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Journal of Economic Entomology, 116(6), 2023, 2193–2200
https://doi.org/10.1093/jee/toad178
Advance Access Publication Date: 12 October 2023
Short Communication

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Evaluation of identification methods for cryptic *Bactrocera dorsalis* (Diptera: Tephritidae) specimens: combining morphological and molecular techniques

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Subject Editor: Kent Shelby

Received on 5 July 2023; revised on 5 July 2023; accepted on 8 September 2023

The potential for population genomics to elucidate invasion pathways of a species is limited by taxonomic identification issues. The Oriental fruit fly pest, *Bactrocera dorsalis* (Hendel) belongs to a complex in which several sympatric species are attracted to the same lure used in trapping and are morphologically cryptic and/or reported to hybridize. In this study, we evaluated the taxonomic ambiguity between *B. dorsalis* and 2 major cryptic species, based on morphological expertise and 289 target specimens sampled across the whole distribution range. Specimens were then subjected to DNA sequence analyses of the COI mitochondrial barcode and the EIF3L nuclear marker to evaluate the potential for molecular identification, in particular for specimens for which morphological identification was inconclusive. To this aim, we produced reference datasets with DNA sequences from target specimens whose morphological identification was unambiguous, which we complemented with 56 new DNA sequences from closest relatives and 76 published and curated DNA sequences of different species in the complex. After the necessary morphological observation, about 3.5% of the target dataset and 47.6% of the specimens from Southeast Asian islands displayed ambiguous character states shared with *B. carambolae* and/or *B. occipitalis*. Critical interpretation of DNA sequence data solved morphological ambiguities only when combining both mitochondrial and nuclear markers. COI discriminated *B. dorsalis* from 5 species; EIF3L and ITS from another species. We recommend this procedure to ensure correct identification of *B. dorsalis* specimens in population genetics studies and surveillance programs.

Key words: mtDNA, nDNA, haplotype diversity, ambiguous identification

Introduction

Over the last 2 decades, the Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), has emerged as one of the most invasive and destructive insect pests of tropical and subtropical fruits and vegetables, particularly in sub-Saharan Africa and the Indian Ocean (Drew and White 2005, Schutze et al. 2015). *Bactrocera dorsalis* is highly polyphagous, with more than 300 host plants (Allwood et al. 1999, Clarke et al. 2005, Ekesi and Billah 2007), although its major host is mango (*Mangifera indica*) (Sileshi et al. 2019, Ndlela et al. 2022). Population genetics has been, and increasingly is, a powerful tool for providing new insights into its invasion biology, in particular introduction sources, invasion success, and colonization dynamics (Aketarawong et al. 2007, Khamis et al. 2009, Wan et al. 2011, Li et al. 2012, Shi et al. 2012, Choudhary et al. 2016, Kim et al. 2021). Most previous studies omitted to detail the method used for specimen identification, despite a clearly articulated need to do so as outlined by Schutze et al. (2017) advocating an integrative taxonomic approach to tephritid fruit fly species delimitation and diagnoses (but see Choudhary et al. 2016, Qin et al. 2018, Zhang et al. 2022). There is a risk of confusion with several sympatric and morphologically cryptic species, since *B. dorsalis* belongs to a complex of about 90 species including roughly 30 species attracted to methyl-eugenol (ME), a male-targeted chemical lure used for sampling in population genetics studies and surveillance programs. The undetected presence of a species closely related to *B. dorsalis* in population genetics analyses skews estimations of genetic structure and

diversity, and thereby compromises the understanding of underlying processes or induces discrepancies between studies.

Bactrocera dorsalis identification based on morphological characters alone is sometimes difficult because its intraspecific variability overlaps with other members of the complex, especially 2 other important pests attracted to ME: *B. carambolae* Drew & Hancock and *B. occipitalis* (Bezzi) (Leblanc et al. 2015, Pieterse et al. 2017, Taddei et al. 2023). Moreover, cases of hybridization have been reported between *B. dorsalis* and *B. carambolae, B. kandiensis* Drew & Hancock, *B. occipitalis*, and *B. raiensis* Drew & Hancock (Wee and Tan 2005, Schutze et al. 2013, Schutze, Mahmood et al. 2015, Nugnes et al. 2018, San Jose et al. 2018, Zhang et al. 2022, Doorenweerd et al. 2020). Since accurate identification is crucial for surveillance of *B. dorsalis,* standard diagnostic protocols (e.g., EPPO 2021) incorporated a DNA barcode approach, often based on the single mitochondrial Cytochrome c Oxidase subunit I (COI), which has become a widely used identification tool (Hebert et al. 2003). However, the ability of the mtDNA barcode to distinguish *B. dorsalis* from other species within the complex remains controversial and its issues relating to tephritid diagnoses are well documented (Krosch et al. 2019, 2020), some studies indicating a substantial overlap in intra- and interspecific sequence variation for many species in the complex (e.g., Jiang et al. 2014 and references within; San Jose et al. 2018, Doorenweerd et al. 2020). The contradictory and unclear results may be explained by differences in taxonomic coverage and intraspecific sampling effort, in particular of the target species, and by identification errors, in particular when DNA entries from public databases are used (e.g., Asadi et al. 2019).

As a consequence, *B. dorsalis* identifications could possibly be validated by combining the mtDNA barcode with independent nDNA markers. Finding appropriate nuclear barcodes is challenging, however, since nuclear genes often lack sufficient species-level diagnostic variability. In this context, IPPC (2019) recommends using the nuclear Internal Transcribed Spacer 1 (ITS1) (Boykin et al. 2014), which discriminates *B. dorsalis* from *B. carambolae* (only) via length polymorphism. Furthermore, Plant Health Australia (2020) provides reference sequences for 4 nuclear markers, including the Eukaryotic translation Initiation Factor 3 subunit L gene (EIF3L), developed from anchored hybrid enrichment research and which seems the most promising for discriminating the species closest to

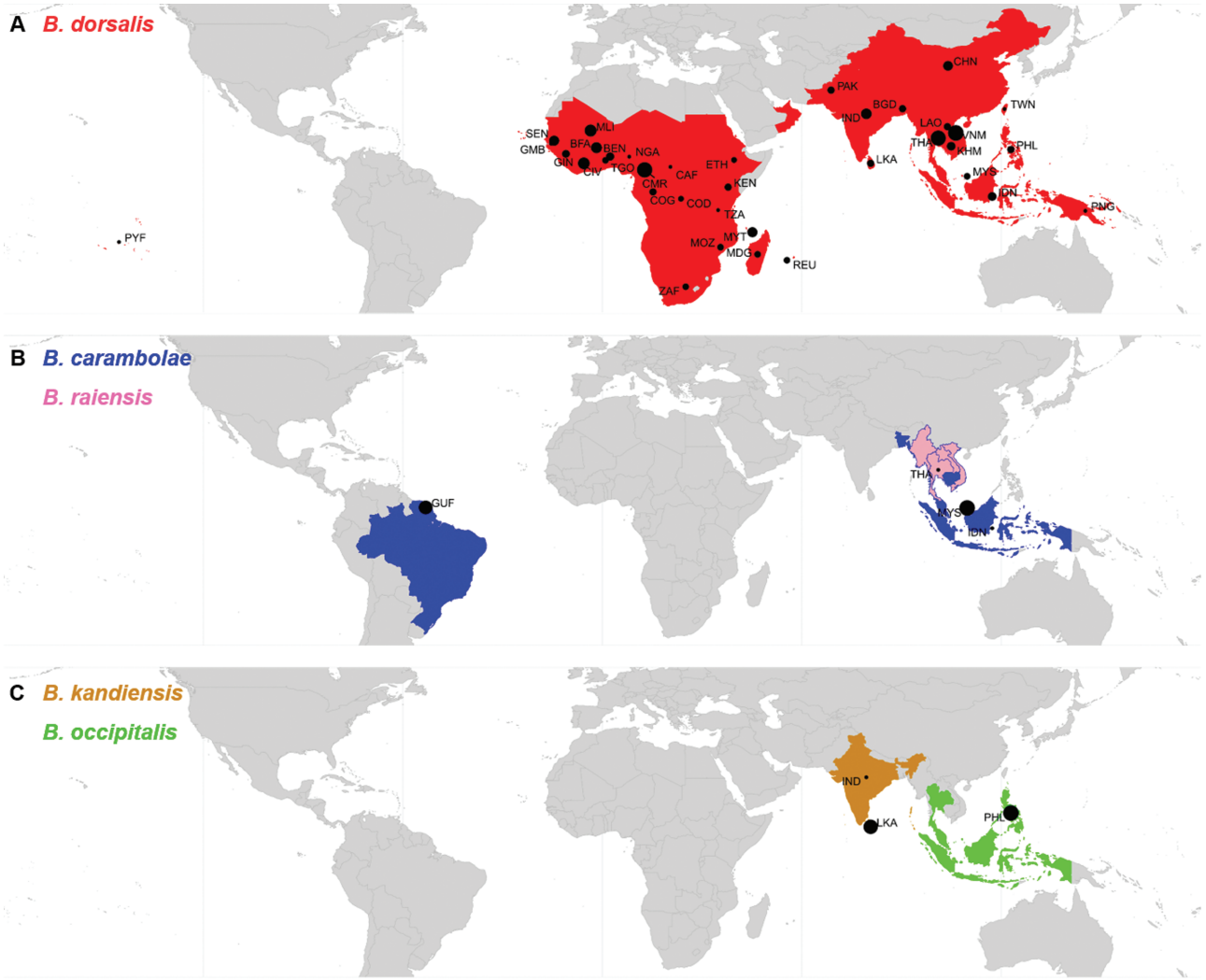

Fig. 1. Distribution maps and sampling points for *B. dorsalis* (A), *B. carambolae* and *B. raiensis* (B), and *B. kandiensis* and *B. occipitalis* (C). The maps are adapted from the April 2023 EPPO database, completed with the addition of India for *B. kandiensis*. The size of dots is proportional to the number of samples collected in each country. (B) Sampling dot in Thailand is for *B. raiensis*. Note that the Plant Health Australia collection samples are not represented here because no locality information was provided with the reference sequences.

Table 1: Final identification for the ten specimens with ambiguous morphological characters, with pieces of information from morphology and each molecular marker. Six lesser-known species not attracted by methyl-eugenol are marked with an asterisk.

Sample ID	Ambiguous morphological characters	Possible species confusion	COI identification	EIF3L identification	ITS1 identification	Final identification
IDN_322	- Costal band confluent with R2 + 3 vein, very broad around apex of wing. - Thin "T" pattern on abdomen. - Rectangular anterolateral black markings on tergite 4.	*B. dorsalis*, *B. occipitalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*NA*	Not *B. carambolae* (~500bp)	*B. dorsalis*
IDN_323	- Costal band confluent with R2 + 3 vein, very broad around apex of wing.	*B. dorsalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. carambolae*	*B. carambolae* (~550bp)	*B. carambolae*
IDN_331	- Costal band slightly overlapping R2 + 3 vein.	*B. dorsalis*, *B. occipitalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. cacuminata*, *B. endiandrae*, *B. kandiensis*, *B. dorsalis*, *B. latilineola*, *B. occipitalis*, *B. opiliae*, *B. raiensis*	Not *B. carambolae* (~500bp)	*B. dorsalis*
IDN_373	- Intermediate costal band between confluent and overlapping with R2 + 3 vein. - Rectangular anterolateral black markings on tergite 4. - Fulvous femora and tibia, with dark spot on fore femora and dark half of the tibia of the mid leg.	*B. dorsalis*, *B. occipitalis*, *B. carambolae*, *B. irvingiae**	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. cacuminata*, *B. endiandrae*, *B. kandiensis*, *B. dorsalis*, *B. latilineola*, *B. occipitalis*, *B. opiliae*, *B. raiensis*	Not *B. carambolae* (~500bp)	*B. dorsalis*, *B. irvingiae**
IDN_376	- Costal band slightly overlapping R2 + 3 vein. - Dark spot on the fore femora. - Large "T" pattern on abdomen.	*B. dorsalis*, *B. carambolae*, *B. propinqua**	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. cacuminata*, *B. endiandrae*, *B. kandiensis*, *B. dorsalis*, *B. latilineola*, *B. occipitalis*, *B. opiliae*, *B. raiensis*	Not *B. carambolae* (~500bp)	*B. dorsalis*, *B. propinqua**
MYS_352	- Costal band confluent with R2 + 3 vein, broad around apex of wing. - Rectangular anterolateral black markings on tergite 4.	*B. dorsalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. carambolae*	*B. carambolae* (~550bp)	*B. carambolae*
MYS_354	- Costal band confluent with R2 + 3 vein, broad around apex of wing. - Rectangular anterolateral black markings on tergite 4.	*B. dorsalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. carambolae*	*B. carambolae* (~550bp)	*B. carambolae*
MYS_356	- Costal band confluent with R2 + 3 vein, broad around apex of wing. - Rectangular anterolateral black markings on tergite 4.	*B. dorsalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. carambolae*	*B. carambolae* (~550bp)	*B. carambolae*

Table 1. Continued

Sample ID	Ambiguous morphological characters	Possible species confusion	COI identification	EIF3L identification	ITS1 identification	Final identification
PHL_268	- Costal band slightly overlapping R2 + 3 vein and moderately broad around apex of wing. - Rectangular anterolateral black markings on tergite 4. - Fulvous femora.	*B. dorsalis*, *B. occipitalis*, *B. carambolae*	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. cacuminata*, *B. endiandrae*, *B. kandiensis*, *B. dorsalis*, *B. latilineola*, *B. occipitalis*, *B. opiliae*, *B. raiensis*	Not *B. carambolae* (~500bp)	*B. dorsalis*
PHL_269	- Costal band overlapping R2 + 3 vein, broad around apex of wing. - Not easy to tell if there are rectangular anterolateral black markings on tergite 4. - Shining spots on abdomen neither light nor dark.	*B. dorsalis*, *B. occipitalis*, *B. carambolae*, *B. fuscitibia**, *B. bimaculata**, *B. involuta**, *B. dongniae**	*B. carambolae*, *B. dorsalis*, *B. kandiensis*, *B. raiensis*	*B. cacuminata*, *B. endiandrae*, *B. kandiensis*, *B. dorsalis*, *B. latilineola*, *B. occipitalis*, *B. opiliae*, *B. raiensis*	Not *B. carambolae* (~500bp)	*B. dorsalis*, *B. fuscitibia**, *B. bimaculata**, *B. involuta**, *B. dongniae**

B. dorsalis within the complex. While this region has proved useful in evolutionary analyses for the broader tribe (Starkie et al. 2022), further research is needed to evaluate this locus utility as part of an integrated molecular approach to differentiate *B. dorsalis* from morphologically cryptic congeners.

Here, we evaluate taxonomic ambiguity associated with conventional morphological characterization of *B. dorsalis* specimens and propose a protocol integrating 2 types of molecular information to ensure an authoritative identification of equivocal specimens: the mitochondrial COI barcode and the nuclear EIF3L and ITS markers. Particular care went into sample sizes and geographical ranges to ensure a rigorous validation of DNA barcodes, at least for the target species and its closest relatives (i.e., species morphologically similar and/or reported to hybridize).

Materials and Methods

We sampled 289 adult fruit fly specimens across the whole distribution range of *B. dorsalis* (Fig. 1, Supplementary Table S1): 213 specimens from the collection of the French Agency for Food, Environmental and Occupational Health & Safety, Plant Health Laboratory (ANSES – LSV), reared from larvae intercepted at European borders on infested imported fruits, and 76 specimens collected worldwide in orchards using ME traps, including 3 specimens from the Queensland Primary Industries Insect Collection (QDPC). Specimens were identified morphologically using IPPC (2019), completed with Drew and Romig (2013, 2016) and White and Elson-Harris (1992), by at least 1 expert in taxonomic entomology and/or person trained in morphological techniques. Following Schutze, Aketarawong et al. (2015), we considered *B. invadens*, *B. papayae*, and *B. philippinensis* as synonyms of *B. dorsalis*.

We used nondestructive DNA extraction protocols on whole specimens to conserve morphological characters, as detailed in Supplementary Table S1. The 658 bp 5' standard "barcoding region" of the COI gene (Hebert et al. 2003) and the 581 bp of the EIF3L gene (Plant Health Australia 2020) were amplified using polymerase chain reactions (PCRs) performed as detailed in Supplementary Table S2 and sequenced by Eurofins Genomics. All nucleotide sequences were checked for ambiguous bases, the presence of frame shift mutations and stop codons, edited using CodonCode Aligner v.10.0.2 and aligned using Seaview v5.0.5 (Gouy et al. 2021). PCR products of the ITS1 marker (Boykin et al. 2014), obtained as detailed in Supplementary Table S2, were run in 2.5% electrophoresis agarose gel for 90 min to double blind read the fragment size, expected to be 544 bp for *B. carambolae* and 500 bp for other *B. dorsalis* complex species.

For specimens for which morphological identification was inconclusive, molecular identification was achieved by visualizing the position of their COI and EIF3L sequences in tree graphics relatively to curated sequences of species of the *B. dorsalis* complex. The reference datasets included 406 (COI) and 309 (EIF3L) sequences from target specimens with unambiguous morphological identifications, specimens of the *B. dorsalis* species complex published by Plant Health Australia (2020) (Supplementary Table S1), and additional specimens from species morphologically confusable and/or with reported hybridizations with *B. dorsalis* (see Fig. 1, Supplementary Tables S1 and S3 for sample size details). Maximum likelihood (ML) trees were constructed on unique haplotypes, recovered with the *sidier* package (Muñoz-Pajares 2013), using MEGA v.X (Kumar et al. 2018) and the T92+G mutational model (Tamura 1992). We performed 500 bootstrap replications and condensed the ML trees to show clades supported by bootstrap values over 50%. For the

haploid COI barcode, we also constructed a minimum spanning network among haplotypes using Popart v.1.7 (Leigh and Bryant 2015).

Results and Discussion

Based on morphological keys, we confidently identified 279 specimens as *B. dorsalis* out of the 289 studied. The 10 remaining specimens, ~3.5% of the target dataset, possessed character states intermediate between *B. dorsalis* and other species of the complex, including *B. carambolae*, *B. occipitalis* and several little-known species rarely cited in literature and indifferent to ME (but attracted to Cue-lure, except *B. irvingiae* Drew & Hancock, for which no lure is known), preventing conclusive morphological identification (Table 1). All these specimens originated from either Indonesia, Malaysia, or the Philippines, where the rate of ambiguous individuals reaches 47.6%. The characters that diverged from the conventional morphological description of *B. dorsalis* were mainly the extent of the wing costal band and apex spot (Fig. 2G and H) and the abdominal color pattern (Fig. 2I and J).

COI barcoding confidently discriminated *B. dorsalis* from *B. cacuminata* (Hering), *B. endiandrae* (Perkins & May), *B. latilineola* Drew, *B. occipitalis*, and *B. opiliae* (Drew & Hardy) (Fig. 3A) whereas *B. carambolae* and *B. raiensis* specimens branched individually among *B. dorsalis* individuals in the ML tree without clustering according to species. The minimum spanning network of the mitochondrial haplotypes from *B. dorsalis* and its closest relatives was largely congruent with the ML tree: while the most frequently observed haplotypes clearly segregated according to species, the star-like shapes of the *B. dorsalis*, *B. carambolae*, and *B. raiensis* haplogroups overlapped to the point of preventing species resolution (Supplementary Fig. S1).

In addition, 7 specimens from India, Sri Lanka, Cameroon and the Congo that were confidently identified morphologically and with the EIF3L marker (see Fig. 3B and text below) as *B. dorsalis* clustered in the ML tree with *B. kandiensis* reference mitochondrial sequences (Fig. 3A). These represented 2.4% of the target specimens, 23.5% of which originated from the *B. kandiensis* range, and 2.3% of those from the invaded African continent. The minimum spanning network showed not only these *B. dorsalis* haplotypes in the *B. kandiensis* haplogroup, but also 1 distant haplotype in the *B. carambolae* haplogroup, and *B. raiensis* haplotypes in the *B. dorsalis* haplogroup (Supplementary Fig. S1). This supports previous reports of mitochondrial introgression events between *B. dorsalis* and *B. carambolae*, *B. kandiensis*, and *B. raiensis*.

Regarding the EIF3L marker, 34 specimens out of 272 (12.5%) failed the PCR amplification step due to poor DNA quality (Supplementary Table S1). Among those that worked, EIF3L confidently discriminated *B. carambolae* and *B. endiandrae* from *B. dorsalis* (Fig. 3B). However, *B. cacuminata*, *B. kandiensis* (1 specimen, Bactrocera_kandiensis_LKA_154, with heterozygous sites on diagnostic bases; see below), *B. latilineola*, *B. occipitalis*, *B. opiliae*, and *B. raiensis* could not be discriminated from *B. dorsalis*. Concerning ITS1, length polymorphism differentiated *B. carambolae* from other species in the complex and PCR amplification was more successful than with EIF3L (~99%) (Supplementary Table S1).

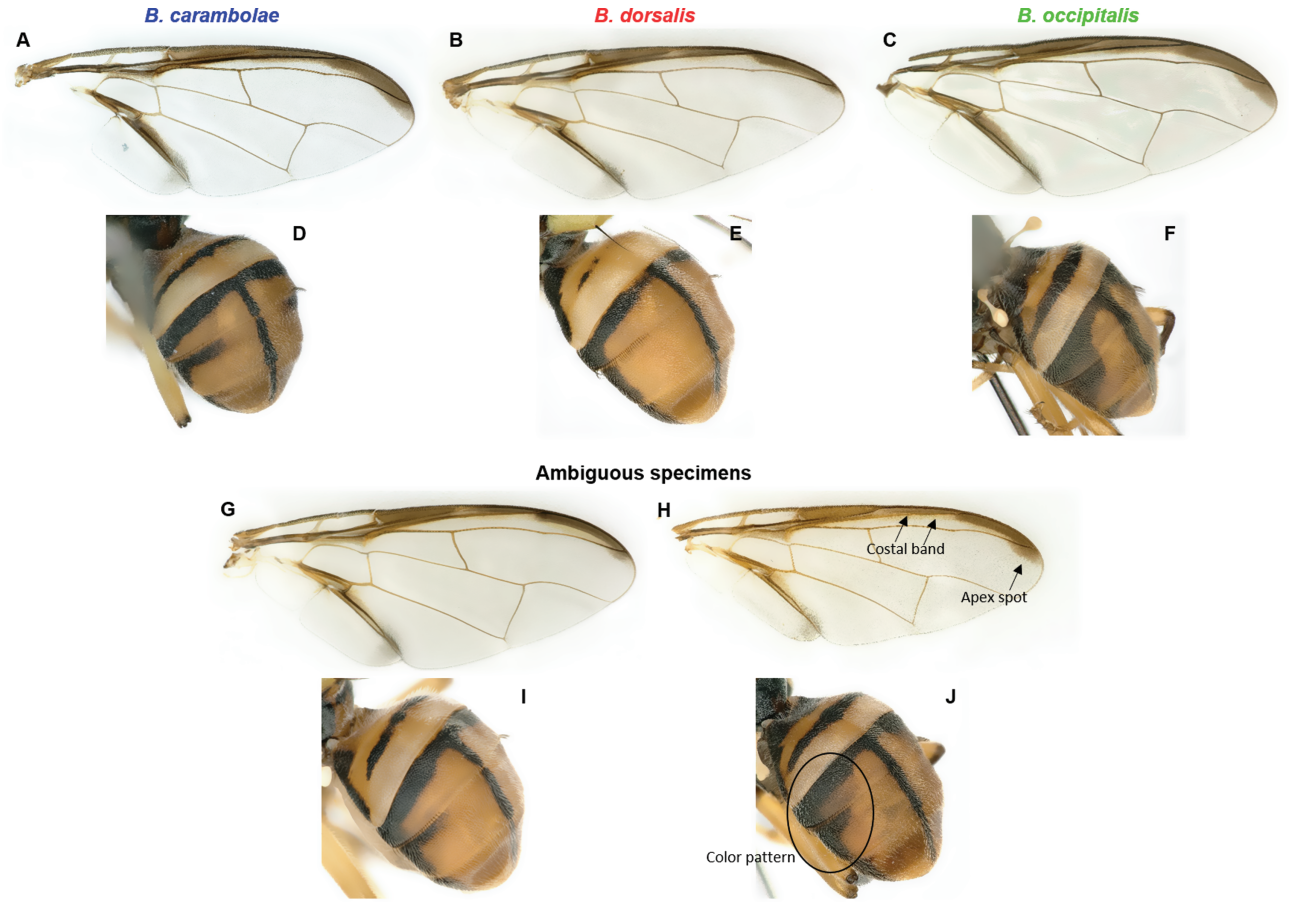

Fig. 2. Photographs of the wing (above) and abdomen (below) from typical *B. carambolae* (A, D), *B. dorsalis* (B, E), and *B. occipitalis* (C, F) specimens and from specimens with morphological traits intermediate between *B. carambolae* and *B. dorsalis* (G, I) and between *B. carambolae, B. dorsalis* and *B. occipitalis* (H, J).

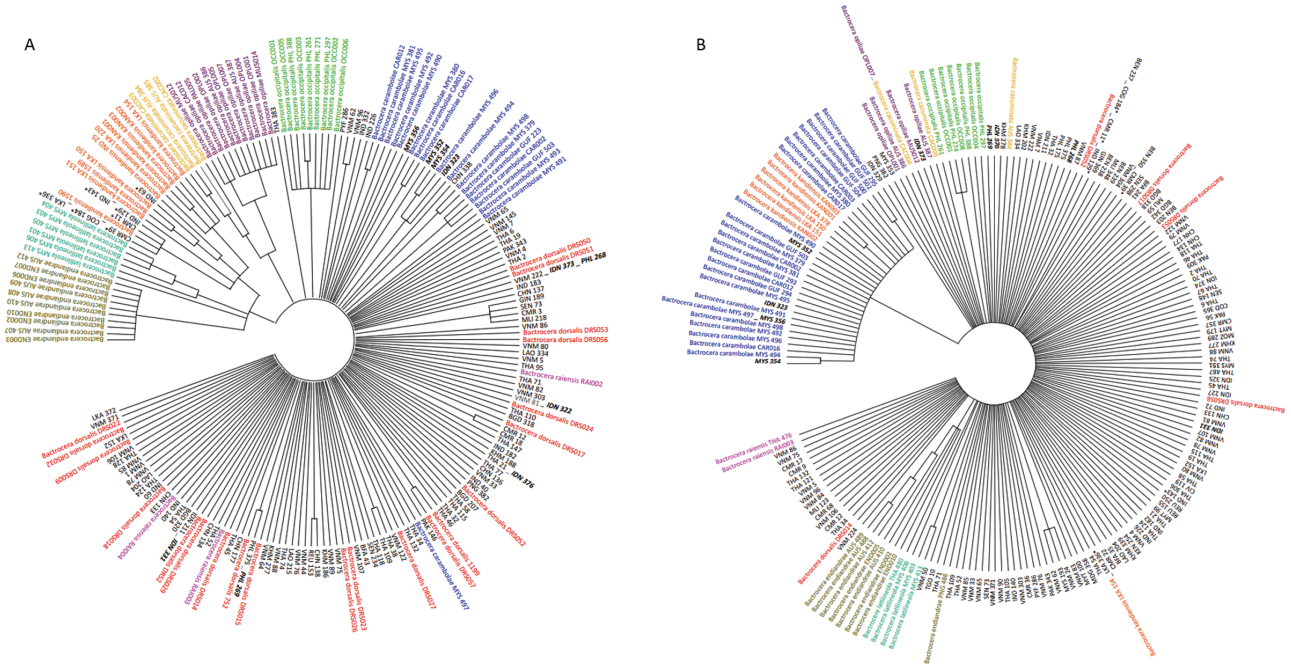

Fig. 3. Consensus Maximum Likelihood (ML) unrooted tree from (A) 192 COI sequences, and (B) 190 EIF3L sequences of *B. dorsalis* complex species. Colors reflect species of reference sequences. Haplotypes in black correspond to the target dataset. Specimens in bold and italics are the 9 ambiguous specimens. Specimens flagged with a star are *B. dorsalis* specimens with a *B. kandiendis* mitochondria.

Table 2: Success (√) and fail (X) of each morphological and molecular tool to discriminate *B. dorsalis* from other species in the complex, to highlight the importance of a multidisciplinary approach when studying the *B. dorsalis* complex.

	Morphology	COI	EIF3L/ ITS1
B. cacuminata	√	√	X
B. carambolae	X	X	√
B. endiandrae	√	√	X
B. kandiensis	√	X	X
B. latilineola	√	√	X
B. occipitalis	X	√	X
B. opiliae	√	√	X
B. raiensis	√	X	X

Two molecular markers (COI + EIF3L or ITS1) thus successfully discriminated *B. dorsalis* from 6 species of the complex, including the morphologically similar pest species *B. carambolae and B. occipitalis* (Table 2). *Bactrocera dorsalis* had bases different from *B. carambolae* at positions 99 and 348 for EIF3L, and from *B. occipitalis* at positions 178 and 530 for COI. In both species pairs, the different bases were rare and often not borne by all 278 *B. dorsalis* specimens. Identification of the 10 ambiguous specimens was thus achieved using the ML trees constructed from each marker (see list in Table 1): the COI barcode confirmed IDN_322, IDN_331, IDN_373, PHL_268, and PHL_269 as not *B. occipitalis* (Fig. 3A), and the EIF3L marker and ITS1 length polymorphism confirmed IDN_323, MYS_352, MYS_354, and MYS_356 as *B. carambolae* and IDN_322, IDN_331 and PHL_268 as *B. dorsalis* (Table 1; Fig. 3B). Specimens IDN_373, IDN_376, and PHL_269 were neither *B. occipitalis* nor *B. carambolae* according to COI and EIF3L (Fig. 3), but they could be either *B. dorsalis* or 1 of the 6 lesser-known species unattracted to ME (Table 1).

B. kandiensis and *B. raiensis* could not be discriminated from *B. dorsalis* with COI nor EIF3L (Fig. 3), but their morphology is sufficient for identification (Table 2). The limited individual diagnostic capacity of these molecular markers was confirmed by the considerable overlap between the distributions of intraspecific and interspecific distances (Supplementary Fig. S2) and the low sampling coverage of haplotype diversity (Supplementary Fig. S3). Using next-generation sequencing will produce large genomic resources with which to target diagnostic nuclear loci. However, we demonstrate that combining a mtDNA (i.e., COI) with a nDNA (i.e., EIF3L) marker is effective for identifying *B. dorsalis* among morphologically similar specimens of sibling and other closely related taxa. To assign taxonomic status to specimens, we therefore recommend combining expert morphological description with both mitochondrial and nuclear DNA barcodes, particularly when studying *B. dorsalis* in Southeast Asian islands.

Acknowledgments

This work was funded by the French Agricultural Research Centre for International Development (CIRAD) and the French Agency For Food, Environmental and Occupational Health & Safety (ANSES) (project BACTRACK). We are thankful to the many collaborators named in Supplementary Table S1 for providing us with fruit fly specimens, the phytosanitary border inspectors at Roissy airport, and Anaïs Chailleux in particular for coordinating sampling in Africa. We thank the CBGP (Centre de Biologie pour la Gestion des Populations) for giving us access to Molecular Biology and Collection platforms. We express our deeply felt gratitude to Emma Artige (INRAE) for having taken on the colossal administrative workload to obtain the legal agreements between CIRAD and 21 different government agencies required by the Nagoya Protocol for the Convention of Biological Diversity, and Servane Baufumé (CIRAD) for her assistance and insightful tips.

Author Contributions

Emeline Charbonnel (Conceptualization [Equal], Data curation [Lead], Formal analysis [Equal], Investigation [Equal], Methodology [Equal], Validation [Equal], Visualization [Equal], Writing – original draft [Equal]), Marie Pierre Chapuis (Conceptualization [Lead], Funding acquisition [Lead], Methodology [Lead], Project administration [Lead], Resources [Equal], Supervision [Lead], Validation [Equal], Writing – original draft [Equal]), Andrea Taddei (Data curation [Supporting], Resources [Supporting], Writing – original draft [Supporting]), Mark Schutze (Data curation [Supporting], Resources [Supporting], Writing – original draft [Supporting]), M. L. Starkie (Data curation [Supporting], Resources [Supporting]), Laure Benoit (Data curation [Supporting], Resources [Equal], Writing – original draft [Supporting]), Raphaëlle Mouttet (Data curation [Supporting], Writing – original draft [Supporting], Resources [Supporting], Writing – original draft [Supporting]), and David Ouvrard (Conceptualization [Equal], Funding acquisition [Lead], Methodology [Supporting], Project administration [Equal], Resources [Equal], Supervision [Lead], Validation [Equal], Writing – original draft [Equal])

Data Availability

All COI and EIF3L sequences produced in this study were deposited in GenBank under accession numbers OR208262–OR208544, OQ547452–OG547739, OR554265, and OR197650–OR197700.

Supplementary Material

Supplementary material is available at *Journal of Economic Entomology* online.

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