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RAPID COMMUNICATION

Assessing the applicability of nuclear 18S and ITS2-28S rRNA genes for diagnostics of *Bactrocera dorsalis* (Diptera: Tephritidae) in the Philippines

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Abstract

Fruit flies belonging to the *Bactrocera dorsalis* species complex pose a significant threat to mangoes and other crops in the Philippines and worldwide. Identifying cryptic species within this complex is challenging, particularly when relying solely on morphological analysis. In this study, we sequenced two fragments of the nuclear 18S and ITS2-28S rRNA genes from specimens of *Bactrocera dorsalis* Hendel collected in the Philippines to assess their applicability for species diagnostics. Subsequent sequencing and analysis revealed that the 18S and 28S rRNA gene fragments matched *B. dorsalis* sequences in NCBI but also displayed high similarity with other *Bactrocera* and insect species. On the other hand, sequences of the ITS2 segment showed hits specific to *B. dorsalis*. Further analysis of the 18S rRNA gene in fruit flies collected from various sources and host plants in the country suggests conserved sequences among *Bactrocera* samples, irrespective of collection site and host plant species. In conclusion, our findings suggest that, among the tested nuclear DNA fragments, only the ITS2 demonstrates sufficient species-level nucleotide variation for effective use as a molecular diagnostic marker for *B. dorsalis* identification.

Keywords: molecular diagnostics, nuclear genes, oriental fruit fly, quarantine pest

Fruit flies in the *Bactrocera dorsalis* species complex are among the most damaging insect pests of fruit and other crops worldwide. Proper identification of cryptic species within a species complex is essential but remains a huge challenge, especially when morphological analysis is insufficient for species-level identification. Thus, the *Bactrocera dorsalis* species complex has been subjected to taxonomic studies that aim to delineate its members using molecular tools (Schutze *et al.* 2014). Mitochondrial genes, including cytochrome oxidase (cox) and NADH dehydrogenase (nad), have been demonstrated to be useful for species diagnostics, identification, and phylogenetic analysis of *Bactrocera* species (Boykin *et al.* 2014; Choudhary *et al.* 2016; Qin *et al*. 2018; Cortaga and Sison 2021). Meanwhile, nuclear genes such as the 18S and 28S rRNA genes, and the internal transcribed spacer 2 (ITS2) segment, which are also used in insect diagnostics and phylogenetic studies (Kjer 2004; Latina *et al.* 2022), are relatively less examined in *Bactrocera*. Thus, in this paper, we sequenced two fragments of the nuclear 18S and ITS2-28S rRNA genes of *B. dorsalis* Hendel from the Philippines to assess their applicability for species diagnostics.

Bactrocera dorsalis from infested mango fruits at the Institute of Plant Breeding, University of the Philippines Los Baños were reared in the laboratory as described by Sison *et al.* (2020). Adults were examined using the morphological keys by White and Hancock (1997) and Iwahashi (1999). DNA was extracted from five adult insects through a modified Dellaporta extraction method (Dellaporta *et al*. 1983). The PCR protocol was performed as described by Sison *et al.* (2020), using the designed forward and reverse primers (Table 1) to amplify two fragments found at the 5' regions of the nuclear genes 18S and 28S rRNA,

and one fragment found at the 3' region of ITS2 (Fig. 1). PCR products were resolved in 1.5% agarose gel in 0.5x TAE buffer and viewed using Gel Doc™ XR+ Gel Documentation System (Bio-Rad Laboratories, Inc.). The amplified gene fragments were sequenced for the forward and reverse strands (1st BASE Laboratories, Malaysia) and the sequences were processed using Geneious (version 2019.0.4). After trimming the low-confidence end sequences, we obtained the final sequence length for analysis (Table 2). The two fragments of the 18S rRNA gene include a 715 bp segment (amplified by 18S-1F/R primers) and a 777 bp segment (amplified by 18S-2F/R primers) located at the 5' region (Tables 1, 2; Fig. 1). For 28S, we tested the combination of ITS2 and 28S rRNA as these are adjacent segments (Tables 1, 2; Fig. 1). For this, the forward primer was designed at the 3' region of ITS2 (315 bp) and the reverse primer was designed at the adjacent 5' start of 28S (340 bp), producing the ITS2-28S fragment with a total sequence length of 655 bp (amplified by ITS2-1F/28S-1R primers) (Tables 1, 2; Fig. 1). A second 28S fragment at the 5' region was also amplified (749 bp) using the 28S-2F/R primers (Tables 1, 2; Fig. 1). Multiple sequence alignment via ClustalW of adult *B. dorsalis* insects showed no nucleotide variation, thus, a consensus sequence was obtained in each gene fragment. The consensus sequences were analyzed through nucleotide BLAST (BLASTn) and results were filtered with 98–100% identity to display only significant hits with high sequence similarity.

BLASTn analysis of the DNA fragments from 18S and 28S rRNA genes amplified from laboratory reared

Table 1. Primers designed for amplification of two fragments of nuclear 18S and ITS2-28S rRNA genes of *Bactrocera dorsalis*

Gene	Primer name	Primer sequence (5′ → 3′)	Expected band size [bp]
18S rRNA	18S-1F	CTGGTTGATCCTGCCAGTAGT	723
	18S-1R	GAACTCCACCGGTAATACGCT	
	18S-2F	CGCGGTAATTCCAGCTCCAA	799
	18S-2R	TCCACGAACTAAGAACGGCC	
ITS2 and 28S rRNA	ITS2-1F	TCTTGAATACCTCATATTTGAACGAAA	751
	28S-1R	ACTTTCCCTCACGGTACTTGT	
	28S-2F	GCCAGGCCCGTATAACGTTA	758
	28S-2R	GCTCAAGGTACGCTCCAGTT	

Table 2. Significant BLASTn hits with high similarity to the 18S and ITS2-28S rRNA gene fragments of *Bactrocera dorsalis*

Primer pair (and sequence length analyzed)	Species	% Query Cover	E-value	% Identity	No. of significant hits
18S-1F/R (715 bp)	*Bactrocera dorsalis*	100	0	100	9
	Bactrocera tryoni	100	0	98.18 to 100	10
	Zeugodacus depressus (syn. *Bactrocera depressa*)	95	0	99.85	1
	Zeugodacus cucurbitae (syn. *Bactrocera cucurbitae*)	99 to 100	0	98.88 to 99.72	212
	Ceratitis capitata	97	0	99.71	1
	Anastrepha obliqua	100	0	98.74	268
	Melieria crassipennis	100	0	98.74	1
	Rhagoletis zephyria	100	0	98.61	6
	Rhagoletis mendax	100	0	98.61	1
	Rhagoletis pomonella	100	0	98.33 to 98.61	9
	Rhagoletis cornivora	100	0	98.61	2
	Anastrepha fraterculus	100	0	98.60	1
	Anastrepha ludens	100	0	98.19	1
	Heleomyzidae sp. Hele_H1	98	0	98.16	1
	Eristalis pertinax	100	0	98.32	1

Table 2. Significant BLASTn hits with high similarity to the 18S and ITS2-28S rRNA gene fragments of *Bactrocera dorsalis – continued*

Primer pair (and sequence length analyzed)	Species	% Query Cover	E-value	% Identity	No. of significant hits
18S-2F/R (777 bp)	*Bactrocera dorsalis*	100	0	100	9
	Ceratitis capitata	100	0	99.74 to 100	2
	Bactrocera tryoni	100	0	99.23 to 99.87	10
	Anastrepha obliqua	100	0	99.74	405
	Anastrepha ludens	100	0	99.49 to 99.74	84
	Zeugodacus cucurbitae (syn. *Bactrocera cucurbitae*)	100	0	98.22 to 99.61	221
	Melieria crassipennis	100	0	99.61	1
	Bactrocera correcta	100	0	99.36	1
	Rhagoletis cornivora	100	0	99.74	2
	Rhagoletis zephyria	100	0	99.36	6
	Rhagoletis mendax	100	0	99.36	1
	Rhagoletis pomonella	100	0	99.23 to 99.36	9
	Anastrepha fraterculus	100	0	98.08	1
	Chamaepsila rosae	99	0	98.97	1
	Spelobia bifrons	100	0	98.71	1
	Coremacera marginata	100	0	98.46	1
	Sepsis cynipsea	100	0	98.07	1
	Minettia flaveola	99	0	98.19	1
	Pseudogonia rufifrons	100	0	98.33	1
	Gonia chinensis	100	0	98.07	1
	Thecocarcelia acutangulata	100	0	98.07	1
	Eristalis arbustorum	100	0	98.07	1
	uncultured eukaryote	100	0	98.58 to 98.97	14
ITS2-1F/28S-1R (655 bp)	*Bactrocera dorsalis*	51 to 56	0 to 2e-174	99.20 to 100	3
	Zeugodacus cucurbitae (syn. *Bactrocera cucurbitae*)	51	2e-174 to 3e-172	99.71 to 100	108
	Galleria mellonella	51	2e-174	100	1
	Anastrepha ludens	51	2e-168 to 3e-171	98.83 to 99.41	91
	Anastrepha obliqua	51	2e-169 to 7e-168	98.82 to 99.41	275
	Bactrocera tryoni	51	2e-169 to 9e-173	98.82 to 100	4
	Rhagoletis pomonella	51	7e-173	99.71	1
	Palloptera scutellata	52	7e-168	98.27	1
3′ region of ITS2 (315 bp)	*Bactrocera dorsalis*	56 to 100	1e-107 to 9e-129	98.82 to 100	89
5′ start of 28S (340 bp)	*Bactrocera dorsalis*	99	7e-175	100	2
	Zeugodacus cucurbitae (syn. *Bactrocera cucurbitae*)	99	1e-172 to 7e-175	99.71 to 100	108
	Bactrocera tryoni	99	1e-172 to 7e-175	98.82 to 100	4
	Galleria mellonella	99	7e-175	100	1
	Rhagoletis zephyria	100	1e-173	99.71	6
	Rhagoletis mendax	100	1e-173	99.71	1
	Rhagoletis pomonella	99 to 100	1e-173 to 4e-172	99.41 to 99.71	8
	Rhagoletis cornivora	100	1e-173	99.71	2
	Anastrepha ludens	99	1e-168 to 2e-171	98.83 to 99.41	91
	Anastrepha obliqua	99	2e-171 to 7e-170	98.54 to 99.41	276
	Melieria crassipennis	100	2e-166	98.53	1

Table 2. Significant BLASTn hits with high similarity to the 18S and ITS2-28S rRNA gene fragments of *Bactrocera dorsalis – continued*

Primer pair (and sequence length analyzed)	Species	% Query Cover	E-value	% Identity	No. of significant hits
28S-2F/R (749 bp)	*Bactrocera dorsalis*	100	0	100	2
	Bactrocera tryoni	100	0	98.93 to 99.60	4
	Bactrocera tryoni complex sp. PHEL-12	77	0	99.48	1
	Bactrocera tryoni complex sp. PHEL-9	77	0	99.31	1
	Bactrocera tryoni complex sp. PHEL-10	77	0	98.97	1
	Galleria mellonella	100	0	99.33	1

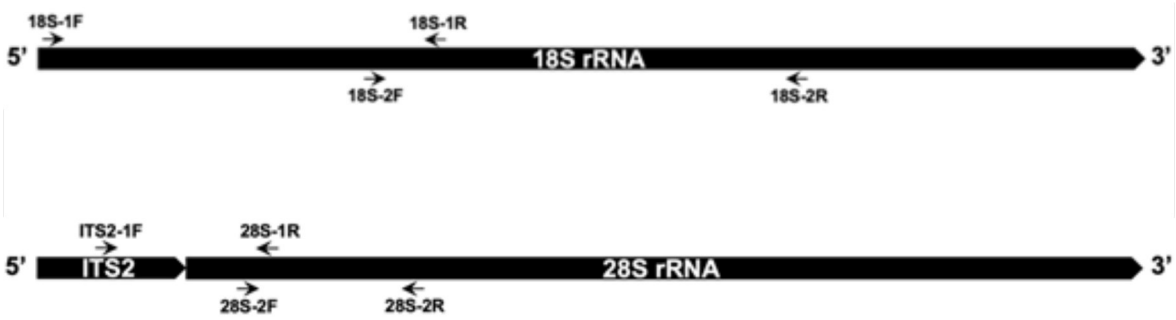

Fig. 1. Position of primers amplifying the 18S and ITS2-28S rRNA gene fragments of *Bactrocera dorsalis*

B. dorsalis showed matches with *B. dorsalis* sequences in NCBI. However, high similarity in one or both genes was also consistently observed from sequences of other *Bactrocera* species (such as *B. tryoni*, *B. cucurbitae*, and *B. correcta*) and other insect species such as *Rhagoletis* sp., *Anastrepha* sp., *Ceratitis capitata*, *Melieria crassipennis*, among others (Table 2). Notably, the insect species identified through BLASTn (excluding *Galleria mellonella*) are generally classified under the taxonomic order Diptera, suggesting that the 18S and 28S rRNA genes are relatively conserved within this order. On the other hand, the ITS2-28S fragment (655 bp) amplified by the ITS2-1F/28S-1R primer pair showed sequence hits from *B. dorsalis*, including other insect species such as *B. tryoni*, *G. mellonella*, and others (Table 2). However, when the 3' region of ITS2 sequence (315 bp) located upstream of the 28S sequence was isolated and analyzed, many highly similar sequences were observed from *B. dorsalis* with more than 80 hits (Table 2).

The results obtained indicate that among the fragments analyzed, only the ITS2 harbors adequate nucleotide variations which makes it a useful molecular diagnostic marker for *B. dorsalis*. Since both 18S and 28S rRNA genes displayed similar results, the 18S rRNA gene was further analyzed to check for nucleotide variation in this gene through collection and sequencing of 40 fruit flies. For this, we analyzed the first 18S fragment (amplified by 18S-1F/R primers) or the 18S-1 segment of two sympatric fruit fly pests of mango in the Philippines, i.e., *B. occipitalis* Bezzi and *B. dorsalis* (syn. *B. philippinensis* Drew & Hancock) collected from diverse sources. The fruit fly-infested fruits of mango and other host plants (such as starfruit and guava) were collected from different provinces in the Philippines, namely, Zambales, Laguna, Quezon, Oriental Mindoro, Guimaras, Cebu, and Cotabato (Table 3). The insect rearing method, PCR, DNA sequencing, and data analysis were performed as previously described. Multiple sequence alignment

Table 3. Number of *Bactrocera dorsalis* and *Bactrocera occipitalis* collected from various sources used for 18S-1 analysis

Location	Host Plant	*B. dorsalis*	*B. occipitalis*
Castillejos, Zambales	Mango	5	
Los Baños, Laguna	Mango	5	
	Guava	5	
	Starfruit	5	
Tiaong, Quezon	Mango	3	2
Calapan City, Oriental Mindoro	Mango	5	
Jordan, Guimaras	Mango	4	
Cebu City, Cebu	Mango		1
Kidapawan City, Cotabato	Mango	4	1
Total = 40 insects			

displayed no intra- and inter-specific variation in the 18S-1 sequence of *B. dorsalis* and *B. occipitalis* samples collected, thus showing similar sequences regardless of collection site, source host plant, and species.

In summary, this study showed that the 18S and 28S rRNA sequences of *B. dorsalis* exhibit high similarity with those of other *Bactrocera* and insect species. On the other hand, the ITS2 sequences were able to identify and delineate the *B. dorsalis* from other *Bactrocera* and insect species. These suggest that among the nuclear DNA fragments analyzed, only the ITS2 is amenable for potential use as a marker for diagnostics and other genetic studies (e.g., genetic diversity and structure, phylogenetic analysis, etc.) of *B. dorsalis*, while the 18S and 28S rRNA genes are relatively conserved and lack sufficient species-level nucleotide variations.

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