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Authors: Barr, Norman B., Hauser, Martin, Belcher, Jennifer, Salinas, David, Schuenzel, Erin, et al.

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Use of ITS-1 to identify *Bactrocera dorsalis* and *Bactrocera occipitalis* (Diptera: Tephritidae): a case study using flies trapped in California from 2008 to 2018

Norman B. Barr[1,], Martin Hauser[2], Jennifer Belcher[1,3], David Salinas[3], Erin Schuenzel[3], Peter Kerr[2], and Stephen Gaimari[2]*

Abstract

Molecular methods are necessary to diagnose immature life stages of the agricultural pest fruit fly *Bactrocera dorsalis* (Hendel), and are useful to corroborate identifications based on adults because morphological variation within the species can overlap with congeners. DNA sequencing of the nuclear ribosomal internal transcribed spacer 1 (ITS-1) has been adopted by the International Plant Protection Convention as an internationally accepted method to distinguish between the 2 pestiferous fruit fly species *Bactrocera dorsalis* and *Bactrocera carambolae* (Drew & Hancock). Reported ITS-1 sequences also are distinct and diagnostically informative to distinguish several other *Bactrocera* species related to *B. dorsalis*. In this study, we applied DNA sequencing of ITS-1 to a collection of 513 adult flies trapped in California, USA, in the yr 2008 to 2018. Internal transcribed spacer 1 sequences were successfully recovered from 504 (98%) of these flies. One fly had an ITS-1 sequence that matched *B. occipitalis* (Bezzi) records. Re-examination of that fly using cytochrome c oxidase I, elongation factor 1-alpha, and morphology supports it as the second record of *B. occipitalis* trapped in California. The other 503 flies had ITS-1 sequences consistent with *B. dorsalis*. Six unique ITS-1 sequences (or DNA types) were observed in the collection of 503 *B. dorsalis*. Three of the ITS-1 sequences (types A, B, and C) were present in 84% of the 503 flies and match ITS-1 records reported in prior publications on *B. dorsalis*. The other 3 sequences (types D, E, and F) observed in 4% of the 503 *B. dorsalis* have not been reported in publications. Ambiguous nucleotides were observed from 12% of the 503 *B. dorsalis* flies, precluding designation of a sequence type. Including the 3 new types from the current study, a total of 15 unique ITS-1 sequences now are known for *B. dorsalis.* The study, therefore, documents additional intraspecific variation of ITS-1 that aids in future applications for species identification.

Key Words: *dorsalis* complex; internal transcribed spacers; diagnostics; invasive species

Resumen

Los métodos moleculares son necesarios para diagnosticar los estadios de vida inmaduras de la plaga agrícola mosca de la fruta *Bactrocera dorsalis* (Hendel) y son útiles para corroborar identificaciones basadas en adultos por la variación morfológica dentro de la especie puede superponerse con congéneres. La secuenciación del ADN del espaciador transcrito interno ribosómico nuclear 1 (ITS-1) ha sido adoptada por la Convención Internacional de Protección Fitosanitaria como un método aceptado internacionalmente para distinguir entre las dos especies de moscas de la fruta, *Bactrocera dorsalis* y *Bactrocera carambolae* (Drew & Hancock). Las secuencias de ITS-1 notificadas también son distintas y proporcionan información diagnóstica para distinguir varias otras especies de *Bactrocera* relacionadas con *B. dorsalis*. En este estudio, aplicamos la secuenciación de ADN de ITS-1 a una colección de 513 moscas adultas atrapadas en California, EE. UU. desde el 2008 hasta el 2018. Se recuperaron las secuencias espaciadoras transcritas internas1 con éxito de 504 (98%) de estas moscas. Una mosca tenía una secuencia ITS-1 que coincidía con los registros de *B. occipitalis* (Bezzi). El reexamen de esa mosca usando la citocromo c oxidasa I, el factor de elongación 1-alfa y la morfología lo respalda como el segundo registro de *B. occipitalis* atrapada en California. Las otras 503 moscas tenían secuencias de ITS-1 compatibles con *B. dorsalis*. Se observaron seis secuencias únicas de ITS-1 (o tipos de ADN) en la colección de 503 *B. dorsalis*. Tres de las secuencias de ITS-1 (tipos A, B, y C) estaban presentes en el 84% de las 503 moscas y coinciden con los registros de ITS-1 informados en publicaciones anteriores sobre *B. dorsalis*. Las otras 3 secuencias (tipos D, E, y F) observadas en el 4% de las 503 *B. dorsalis* no han sido reportadas en publicaciones. Se observaron nucleótidos ambiguos en el 12% de las 503 moscas *B. dorsalis*, lo que excluye la designación de un tipo de secuencia. Incluyendo los 3 nuevos tipos del estudio actual, ahora se conocen un total de 15 secuencias ITS-1 únicas para *B. dorsalis*. Por lo tanto, el estudio documenta una variación intraespecífica adicional de ITS-1 que ayuda en futuras aplicaciones para la identificación de especies.

Palabras Claves: complejo dorsal; espaciadores internos transcritos; diagnósticos; especies invasivas

[1]USDA APHIS PPQ Mission Laboratory, 22675 North Moorefield Road, Edinburg, Texas 78541, USA; E-mail: Norman.B.Barr@usda.gov (N. B. B.), Jennifer.Belcher@usda.gov (J. B.)

[2]California State Collection of Arthropods, Plant Pest Diagnostics Branch, California Department of Food and Agriculture, 3294 Meadowview Road, Sacramento, California 95832–1448, USA; E-mail: martin.hauser@cdfa.ca.gov (M. H.), peter.kerr@cdfa.ca.gov (P. K.), sgaimari@gmail.com (S. G.)

[3]Department of Biology, University of Texas Rio Grande Valley, 1201 West University Drive, Edinburg, Texas 78539, USA; E-mail:dasalinas92@protonmail.com (D. S.), erin.schuenzel@utrgv.edu (E. S.)

*Corresponding author; E-mail: Norman.B.Barr@usda.gov

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Accurate identification of agricultural pests that are trapped during surveillance or intercepted during inspections provides important information that may be used for trend and risk analysis (Garzón-Orduña et al. 2020; Lyal & Miller 2020). For example, identification of intercepted insects may be used to evaluate high risk pathways, and the level of diagnosis of these intercepted insects (e.g., to species, genus, or family level) may impact interpretation of the data (Liebhold et al. 2006). Identification of a specimen to species is necessary to examine its provenance. Population genetic studies require knowledge of species identity to complete source estimation based on correct pest distribution records and reference data (Barr et al. 2014b). Failure to identify a species correctly would result in less-than-optimal decision making regarding surveillance and management (Lyal & Miller 2020). For example, Clarke and Schutze (2014) review an instance when failure to quickly recognize the presence of the fly *Bactrocera musae* (Tryon) (Diptera: Tephritidae) on the Gazelle Peninsula of East New Britain, Papua New Guinea, contributed to its spread.

Fruit flies in the family Tephritidae include some of the world's most destructive agricultural pests and several of these species share similar, overlapping, or identical morphology, thereby impeding or precluding reliable morphological identification (DeMeyer et al. 2015). Species that appear nearly identical still may exhibit different behaviors, host ranges, tolerances, and physiologies (Gilchrist & Ling 2006; Condon et al. 2008; Gómez-Cendra et al. 2016; Virgilio et al. 2019). These factors are important when determining sensitivities to attractants, predicting demographic parameters for life expectancy and degree d models, generating lists of affected hosts in a quarantine, and effectively deploying the proper species and lab strains in sterile insect technique programs (DeMeyer et al. 2015). In cases where morphology of the adult or immature life stage is insufficient to complete an identification, molecular techniques often are employed as alternative diagnostic methods (Armstrong et al. 1997; Armstrong & Cameron 2000). Unfortunately, molecular methods are not available for all economically important fruit fly species.

The oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), is an invasive pest capable of using a wide range of fruits and vegetables as hosts to complete its development (Clarke et al. 2005; Vargas et al. 2015; McQuate & Liquido 2017; USDA 2020). Native within a wide distribution in Asia (Drew & Hancock 1994; Clarke et al. 2019), its specific ancestral range is the subject of ongoing molecular genetic investigations (Aketarawong et al. 2007; Wan et al. 2012; Krosch et al. 2013). It has successfully invaded many tropical and subtropical regions around the world in the past century where it causes significant economic damage (Stephens et al. 2007; Vargas et al. 2015; Steck et al. 2019). This pest has the potential to spread further within countries where it is present currently (Wang et al. 2014; Qin et al. 2019).

Traditionally, identification of *Bactrocera* species is focused on color characters and wing patterns, with less emphasis on morphological structures like the male genitalia, female aculeus, and setal patterns (Drew & Hardy 1981; Drew & Romig 2013, 2016). Although *B. dorsalis* possesses variable color patterns, this is not true for all species in the genus (Leblanc et al. 2015; IPPC 2019). *Bactrocera dorsalis* has been the subject of numerous taxonomic and systematic investigations because of its similar appearance and close genetic relationship to other members of its genus (Krosch et al. 2013; San Jose et al. 2013; Boykin et al. 2014). Recently, these studies have resulted in several other species being placed in synonymy with *B. dorsalis* (Drew & Romig 2013; Schutze et al. 2015a, 2017). *Bactrocera dorsalis* is a member of a species complex that includes several important pests (Clarke et al. 2005). That complex is named the "*Bactrocera dorsalis* complex," but to avoid confusion with the species, it will be referred to hereafter as the "*dorsalis* complex." The *dorsalis* complex is an informal taxonomic grouping of over 75 species (Clarke et al. 2005; Doorenweerd et al. 2018) that do not form a monophyletic lineage (Leblanc et al. 2015). Unlike cryptic species complexes (Clarke & Schutze 2014), many of the species of the *dorsalis* complex are distinguishable using adult morphology. However, there are several species within the complex that are very difficult to distinguish from *B. dorsalis* itself. High morphological variation and intergradation of character states among *B. dorsalis*, and some species of the *dorsalis* complex, can make reliable identification using keys and descriptions very difficult, even for scientists with taxonomic expertise and experience working with the group (e.g., Drew & Romig 2016; IPPC 2019).

In California, a trapping program for *B. dorsalis* and other exotic fruit flies is ongoing to support early detection of pests. The first reported *B. dorsalis* in the state was collected in 1960 and the pest has been trapped there in most yr since 1966. The California Department of Food and Agriculture's Plant Pest Diagnostics Laboratory routinely identifies suspect *B. dorsalis* specimens as "*B. dorsalis* group" based on morphology. This designation is a pragmatic definition to support California State eradication efforts. This group includes *B. dorsalis*, *Bactrocera carambolae* Drew & Hancock, *Bactrocera caryeae* (Kapoor), *Bactrocera kandiensis* Drew & Hancock, *Bactrocera raiensis* Drew & Hancock, and *Bactrocera occipitalis* (Bezzi) (all Diptera: Tephritidae). These 6 species share similar morphology and are all attracted to methyl eugenol. Molecular methods to identify *B. dorsalis* and these related species have been explored but diagnosis of these pests has not been completely resolved using DNA (Jiang et al. 2014).

DNA sequencing of the mitochondrial cytochrome c oxidase I (COI) gene has proved useful to distinguish *B. dorsalis* from other *Bactrocera* species that are not closely related (Armstrong & Ball 2005; Jiang et al. 2014; Leblanc et al. 2015). However, closely related species in the *B. dorsalis* group often cannot be diagnosed using COI alone because species share identical sequences or overlap in sequence variation (Armstrong & Ball 2005; Frey et al. 2013; Jiang et al. 2014; San Jose et al. 2018). Examination of nuclear ribosomal internal transcribed spacer (ITS) DNA has been useful in discriminating some closely related species (Armstrong et al. 1997; Armstrong & Cameron 2000; Boykin et al. 2014), and a method to separate *B. dorsalis* and *B. carambolae* using ITS-1 sequences has been adopted by the International Plant Protection Convention (IPPC 2019).

In this study, all fruit flies trapped in California over an 11-yr period from 2008 to 2018 and identified as *B. dorsalis* group were analyzed using ITS-1 DNA sequencing. The specific goals were to (1) measure success rate of the ITS-1 protocol when applied to field trapped fruit flies in California, (2) identify captured flies to species using both genetic and morphological examination, and (3) document variation in ITS-1 for flies with *B. dorsalis*-like sequence identities. Documenting protocol performance and observed ITS-1 variation for *B. dorsalis* will support future use of the method.

Materials and Methods

SAMPLES AND DNA EXTRACTIONS

A total of 513 adult fruit flies were trapped in California from 2008 to 2018 and morphologically identified to the *B. dorsalis* group at the California Department of Food and Agriculture's Plant Pest Diagnostics Laboratory by Martin Hauser, Jason Leathers, Peter Kerr, and Stephen Gaimari. This includes all 159 flies collected from 2008 to 2012 that were previously analyzed by Barr et al. (2014a) to compare COI sequences. The first fly detection in 2008 was in Jun and the last detection in 2018 was in Nov. A leg from each fly was used for nucleic acid ex-

traction. Legs were removed from flies at the California Department of Food and Agriculture laboratory immediately after identification, then shipped to the Plant Protection and Quarantine laboratory in Edinburg, Texas, USA, for DNA extraction upon arrival, or storage at −20 °C until DNA extraction was performed within a wk of arrival. Flies collected between 2008 and 2012 had been processed previously for the Barr et al. (2014a) study using either KingFisher Flex model 711 (ThermoFisher Scientific Inc., San Jose, California, USA) 96-well plate-based magnetic bead extraction instrument and InviMag Tissue DNA Mini Kit/KF96 (STRATEC Molecular, Berlin, Germany) or DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) following the description of Barr et al. (2012). Legs of flies collected in 2013 to 2018 were extracted using the DNeasy method either at the Texas Plant Protection and Quarantine laboratory or at the California Department of Food and Agriculture laboratory. Vouchers of all flies are maintained at the California Department of Food and Agriculture laboratory and collection information is provided in Table S1.

PCR AND DNA SEQUENCING OF ITS-1

Polymerase chain reaction (PCR) was performed on DNA extractions using the primers ITS7 (5-GAATTTCGCATACATTGTAT) (Boykin et al. 2014) and ITS6 (5-AGCCGAGTGATCCACCGCT) (Armstrong & Cameron 2000). Reactions were performed in 25 µL volumes containing 1 µL of template (or water), 2.5 µL 10X buffer (Takara Bio Inc., Kyoto, Japan), 2 µL dNTP (2.5 mM each, Takara Bio Inc.), 0.125 µL Ex Taq HS DNA polymerase (5U per µL, Takara Bio Inc.), 1 µL primer ITS7 (10 µM), 1 µL primer ITS6 (10 µM), and 17.375 µL sterile water. Amplifications were performed in Applied Biosystems (Foster City, California, USA) GeneAmp PCR system 9700. Cycling conditions for amplification were 3 min of denaturation at 94 °C followed by 35 cycles of 20 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C, and a final extension step for 5 min at 72 °C.

Polymerase chain reaction products were visualized using 1.2% agarose gels of TAE buffer (BioRad, Hercules, California, USA) prestained with ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA). The size of products was compared to TriDye 100 base pairs ladder (New England Biolabs, Beverly, Massachusetts, USA) to inspect fragment size for the expected 500 base pairs amplicon of *B. dorsalis*. Polymerase chain reaction products were purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA) prior to DNA sequencing. The amplicons were sequenced using the two PCR primers and ABI BigDye® Terminator v.3.1 chemistry at commercial centers Functional Biosciences (Madison, Wisconsin, USA) or GeneWiz (South Plainfield, New Jersey, USA). All sequences were edited and assembled into contigs using the program Sequencher v5 (Genecodes, Ann Arbor, Michigan, USA) and aligned using MEGA7 (Kumar et al. 2016).

ANALYSIS OF SEQUENCES

A reference data set of 220 *B. dorsalis* ITS-1 sequences was compiled from GenBank records including 133 records from Boykin et al. (2014), 80 records from Schutze et al. (2015b), 4 records from the Philippines (MK184640, MK184649, MK184685, MK184691), and 3 records of flies collected in Italy and identified as *B. dorsalis* by Nugnes et al. (2018) (MK158099–MK158101). The Accession numbers are: KC446776–KC446780, KC446782–KC446785, KC446792–KC446805, KC446807–KC446816, KC446818–KC446835, KC446856–KC446870, KC446891–KC446893, KC446895–KC446897, KC446899, KC446901–KC446904, KC446906–KC446937, KC446938–KC446952, KC446973–KC446980, KC446982, KM453329–KM453348, KM453349–KM453368, KM453369–KM453372, KM453373–KM453382, KM453391–KM453397, KM453398–KM45407, KM453408–KM453416, MK158099–MK158101, MK184640, MK184649, MK184685, and MK184691.

The 220 record reference data set was aligned with ITS-1 records generated for the California flies. Unique genetic types of ITS-1 from the aligned sequences were identified using DNAsp v5.10 (Librado & Rozas 2009) treating gaps as characters and MEGA7 to visually confirm differences. The number of flies per unique type were recorded to measure the frequency of ITS-1 diversity. The ITS-1 sequences of California flies were submitted to GenBank: MT602638–MT603141. The accession codes are provided in Table S1 for each specimen.

SPECIES IDENTIFICATION USING ITS-1

Following the methods reported in ISPM27 (IPPC 2019), the ITS-1 sequences of California flies were compared to those of *B. carambolae* (58 records, Boykin et al. 2014) and *B. dorsalis* (220 records) from GenBank to determine (1) if the sequences were 99% identical to these species, and (2) if a 44-base pairs insertion characteristic of *B. carambolae* (e.g., KC446737) was present. Absence of the insertion supports identification of a fly as *B. dorsalis* or possibly another closely related species. The flies that were less than 99% similar to *B. dorsalis* using NCBI BLAST (https://blast.ncbi.nlm.nih.gov) (Johnson et al. 2008) were examined further for best sequence match in the GenBank database.

PCR AND SEQUENCING OF OTHER GENES

In order to further examine genetic similarity of fruit flies in the study, a subset of California flies also was amplified and sequenced for the COI gene and elongation factor 1-alpha (EF1α) gene. Primers for sequencing the first half of the COI gene used for DNA barcoding were LCO-1490 (5-GGTCAACAAATCATAAAGATATTGG) and HCO-2198 (5-TAAACTTCAGGGTGACCAAAAAATCA) (Folmer et al. 1994). Those primers for the 3-prime region (aka C3p790 fragment in Barr et al. 2014a) were HCO-2198rc (5-TGATTTTTTGGTCACCCTGAAGTTTA) (San Jose et al. 2013) and PAT-K508 (aka TL2-N-3014) (5-TCCAATGCACTAATCTGCCATATTA) (Simon et al. 1994). Primers for amplification and sequencing a fragment of the EF1α gene were M46-1 (5-CAGGAAACGCTATGACCGAGGAAATYAARAAGGAAG) and M4rc (5-TGTAAAACGACGGCCAGTACAGCVACKGTYTGYCTCATRTC) (Cho et al. 1995). Reactions for 2 COI fragments and EF1α were performed each in 25 µL volumes as described for ITS-1. Cycling conditions for amplification of COI fragments were 3 min at 94 °C followed by 39 cycles of 20 s at 94 °C, 20 s at 53 °C, 30 s at 72 °C, and a final extension of 5 min at 72 °C. Cycling conditions for amplification of EF1α fragment were 3 min at 94 °C followed by 39 cycles of 60 s at 94 °C, 60 s at 55 °C, 60 s at 72 °C, and a final extension of 5 min at 72 °C. Gels were inspected and sequencing was performed using the aforementioned methods for ITS-1. Further details on the COI and EF1α protocols are available in Barr et al. (2014a) and San Jose et al. (2013), respectively. The COI (MT597040–MT597049, MT597056) and EF1α (MT602095–MT602100) sequences generated in the study were submitted to GenBank.

PHYLOGENETIC ANALYSIS

The COI and EF1α data generated from California fruit flies were aligned with published records to examine similarity to *B. dorsalis* and closely related species. These records were from publications of San Jose et al. (2013) and Leblanc et al. (2015) with additional COI submissions of flies from the Philippines (MT597041–MT597049). Excluding flies from California, the COI (C3p790) data set included 93 records: *Bactrocera cacuminata* (Hering) (Diptera: Tephritidae) (n = 10), *B. occipitalis* (n = 6), *B. raiensis* (n = 1), *Bactrocera thailandica* Drew & Hancock (Diptera: Tephritidae) (n = 14), *Bactrocera tuberculata* (Bezzi)

(Diptera: Tephritidae) (*n* = 4), *B. musae* (Tryon) (*n* = 1), *B. kandiensis* (*n* = 1), *B. dorsalis* (*n* = 47), and *B. carambolae* (*n* = 9). Excluding flies from California, the EF1α data set included 61 records: *B. cacuminata* (*n* = 2), *B. occipitalis* (*n* = 2), *B. raiensis* (*n* = 1), *B. thailandica* (*n* = 2), *B. tuberculata* (*n* = 4), *B. kandiensis* (*n* = 1), *B. dorsalis* (*n* = 40), and *B. carambolae* (*n* = 9). MEGA7 (Kumar et al. 2016) was used to align sequences, test goodness-of-fit to models of evolution for each alignment using Bayesian Information Criterion and reconstruct Maximum Likelihood trees. The model was selected as lowest Bayesian Information Criterion value from a Maximum Likelihood search of 24 models starting from a Neighbor Joining tree and using moderate branch swapping option and inclusion of all sites. The Tamura "1992" and Jukes & Cantor models (Nei & Kumar 2000) were selected for COI and EF1α, respectively. The heuristic Maximum Likelihood tree search started with Neighbor Joining tree followed by extensive option of Subtree-Pruning-Re-grafting (SPR level 5) and strong branch swap filter. Bootstrap values were calculated by performing 100 replicates of heuristic search. Trees were rooted using *B. tuberculata* based on its position as sister to the clade including species of interest in the study (Leblanc et al. 2015; San Jose et al. 2018). Branch values below 60% bootstrap support were not shown.

MORPHOLOGICAL RE-EXAMINATION OF FLIES

Because fruit flies are identified initially as "*B. dorsalis* group" by the California Department of Food and Agriculture lab, specimens whose ITS-1 sequences did not match *B. dorsalis* ITS-1 records were re-examined for morphological characteristics based on reference collections and published resources (Drew & Hardy 1981; Drew & Romig 2013, 2016; Leblanc et al 2015; IPPC 2019). In addition, flies were inspected for absence of microtrichia on the thorax along longitudinal middle strip from the anterior margin of the thorax. This characteristic of *B. occipitalis* was first noted by Eric Fisher (unpublished) and subsequently used by California Department of Food and Agriculture.

Results

SEQUENCING SUCCESS AND ITS-1 ALIGNMENT

The ITS-1 protocol generated sequence data for 504 of the 513 flies in the study. Although sequencing success was high, 52 of the 504 flies failed to sequence using both primer directions and were confirmed by sequencing the product twice using the same primer (i.e., the consensus of the 2 sequencing reads were from unidirectional data) (Table S1). DNA sequencing of internal transcribed spacers may be problematic because of secondary structures, A+T rich segments, and regions of nucleotide repeats (Whiting 2002; Sutton et al. 2015). These factors could have contributed to our observed failures. Primer sequencing failure for flies was confirmed by repeating those sequencing reactions and observing failure for a second time.

The expected fragment size of *B. dorsalis* ITS-1 is 500 base pairs using the ITS7 + ITS6 primers: 39 bases for primers and 461 bases in between primers. After trimming the data of primers and sites at ends that were of low confidence, the sequences were aligned. One sequence (MT603053, fly 16V457) from the California data set had several base differences and was removed from the alignment (see below). The resulting alignment of 723 suspect *B. dorsalis* sequences (503 California flies and 220 reference samples from National Center for Biotechnology Information) was 424 base pairs in length. The alignment includes sequences with insertions-deletions, and actual lengths of each sequence varied from 416 to 420 bases. The alignment included 15 unique types that were labelled as A to O (Table 1). The types reported from the California study and National Center for Biotechnology Information records were labeled A–C, the types reported only from the California study were labeled types D–F, and the types reported only in National Center for Biotechnology Information records were labeled G–O. The variation in the data set is characterized by 8 base substitution sites and 6 insertions-deletions of 1 or 3 base sites in length (Table 1).

Of the 723 flies with sequences, 74 flies had an ambiguous call at a site that is used to distinguish the 15 specific types. Consequently, these are tracked as ambiguous sequences for intraspecific variation analysis (Table 2). Excluding the ambiguous data, just 3 types represent nearly 90% of the flies: A (76.7%), B (8.4%), and C (4.7%) (Table 2).

IDENTIFICATION USING ITS-1

The 503 California fruit flies included in the alignment have ITS-1 sequences > 99% similar to *B. dorsalis* sequences. These flies did not include the 44 base pairs insertion that is used to diagnose *B. caram-*

Table 1. Fifteen ITS-1 types reported for *Bactrocera dorsalis* reporting length of sequence in alignment, the variable sites, and character states for each type.

		Variable site													
Type	Base pairs	24	65	101	109	160	202	286	310	320	343	363	373–375	418	422
A	419	G	(–)	(–)	(–)	(–)	G	G	(–)	A	T	T	ATT	C	A
B	416	G	(–)	(–)	(–)	(–)	G	G	(–)	A	T	T	(– – –)	C	A
C	419	A	(–)	(–)	(–)	(–)	G	G	(–)	A	T	T	ATT	C	A
D	419	G	(–)	(–)	(–)	(–)	G	G	(–)	G	T	T	ATT	C	A
E	420	G	(–)	(–)	(–)	G	G	G	(–)	A	T	T	ATT	C	A
F	419	G	(–)	(–)	(–)	(–)	G	G	(–)	A	T	T	ATT	C	C
G	419	G	(–)	(–)	(–)	(–)	G	C	(–)	A	T	T	ATT	C	A
H	419	A	(–)	(–)	(–)	(–)	A	G	(–)	A	T	T	ATT	C	A
I	420	G	(–)	(–)	(–)	(–)	G	G	T	A	T	T	ATT	C	A
J	420	G	(–)	(–)	A	(–)	G	G	(–)	A	T	T	ATT	C	A
K	420	G	(–)	A	(–)	(–)	G	G	(–)	A	T	T	ATT	C	A
L	420	G	(–)	(–)	(–)	G	G	G	(–)	A	A	T	ATT	C	A
M	420	G	T	(–)	(–)	(–)	G	G	(–)	A	T	T	ATT	C	A
N	419	G	(–)	(–)	(–)	(–)	G	G	(–)	A	T	C	ATT	C	A
O	419	G	(–)	(–)	(–)	(–)	G	G	(–)	A	T	T	ATT	A	A

Table 2. The frequencies of the 15 ITS-1 types recorded for *Bactrocera dorsalis* according to data sets.

Type	No. CA individuals	No. NCBI GenBank individuals	Frequency of types including ambiguous data	Frequency of types excluding ambiguous data	GenBank type example
A	377	145	72.20%	76.65%	KC446776.1
B	24	33	7.88%	8.37%	KC446914.1
C	21	11	4.43%	4.70%	KC446807.1
D	15	0	2.07%	2.20%	MT602821
E	4	0	0.55%	0.59%	MT602812
F	2	0	0.28%	0.29%	MT603056
G	0	7	0.97%	1.03%	KC446794.1
H	0	2	0.28%	0.29%	KC446803.1
I	0	2	0.28%	0.29%	KC446810.1
J	0	1	0.14%	0.15%	KC446930.1
K	0	1	0.14%	0.15%	KM453350.1
L	0	1	0.14%	0.15%	KM453393.1
M	0	1	0.14%	0.15%	KM453401.1
N	0	1	0.14%	0.15%	KM453406.1
O	0	1	0.14%	0.15%	KC446820.1
Ambiguous	60	14	10.24%	NA	—
Total	503	220	723	649	—

bolae. These flies are consistent with determination as *B. dorsalis*. The California fly 16V457 (collected 26 Jul 2016 in San Martin, Santa Clara County, PDR# SJ0P06327463, BX160805-004) that was removed from the alignment because of noted differences is < 98% similar to *B. dorsalis* records. The best match for this specimen to *B. dorsalis* was to GenBank record KJ545133.1 at 97.61% (search performed on 13 Apr 2019). The fly is a 100% match to ITS-1 sequences from *B. occipitalis*.

ANALYSIS OF COI

The COI DNA barcode sequence of 16V457 (MT597056) is > 99% match to records of *B. occipitalis* and *B. dorsalis*. A search of Barcode of Life Data System records (http://www.boldsystems.org/) did not return a species identification for the query but the highest match was *B. occipitalis*. Similar results were obtained when comparing the 3′ segment of COI (MT597040). The Maximum Likelihood COI tree also grouped 16V457 with *B. occipitalis* (Fig. 1). Five additional *B. dorsalis* flies from California also were included in the analysis because of high similarity with *B. occipitalis* COI records. These 5 flies had the X135–X137 haplotypes (KF801433-35) reported by Barr et al. (2014a). These grouped with 16V457 as well, but COI is not considered a reliable diagnostic for these 2 species.

ANALYSIS OF EF1α

The phylogenetic tree of the EF1α gene also supports placement of fly 16V457 (MT602100) with *B. occipitalis* (Fig. 2). In contrast, the 5 specimens collected in California in 2009 and 2010 with COI sequences similar to *B. occipitalis* have EF1α sequences (MT602095–MT602099) that group them in *B. dorsalis*. The bootstrap branch support for the *B. occipitalis* clade is 74%. In comparison to ITS-1, the EF1α data set includes few individuals of this species ($n = 2$) making identification less certain. But the data suggest 16V457 is *B. occipitalis*.

MORPHOLOGICAL RE-EXAMINATION

The specimen genetically identified as *B. occipitalis* was re-examined for morphological characteristics to confirm that identification. While studying reference specimens in the California Department of Food and Agriculture insect collection, a second pinned Californian specimen of *B. occipitalis* from 1983 was discovered and included in the morphological analyses. The first Californian record of *B. occipitalis* is a male collected in Cupertino, Santa Clara County, on 20 Sep 1983 (Fig. 3, Voucher CSCA 74602). This specimen was identified as *B. occipitalis* by Eric Fisher, the California Department of Food and Agriculture dipterist at the time, and confirmed by R.I. Drew in 1999. The 1983 *B. occipitalis* did not generate ITS-1 sequence data and was not included in the molecular analysis.

The 2 California-collected fruit fly specimens were compared with several *B. occipitalis* individuals from the Philippines as well as other species of the *dorsalis* group with characters from the literature. The California specimens fit the morphological concept of *B. occipitalis*: the apical wing band is bleeding over R_{2+3} (Fig. 4), the ocellar bristles are without dark spots around their bases, and the dark markings on tergite IV are rectangular. The most notable characteristic is the absence of microtrichia on the thorax of *B. occipitalis*, forming a polished longitudinal middle strip from the anterior margin of the thorax to at least the transversal suture (Fig. 5). In most other *dorsalis* group species, this area is covered in dense microtrichia.

The 5 flies with ITS-1 and EF1α sequences matching *B. dorsalis* were re-examined as well because of their similarity to COI sequences found in *B. occipitalis*. Images of 1 male (California Department of Food and Agriculture voucher 09E332) and 2 females (California Department of Food and Agriculture voucher 10F724, 09D379) are provided in Supplemental Figure S2. The relevant characteristics for *B. dorsalis* are expressed weakly in the male (09E332) but are visible and are found also in the other 2 males (09D261 and 10F809). Morphology of these flies is largely consistent with *B. dorsalis* but also exhibits character states that are rarely found in this species. For example, the costal band overlapping R_{2+3}, the dark dorsoapical markings on the protibia, the apically darkened meso- and metafemur, the darkened apical 3 tarsal segments, and the broad lateral yellow markings on the thorax are atypical for *B. dorsalis*. Atypical patterns such as these have been seen before in *B. dorsalis* specimens (C. Doorenweerd and L. Leblanc, personal communication). The aculeus of the female is more elongate (2 mm) than in typical *B. dorsalis* specimens but falls within the range for this species (IPPC 2019).

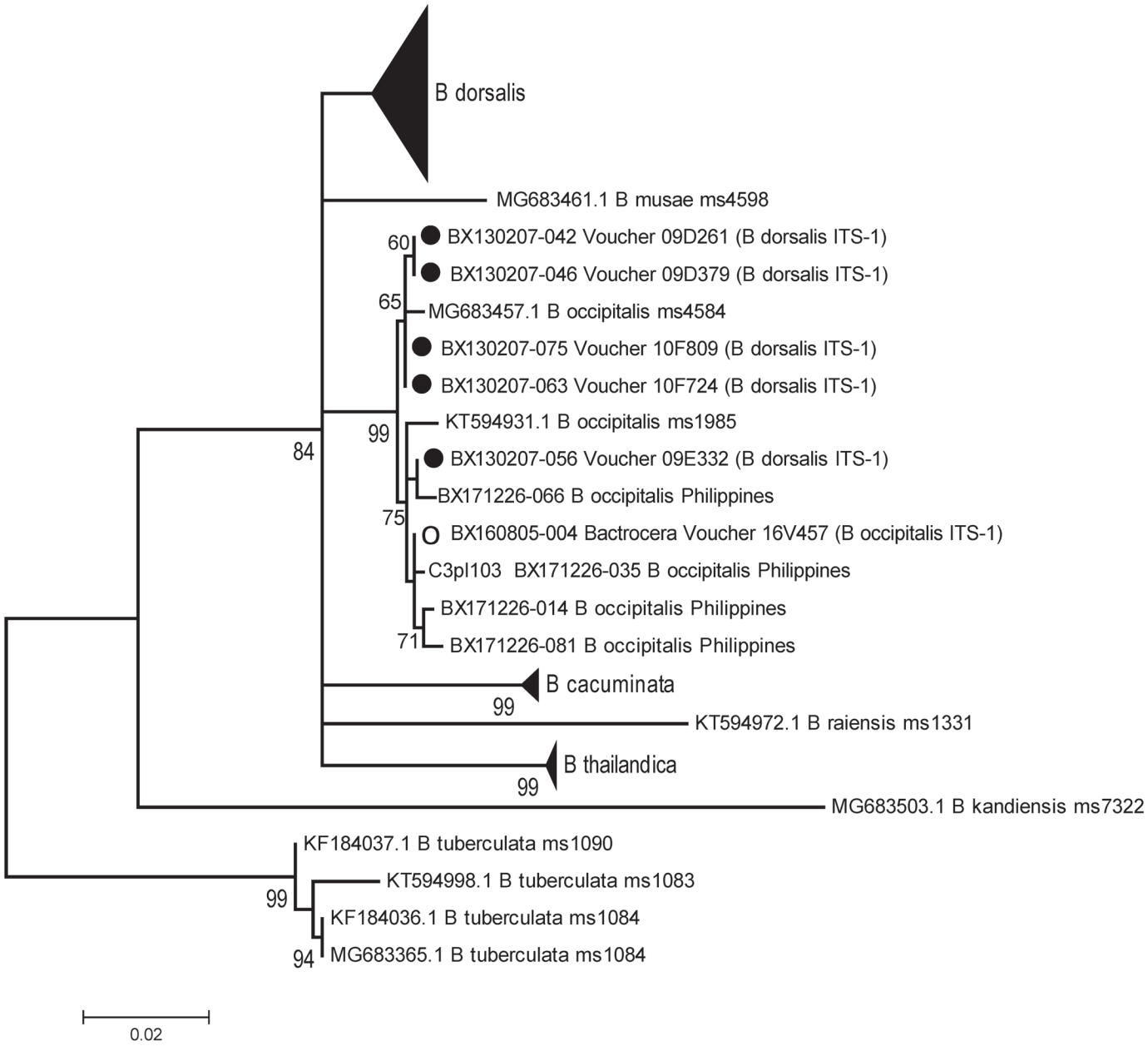

Fig. 1. ML tree (log likelihood –2782.3671) of C3p790 fragment of COI gene based on the Tamura model with Gamma distributed rates and Invariant sites (T92+G+I). The California fly (16V457) with *Bactrocera occipitalis* ITS-1 sequence is marked with an open circle dot. Five flies trapped in California that have ITS-1 sequences that match *Bactrocera dorsalis* and reported in Barr et al. (2014a) are marked with black dots. Operational Taxonomic Units and branches are collapsed for species in clades. The collapsed *B. dorsalis* clade includes both *B. dorsalis* and *B. carambolae* records.

Discussion

In this study we have demonstrated that the ITS-1 sequencing protocol was successful at generating data from 504 of the 513 flies trapped in California. Of the 504 flies with amplified product, nearly 90% were successfully sequenced using both primers. These data indicate that the protocol is appropriate for DNA analysis of flies collected in trapping programs. Of the flies with ITS-1 sequences, the currently available reference *Bactrocera* ITS-1 records support *B. dorsalis* as the identification for 503 flies. To further support that identification, additional records for less studied pests will be needed to document variation for a wider range of species.

One fly (16V457) of the 504 that were successfully sequenced had an ITS-1 sequence that matched *B. occipitalis*. Morphological examination confirmed this identity. This represents the second record of *B. occipitalis* in the state. The first detection of *B. occipitalis* was a single fly trapped in 1983. The time period between detections (1983 and 2016) indicates the fly did not establish in North America.

In 1997, a fly trapped in California (voucher 1187308) was morphologically identified as *B. carambolae* by Eric Fisher, and corroborated by R.I. Drew and Martin Hauser. This is the only record of the invasive pest in the state. Unfortunately, we could not successfully extract DNA from the specimen, likely due to its age. Based on ITS-1 data, *B. carambolae* has not been trapped in California in the 11 yr

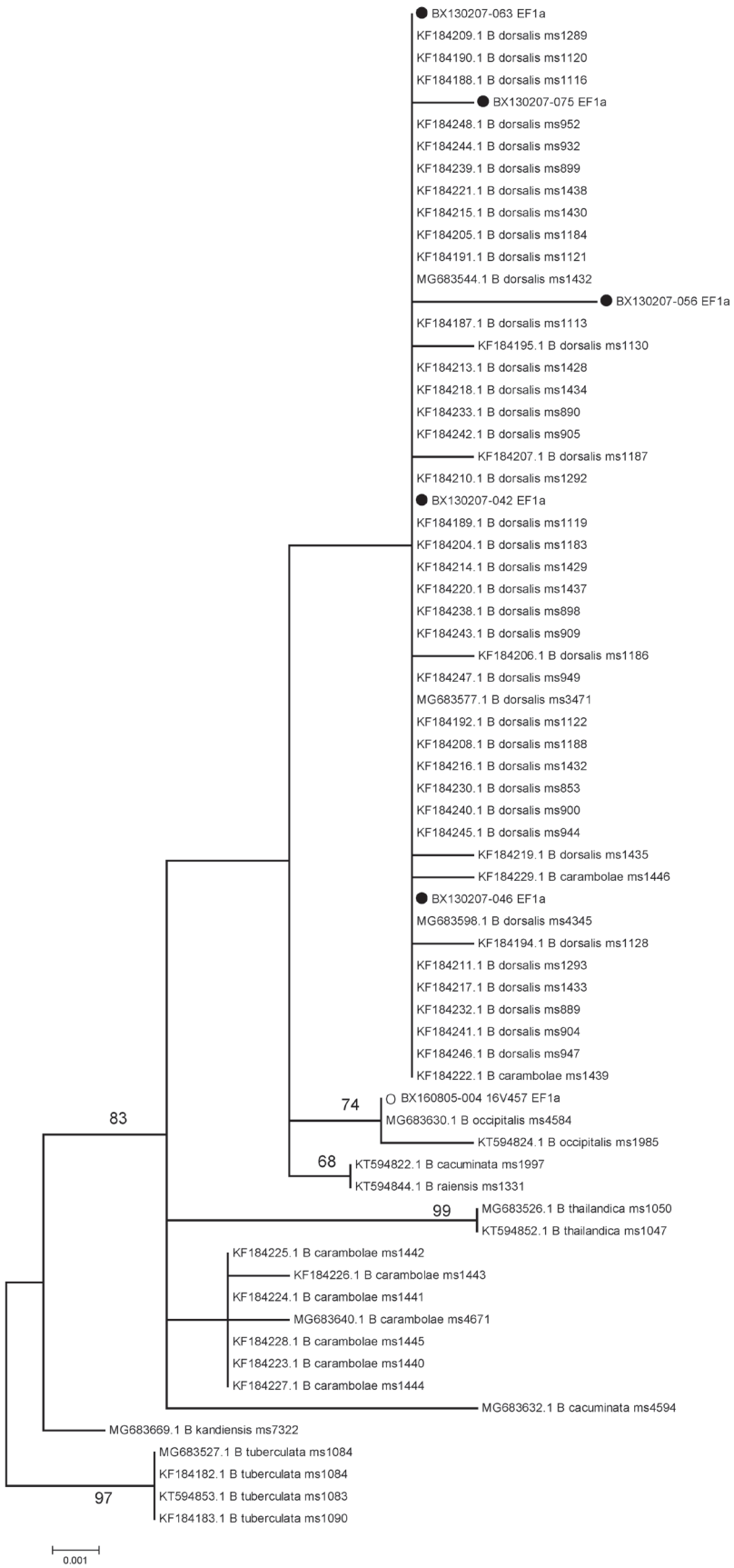

Fig. 2. ML tree (log likelihood –1370.3141) of elongation factor 1-alpha (EF1α) gene based on Jukes-Cantor model. The California fly (16V457) with *Bactrocera occipitalis* ITS-1 sequence is marked with an open circle dot. Five flies trapped in California that have ITS-1 sequences that match *Bactrocera dorsalis* and reported in Barr et al. (2014a) are marked with black dots.

Fig. 3. Image of first *Bactrocera occipitalis* trapped in California in 1983.

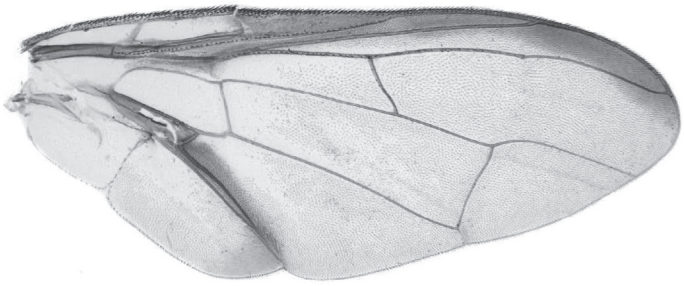

Fig. 4. Image of wing of fly (16V457) with *Bactrocera occipitalis* ITS-1 sequence.

examined in our study nor from flies collected in 2019 to 2020 (N. Barr, unpublished). The molecular methods described here for ITS-1 analysis were applied also to flies trapped in Florida during the 2015 to 2016 outbreak of *B. dorsalis* (Steck et al. 2019) to provide confirmation that *B. carambolae* flies were not present in those detections (N. Barr, unpublished).

The ability of *B. carambolae* and *B. dorsalis* to hybridize under laboratory conditions (Schutze et al. 2013, 2015a) and possibly in nature (Ebina & Ohto 2006; Delomen et al. 2013; Jalani et al. 2014) could complicate use of the ITS-1 genetic identification method because of introgression. Schutze et al. (2015b) also reported evidence of hybridization between *B. dorsalis* and *B. kandiensis*. We did not detect evidence of hybrids based on amplification because only 1 size product of ITS-1 was visible and sequenced in the fruit flies studied. However, we did not screen flies for evidence of introgression at other regions of the genome. Methods using single nucleotide polymorphisms could be applied to the issue of introgression between species (e.g., Anderson et al. 2018) but have not been developed for *B. dorsalis*.

Although the COI gene is not suitable for distinguishing *B. dorsalis* from certain closely related species, the 16V457 fly identified as *B. occipitalis* using ITS-1 had a COI sequence that is common for *B. occipitalis* specimens. Review of other flies in our data set with COI sequences similar to *B. occipitalis* records demonstrates some limitations of that approach to screening for *B. occipitalis*. Five of the flies trapped in California between 2008 and 2012 had COI sequences that are common to *B. occipitalis* specimens, but these flies were supported as *B. dorsalis* based on ITS-1 and EF1α sequences. These 5 had morphologies similar to but atypical for *B. dorsalis* and clearly were different from *B. occipitalis.*

As more *B. dorsalis* complex specimens from additional global collections are sequenced for ITS-1, EF1α, COI, and other genes under investigation (e.g., San Jose et al. 2013; Krosch et al. 2017), the utility of these reference datasets can be confirmed and eventually become formalized as aids to identifiers. The sequences analyzed in this study provided useful information to question or corroborate morphological identifications. The ITS-1 results were used to select specimens for re-examination using morphology and other genes. However, not all pest species related to *B. dorsalis* have genetic profiles for ITS-1 or other DNA markers. For example, ITS-1 records for the pests *B. caryeae* and *Bactrocera pyrifoliae* Drew & Hancock (Diptera: Tephritidae) are lacking. Based on ITS-1 data available from other *Bactrocera* species, it is reasonable to assume the ITS-1 sequences of these pests will not be identical to *B. dorsalis* sequences. However, that assumption needs be tested. Furthermore, lack of records for these known pests complicates interpretation of any new ITS-1 sequences that are generated from intercepted immature flies. Adult specimens still should be examined using morphological methods (e.g., IPPC 2019) to identify these pests.

The ability to distinguish the exotic species *B. carambolae*, *B. occipitalis*, and *B. dorsalis* is significant because it provides accurate data used to track new introductions, track the spread of invasions, determine the true number of flies contributing to a quarantine threshold, and adjust life cycle models appropriately as new exotic fruit flies are found. Identification of species and genetic variants helps to determine if a newly detected fly is part of an existing or an independent infestation. This information then is used to calculate degree d models for tracking local eradication efforts. The results of our current study support inclusion of the majority of trapped flies in California from 2008 to 2018 in future population genetic and pest distribution studies of *B. dorsalis*.

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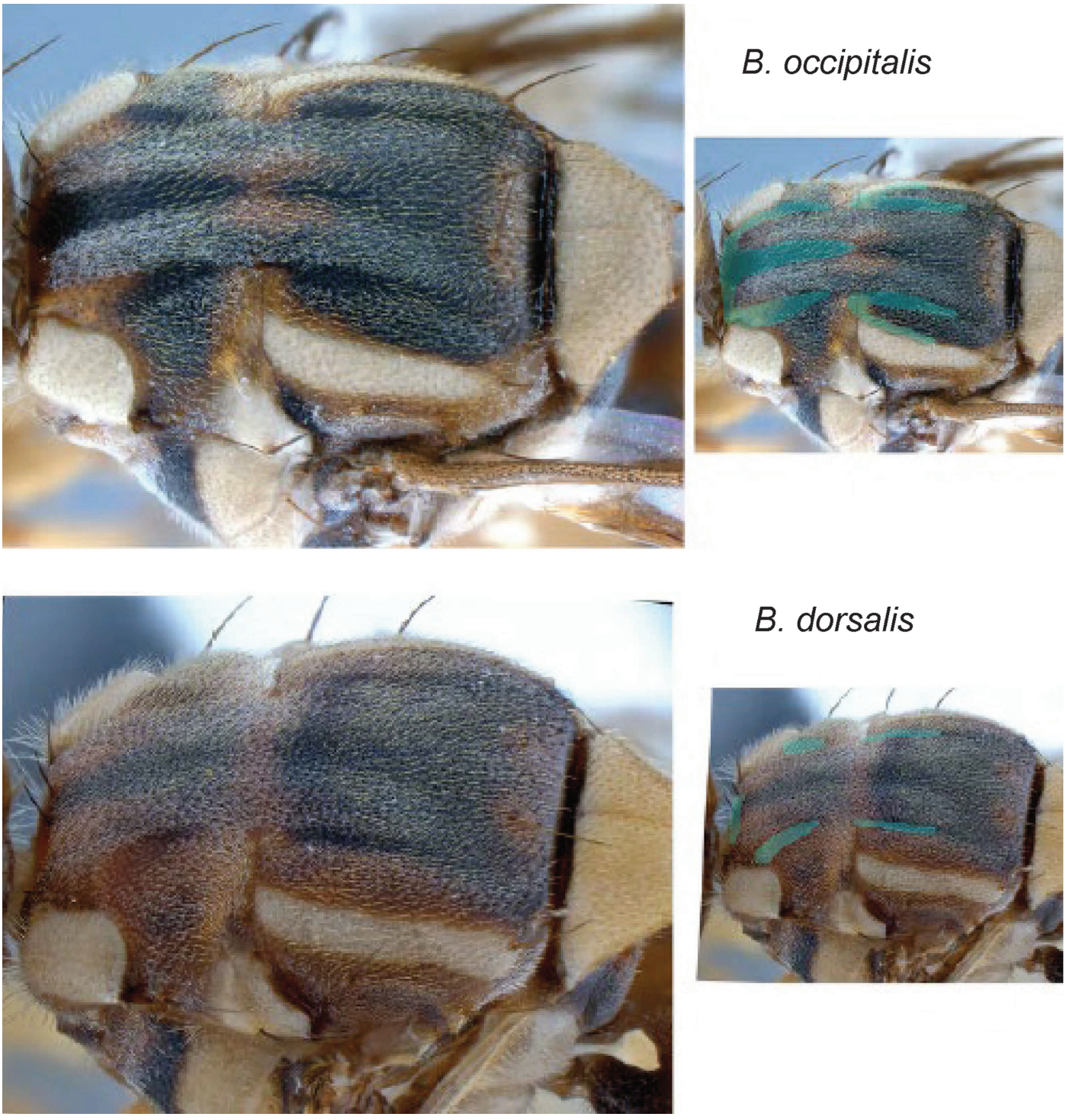

Fig. 5. Images of thoraces of the fly (16V457) with *Bactrocera occipitalis* ITS-1 sequence and a *Bactrocera dorsalis* fly. Areas without microtrichia are highlighted in green in the smaller pictures.

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