adult), GU323778 - *B. zonata* (larva), GU323779 - *B. zonata* (pupa), GU323780 - *B. zonata* (egg) shows that both of the sequences had the maximum hits for the respective species. Alignment of the sequences for various developmental stages, viz., egg, larva, pupa and adult, did not show any differences in the COX-I nucleotide sequences, which clearly proved that molecular identification is not limited by stage of development, polymorphism and sex of the target species. Hence, it also proved that the species-specific marker developed can be extremely useful for molecular identification in any given developmental stage. *Bactrocera* fruit flies are difficult to identify in their immature stages and there is no definitive identification key available for their identification. Given the need to identify species in plant quarantine interceptions at the port of entry, the demonstrated the utility of the present study through use of a species-specific marker in any given developmental stage, in particular for the *B. zonata* and *B. tau*, the 2 species which occur on mango (*Mangifera indica* L.), is significant.

Alignment of the sequences for GU323774 - *B. tau* (adult) and GU323777 - *B. zonata* (adult) in BioEdit V. 7.0 shows that there were variations in 103 nucleotides out of 658 bp; amounting to a 15.65% difference between *B. tau* and *B. zonata* (Fig. 1). The rapid and accurate identification of invasive insect pest species at any given time and for all the developmental stages is important in the aspect of biosecurity and quarantine purpose. The species-specific markers require only conventional PCR (with degenerate primer sequences) which is readily available, rapid and inexpensive for molecular identification of species in question. Out of 12 primer sets identified each for *B. tau* and *B. zonata*. One primer set, viz. Rebi(BT)- F & Rebi(BT)- R and Rebi(BZ)- F & Rebi(BZ)- R, could successfully identify *B. tau* and *B. zonata,* respectively.(Table 2; Fig. 2). These species-specific markers amplified an expected fragment size of 280 bp and 498 bp for *B. tau* and *B. zonata,* respectively. Cloning and sequencing of these products and BLAST search of these sequences shows a maximum hit for the respective species only, which is already sequenced and submitted to NCBI-GenBank. Similarly, same size bands were obtained from test fruit fly specimens collected from mango and guava and was corroborated with morphological markers.

The validation of these species-specific markers, which were developed in the present study, were carried out by employing PCR using both these primers and genomic DNA isolated from the following *Bactrocera* species, viz., *B. correcta, B. caryeae, B. dorsalis,* and *B. cucurbitae,* which

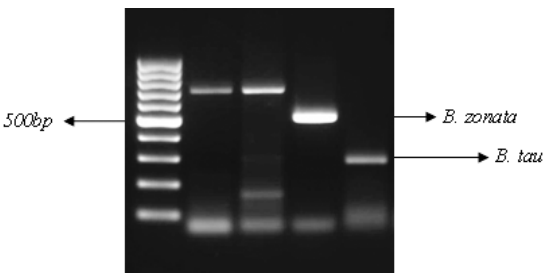

Fig. 2. Validation of species-specific markers for *B. zonata* and *B. tau* (M - 100bp DNA ladder, (Thermo Scientific- Fermentas) 1 & 2 - PCR amplified product (COX-I) *B. zonata and B. tau* respectively, 3 - *B. zonata*- specific marker, 4 - *B. tau*- specific marker.

were morphologically identified and collected from IARI, New Delhi. None of the PCR reactions produced any amplification, including non-specific amplicons, where the PCR mix and PCR cycling conditions were kept same, including the annealing temperature, viz., 62 °C for 45 s, for all the reactions. However the applicability of these primers on other populations of *B. tau* & *B. zonata* within and outside India depend on the variation in the nucleotide sequences both in forward (274-298 and 58-83 for *B. tau* and *B. zonata,* respectively) and reverse primer binding regions (529-550 and 529-556 for *B. tau* and *B. zonata*, respectively). Comparison of forward primer binding regions for *B. tau* (GU323776) with only 1 existing COX-I (658 bp) deposit showed that there were variations at 2 positions viz., at the 276th position 1 accession had T instead of C, and at the 278th position 1 accession had T instead of C. Similarly examination of the reverse primer binding region showed that there was no variation in any of the nucleotide positions. A comparison of the forward primer binding region for *B. zonata* (GU323780) with 4 other NCBI accessions showed that there were variations at 8 positions, viz., at the 59th position 1 accession had G instead of T; at the 60th and 67th positions 1 accession had A instead T; at the 64th and 76th position 1 accession had C instead of T & A, respectively; at the 74th and 79th position 1 accession had T instead of C & A, respectively; at the 73rd position 1 accession had G and another having T instead of A. Similarly in the reverse primer binding region, variations were found at the 529th, 530th and 539th positions, where 2 accessions had T instead of C. In this regard, development of degenerate primers would be a valuable tool in identifying the other populations of *B. tau* and *B. zonata*. Other criteria to be taken into consideration in developing a species-specific marker for *Bactrocera* species are intra- and inter-specific variations (Bayar et al. 2002; Brunner et al. 2004). Color morphs and size variations in *Bactrocera* species are influenced by many factors, which could be impediments for species identification. Hence molecular identification using species-specific markers is an advantage where there is polymorphism in the target species. Development of species-specific markers for *B. tau* and *B. zonata* would be of immense value to identify these invasive pests at any developmental stages like egg, larva, pupa or adult. Hence, the species-specific markers developed in this study could be used for the identification of *B. tau* and *B. zonata* in any stage of development without even sequencing. The species-specific marker is also available tool when there is a problem of polymorphism as it has been shown in the case of *Ceratitis capitata* (Wiedemann) and *Anastrepha fraterculus* (Wiedemann) (Sonvico et al. 1996). In addition to the above, molecular identification could reveal multiple infestations as against the morphological identification, which could be used to separate the species (Barbosa et al. 2005).

In recent years mitochondrial DNA has become the common molecular marker in phylogenetic analysis and population genetic studies in animals (Langor & Sperling 1997), because it has the advantage that rare mutations create new haplotypes. Therefore, 2 individuals that share the same haplotypes are likely to have a common ancestor (Li 1997). Mitochondrial DNA has also been used in analyze phylogenetic relationships among tephritid fruit fly species (Smith et al. 2003, 2005; Zhang et al. 2010), because the COX-I sequences provide slightly better resolution, and also quantitative support in terms of bootstraps and divergence values for species - level identification than was previously possible (Armstrong 1997). Based on the mitochondrial DNA sequence data, the phylogenetic relationship of some tephritid taxa has been resolved (Han 2000) especially at the generic level (Smith & Bush 1997). In this connection, we used a nucleotide sequence of 658 bp variable region of the mitochondrial cytochrome oxidase I (COX-I), for constructing the phylogenetic relationship among the five subgenera of *Bactrocera*. The aligned dataset consisted of 600 nucleotides with 193 variable sites which were parsimoniously informative. The heuristic MP search resulted in 4 equally parsimonious trees with 995 steps (CI = 0.360, RI = 0.511, HI = 0.639). All of the parsimonious trees were similar in topology and one of the trees is shown in Figure 3. The best model of nucleotide substitution according the AIC was GTR + I + G. The Bayesian phylogenetic analysis implementing GTR + I + G resulted in a consensus tree similar in topology to the parsimony analysis except for minor changes in not well supported nodes (figure not shown). The 30 ingroup taxa belonging to *Bactrocera* subgenus. *Bactrocera* were retrieved as a monophyletic group with good statistical support at the nodes (BS = 89% and PP = 1.0: Fig. 3). COX-I sequences of the same region for *Ceratitis capitata* and *Anastrepha ludens* were used in the analysis as out groups.

Researchers have proposed a phylogentic analysis of the *Bactrocera* subgenus groupings based on morphological characters (Drew & Hancock 2000; White 2000). Muraji & Nakahara (2001) used mitochondrial DNA sequences of 18 *Bactrocera* species in 4 subgenera, indicated that *B.* (*Bactrocera*) was paraphyletic and *B.* (*Zeugodacus*) was monophyletic. Drew (1989) divided the subgenera of *Bactrocera* into 4 groups, viz. *Bactrocera* group, *Queenslandacus* group, *Zeugodacus* group and *Melanodacus* group. There are many views on the phylogeny of the subgenus *Bactrocera* whether it is monophyletic or paraphyletic (Zhang et al. 2010). In this regard, White (2000) reported that *Bactrocera* is paraphyletic in

both weighted and unweighted analysis, which was also supported by the work of Muraji & Nakahara (2001) and Zhang et al. (2010). On the contrary, Smith et al. (2003) reported that the subgenus *Bactrocera* is monophyletic based on the analyses of mitochondrial DNA sequences. In the present study, by employing both maximum parsimony and likelihood analyses (as implemented in MrBayes) we have shown that the subgenus *Bactrocera* is monophyletic (Fig. 3).

From the above phylogenetic analysis, our result supports the morphological group classifica-

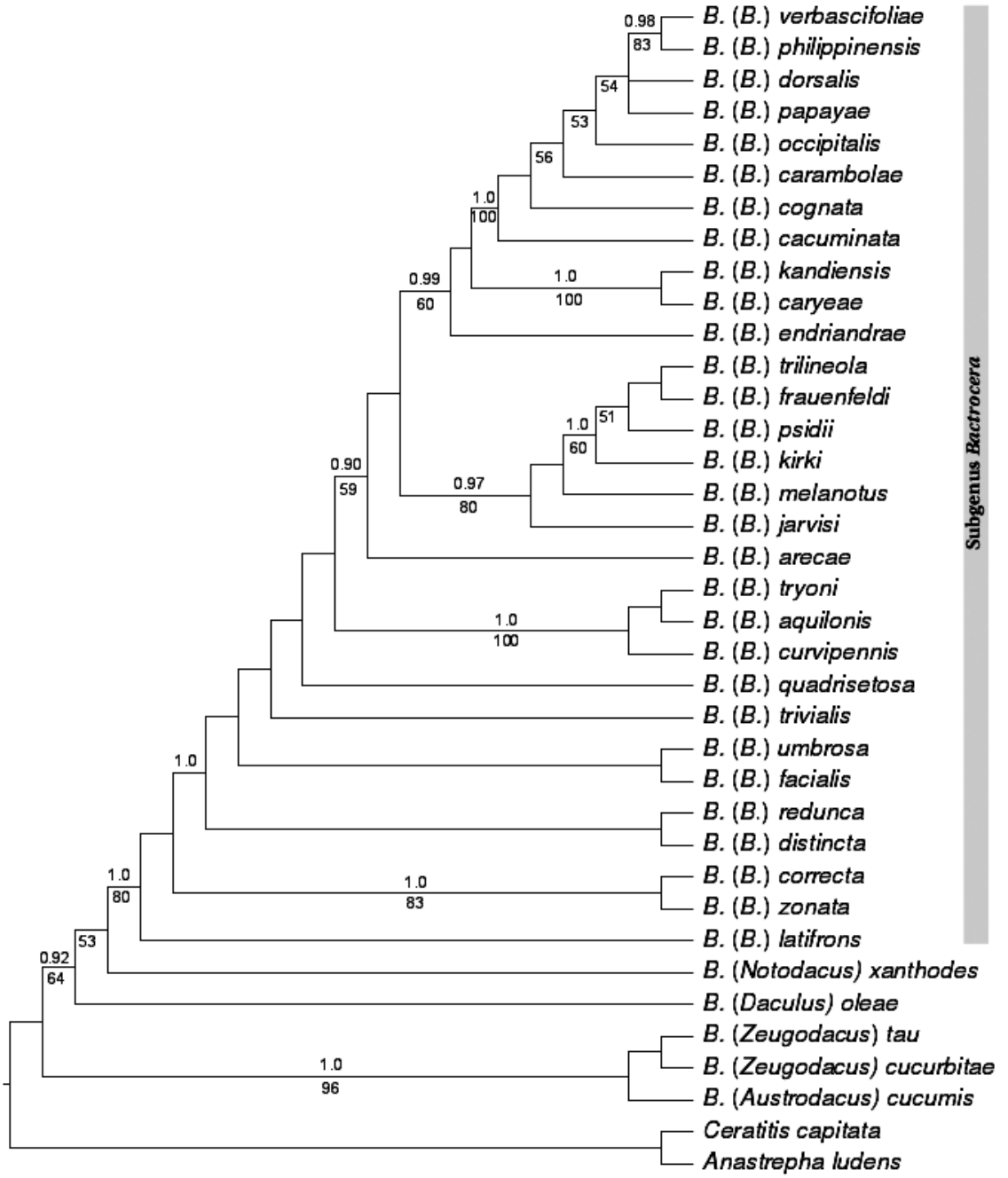

Fig. 3. One of the four equally parsimonious trees obtained from MP analysis of the COX-I dataset. Bayesian posterior probabilities (PP) > 0.9 are indicated above the branches and bootstrap > 50% are indicated below the branches. *Ceratitis capitata* (DQ116371) and *Anastrepha ludens* (DQ116207) were used as out groups.

tion: *Bactrocera* group (*Bactrocera*) and *Zeugodacus* group (*Zeugodacus* + *Austrodacus* + *Notodacus*) (Drew & Hancock 2000). This study showed that the subgenus *Zeugodacus* is not paraphyletic, since *Austrodacus* is not located within the *Zeugodacus* clade because there is a 100% bootstrap separating them. Here the result was against Smith et al. (2003), who proposed that the subgenus *Zeugodacus* is paraphyletic. Whereas Muraji & Nakahara (2001) reported that the subgenus *Zeugodacus* is a monophyletic clade. The subgenus *Daculus* represented by *B.* (*Daculus*) *oleae*, shows different classification positions with maximum parsimony and likelihood analyses (as implemented in MrBayes). Drew (1989) suggested *B* (*Daculus*) *oleae* belongs to the *Melanodacus* group and did not fall into the *Bactrocera* group or *Zeugodacus* group. Whereas Smith et al. (2005) indicated that *B.* (*Daculus*) *oleae* is a sister group to *B. (Bactrocera)* group. Our study indicated subgenera *Daculus* and *Notodacus* are a single lineage by itself, and the subgenus *Austrodacus* is closely related to the subgenus *Zeugodacus* (Fig. 3).

Considering the pest status, it is necessary to analyze the molecular diversity in all 4 important Indian *Bactrocera* species viz. *B. tau*, *B. correcta*, *B. zonata* and *B. dorsalis*. For molecular diversity analysis, all the corresponding species sequences were acquired from NCBI-GenBank. The phylogram for the 19 NCBI accessions of *B. dorsalis* revealed that there were 2 major groups, namely group I, which represents the *B. dorsalis* population from India, Taiwan, China, New Zealand, Tanzania and Vietnam, while the Group II is the population from USA (Fig. 4); whereas analysis of the 7 available NCBI accessions of *B. zonata* revealed that Group I represents India, Russia, Africa and Egypt, while group II is the population from Pakistan (Fig. 5). For *B. correcta* detailed information is lacking, nevertheless the phylogram for the available accessions is shown in Fig. 6.

In the present study we showed the utility of species-specific markers which can be used for the species identification of *B. tau* and *B. zonata* in any of the developmental stages. Thus it will help in timely, accurate and stage non-limiting identification of these 2 species of fruit flies, which will in turn help for better quarantine purpose. Our results indicated that the COX-I gene is useful for inferring the phylogeny of *Bactrocera* taxa included in the present study. Clearly more taxa must be analyzed and more data, particularly from genes that show sufficient information vari-

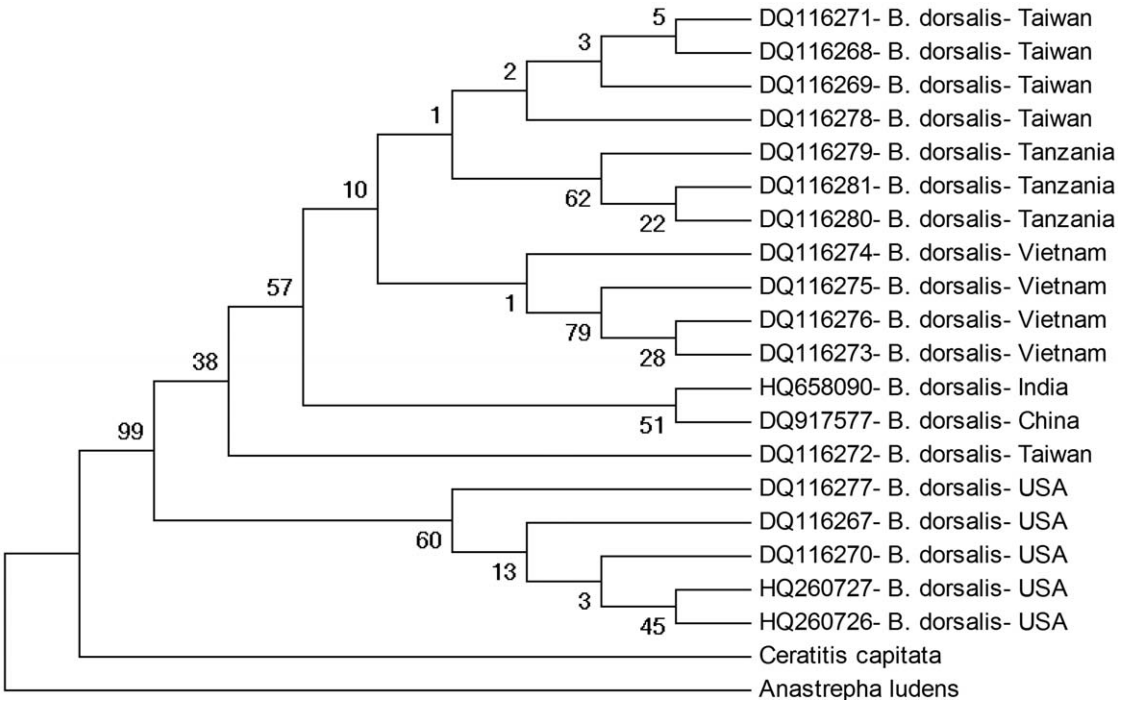

Fig. 4. Maximum- Parsimony (MP) tree (MEGA. 4.0) with bootstrap support (1000 replicates) showing clustering of *B. dorsalis* species for COX-I sequences. Two distinct clades can be seen in which, the Indian populations of *B. dorsalis* clustered with the Taiwan, Vietnam, Tanzania, China and New Zealand population and USA populations stand as a separate clade with 100% bootstrap support. *Ceratitis capitata* (DQ116371) and *Anastrepha ludens* (DQ116207) were used as out groups.

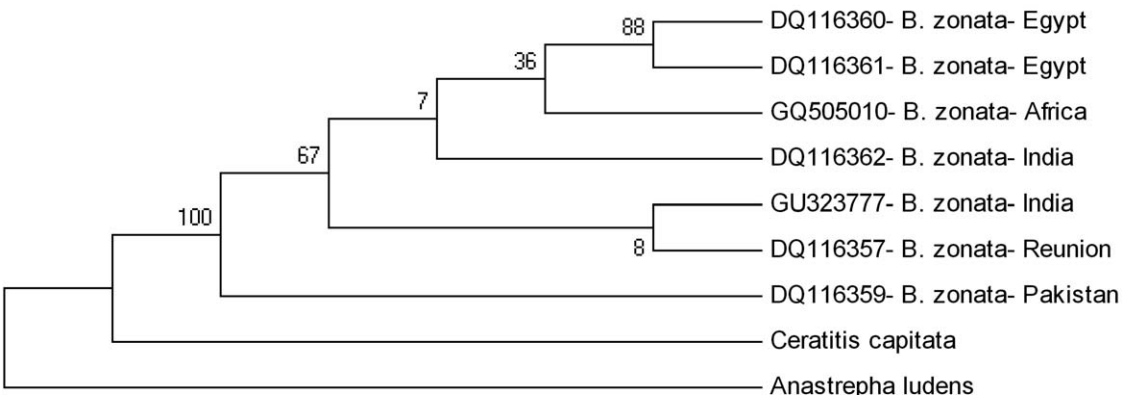

Fig. 5. Maximum- Parsimony (MP) tree (MEGA. 4.0) with bootstrap support (1000 replicates) showing clustering of *B. zonata* species for COX-I sequences from various countries. *Ceratitis capitata* (DQ116371) and *Anastrepha ludens* (DQ116207) were used as out groups.

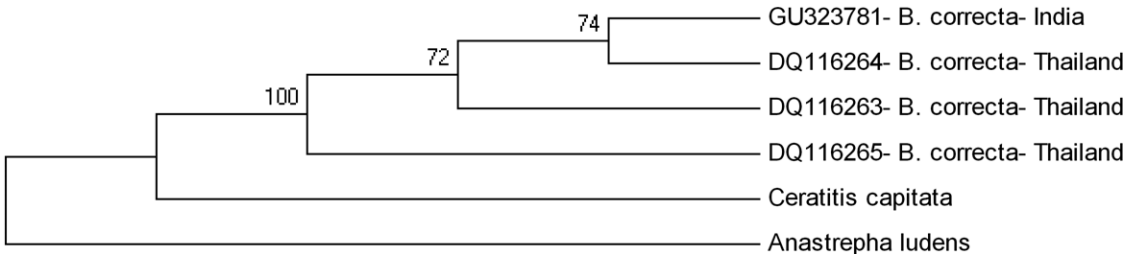

Fig. 6. Maximum- Parsimony (MP) tree (MEGA. 4.0) with bootstrap support (1000 replicates) showing clustering of *B. correcta* species for COX-I sequences from India and Thailand. *Ceratitis capitata* (DQ116371) and *Anastrepha ludens* (DQ116207) were used as out groups.

ation to resolve some of the internal nodes, are needed (Smith et al. 2003). However our maximum parsimony and Bayesian analysis suggested that the subgenus *Bactrocera* is monophyletic.

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