



EURL European Union Reference Laboratory for INSECTS AND MITES



FINAL REPORT

Validation of the morphological and molecular diagnostic protocols for Bactrocera dorsalis

IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: *Bactrocera dorsalis* and EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests

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1. Introduction

The European Reference Laboratory for Insects and Mites has to select, adapt or develop reliable diagnostic protocols for the phytosanitary insect and mite species that are relevant for the European Union (included in the Commission Delegated Regulation (EU) 2019/1702 and in the EURL for Insects and Mites working programmes). One of the tasks of the EURL is to validate available diagnostic protocols before recommending their use to the National Reference Laboratories of the European Union.

According to the ISO/IEC 17025 standard, the validation of a test is defined as the "confirmation by examination and the provision of objective evidence that the particular requirements for a given intended use are met". In fact, this confirmation consists of comparing the values of the performance criteria determined during the test characterization study with those expected or assigned beforehand (limits of acceptability, objectives to be achieved), then declaring the analytical test valid or invalid. In the field of entomology, identification is qualitative, meaning that diagnostic protocols allow the identification at a given taxonomic level providing a response in terms of presence/absence.

The EURL for Insects and Mites focuses on the validation of tests published in international or regional standards, such as those issued by the International Plant Protection Convention (IPPC) or the European and Mediterranean Plant Protection Organization (EPPO).

Bactrocera dorsalis (Hendel, 1912) (Diptera: Tephritidae) is endemic and widespread in tropical areas of Southeast Asia and is part of the *Bactrocera dorsalis* complex, a group of closely related species that comprises 88 described species (Doorenweerd *et al.*, 2018). The complex is named after *Bactrocera dorsalis* because of the importance of this polyphagous commercial fruit pest worldwide. However, the complex as a whole does not represent a monophyletic group (Leblanc *et al.*, 2015).

An ongoing debate is taking place regarding the taxonomy of *B. papayae* and *B. invadens*: some experts consider them indistinguishable from *B. dorsalis* and thus, the same biological species; others consider them as valid taxonomic entities distinct, although extremely similar, from *B. dorsalis* (Clarke *et al.*, 2005; Chen and Hui, 2007; Schutze *et al.*, 2015a, b; Drew & Romig, 2016; Schutze *et al.*, 2017). In this paper, these three species are considered as the same species and treated under the name of *B. dorsalis sensu lato* (*s.l.*). For a list of other *B. dorsalis* synonyms, see Pest information in IPPC, 2019.

Due to its high reproductive and biotic potential, a rapid dispersal ability and a broad host range, *B. dorsalis* is considered a species with a high invasive capacity. Since the first report in Kenya in 2003, the species has rapidly colonized almost the entire African continent. It is locally present in the United States and the recent, repeated interceptions in Italy (2018, 2019), Austria (2014-2019) and France (2019) keep the European Plant Protection Organisations on alert (Egartner *et al.* 2019; CABI, 2021; EPPO, 2021a).

Bactrocera dorsalis (Diptera: Tephritidae) is a European Union regulated species, listed among the EU quarantine pests (Annex II of the Commission Implementing Regulation (EU) 2019/2072) and among the EU priority pests (Commission Delegated Regulation (EU) 2019/1702).

2. Scope of validation and diagnostic protocols

2.1 Scope

The scope of this validation study is to provide objective evidence that the selected diagnostic protocols are suitable to perform routine identification of *Bactrocera dorsalis* by the staff of the EU National Reference Laboratories. Note that in this document, when reference is made to "*Bactrocera dorsalis*" simply, it means "*Bactrocera dorsalis s.l. (sensu lato*)". For a brief explanation of the meaning of *B. dorsalis s.l.*, see the introduction.

2.2 Description of the diagnostic protocols under validation

This validation study is focused on two diagnostic protocols for the morphological and molecular identification of *Bactrocera dorsalis*, i.e.:

- > IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: *Bactrocera dorsalis* (IPPC, 2019), which includes:
 - tables of characters and keys for the morphological identification of adults of the *B. dorsalis* complex
 - a molecular test to distinguish B. carambolae from other species of B. dorsalis complex
- EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b), which includes tests for the DNA barcoding of arthropods.

Validation was conducted according to the EPPO PM7/ 98(4) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity (EPPO, 2019).

2.2.1 <u>Morphological identification of adults</u>

Protocol: IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: Bactrocera dorsalis

The identification at the level of the species for the *Bactrocera dorsalis* complex requires morphological examination of adult flies. The identification is possible both on male and female specimens. The use of a stereomicroscope is needed (\geq 20 magnification).

The protocol provides guidance for the identification of the species for the *Bactrocera dorsalis* complex starting from the subgenus level:

- paragraph 4.2.1, characters for the identification of the **subgenus** *Bactrocera* (*Bactrocera*);
- paragraph 4.2.2, list of characters (Table 2) that are useful for the identification of **the** *B. dorsalis* **complex**. A specimen must have all the characters that match the description provided to be identified as belonging to the *B. dorsalis* complex;
- paragraph 4.2.4, diagnostic key to six economically important species belonging to the *Bactrocera dorsalis* complex. The species included in the key are *B. caryeae*, *B. kandiensis*, *B. occipitalis*, *B. pyrifoliae*, *B. carambolae* and *B. dorsalis s.l.*. Results obtained by means of this key have to be confirmed by checking the list of morphological characters included in Table 3 (paragraph 4.2.3).

The validation planned in this document took into account the list of characters for the identification of the *B. dorsalis* complex (4.2.2), the diagnostic key to six economically important species (4.2.4) and Table 3 – 'Diagnostic morphological characters of adult fruit flies of six economically important species of the *Bactrocera dorsalis* complex' (4.2.3). **The list of characters for the identification of the subgenus** *Bactrocera* (*Bactrocera*), as well as the observation of male and female genitalia, was not subject of this study, due to the following practical reasons:

- the dissection of genitalia must be performed in advance by supervisor (for definition of staff roles, see 5 Time schedule and staff) and, if the whole abdomen has to be removed, that means that the characters of the abdomen are not available anymore for the operators to be checked;
- handling of male genitalia by supervisor and operators risks damaging/breaking the aedeagus, with a considerable impact of repeatability and reproducibility of the analysis;
- as it is stated in the protocol by authors themselves, the aedeagus length "does not always provide a clear diagnosis because of overlap in the range of aedeagus (M) and aculeus (F) size between *B. dorsalis s.l.* and *B. carambolae*" (see 4.2.3).

This choice was supported by the opinion of an internationally renowned expert, Marc de Meyer (Royal Museum for Central Africa, Tervuren, Belgium), directly questioned on the matter. In particular, his opinion was that "No, I don't think that the reliability of an identification to species level is reduced if you cannot confirm the subgenus first. As long as the character state data set used for identification of the species is large enough and allow excluding species, a species identification can be done based on this without knowing the subgenus".

2.2.2 <u>Molecular identification of adults, larvae and pupae</u>

Molecular tests can support morphological identifications of adults. Furthermore, these tests can especially be used when dealing with other developmental stages than adults (e.g. larvae, pupae). Two protocols were validated.

- Protocol: EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b), Appendix 1 – DNA barcoding of arthropods

DNA barcoding is used to identify arthropods at a certain taxonomic level. The chosen marker region is the mitochondrial cytochrome c oxidase I (*COI*) gene. Two different primer sets (LCO1490/HCO2198 and LepF/LepR), targeting this gene, were validated.

- Protocol: IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: Bactrocera dorsalis

In this protocol, molecular tests are not recommended as standalone test in order to discriminate the six economically relevant species mentioned in the standard. When identifying *B. carambolae* and *B. dorsalis s.l.* specimens using this protocol, this

molecular test is necessary for accurate identification whenever adult morphology alone cannot distinguish between the two species.

DNA sequencing of either the internal transcribed spacer 1 (*ITS*1) or 2 (*ITS*2) nuclear DNA regions has been proposed as a reliable way to distinguish between the species *B. carambolae* and *B. dorsalis s.l.* (Boykin et al., 2014; Schutze et al., 2015a) (Paragraph 4.3.2) . The *ITS*1 test as described by Boykin et al. (2014) for distinguishing the two species is included in the current protocol. This test is designed to diagnose a fly as *B. carambolae* based on the presence of a unique DNA insert that is not present in *B. dorsalis s.l.*. The *ITS*1 test in the IPPC protocol has not been tested to distinguish *B. carambolae* from all other *Bactrocera dorsalis* complex species.

2.3 Composition of the sample set

A set of 40 samples was used. It consisted of 40 adult specimens belonging to the target and to the non-target species (11 taxa). Table 1 provides a summary of the sample set. For the detailed composition of the sample set, see Appendix 1 of this document. Target specimens came from 6 different countries (Laos, Mali, Senegal, Taiwan, Thailand and Vietnam). Non-target specimens belonged all to the family Tephritidae and were selected primarily based on the close similarity to the target species and the availability in the partner laboratories reference collections. The origin of the non-target specimens was variable, including Asian, African and European countries. Each sample was re-labelled with a number from 1 to 40 by supervisors, after randomization (for definition of staff roles, see 5 - Time schedule and staff). Original codification of samples was available only to supervisors. All samples were preserved in single tubes, filled with 95% ethanol.

The composition of the set was chosen to allow the evaluation of sensitivity, specificity, repeatability, reproducibility and accuracy of the tests.

For the validation of the molecular protocols additional, smaller sample sets were prepared (see 3.3.2 – Molecular tests).

Species	Number	Provider	Number	Provider	Total Number
B. dorsalis	12	ANSES	2	AGES	14
B. carambolae	5	ANSES	1	AGES	6
B. caryeae	0	ANSES	2	AGES	2
B. kandiensis	5	ANSES	0	AGES	5
B. occipitalis	0	ANSES	2	AGES	2
B. pyrifoliae	0	ANSES	1	AGES	1
Anastrepha suspensa	0	ANSES	2	AGES	2
Anastrepha obliqua	0	ANSES	2	AGES	2
Zeugodacus cucurbitae	2	ANSES	0	AGES	2
Bactrocera oleae	2	ANSES	0	AGES	2
Dacus ciliatus	2	ANSES	0	AGES	2
					40

Table 1: Summary of the composition of the sample set

3. Validation of the diagnostic protocols

3.1 Performance characteristics assessed

According to the guidance given in PM 7/98 (4) (EPPO, 2019) and the definitions given in PM 7/76 (5) (EPPO, 2018a), PM 7/122 (1) (EPPO, 2014) and EPPO PM 7/129 (2) (EPPO, 2021b), validation of diagnostic tests relies on the evaluation of the following performance characteristics: sensitivity, specificity, reproducibility, repeatability and accuracy.

Table 2 shows the criteria that were used to calculate the performance characteristics of the tests.

Performance criteria	Definition	Calculation		
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests) <u>Comments</u> : as far as possible, the evaluation of specificity must include samples from non-target organisms that can be confused with the target species	Diagnostic specificity = true negatives/(true negatives + false positives)		
Analytical spacificity	Inclusivity: The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-		
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-		
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)		
Analytical sensitivity	The smallest amount of target that can be detected reliably. In the case of molecular test, it is referred to as "limit of detection", i.e. the lowest DNA concentration of the target organism that can be reliably detected). For DNA barcoding the limit of detection is the DNA concentration that is sufficient to generate an amplicon which can be sequenced and leading to a HQ consensus sequence (Phred score > 40) of at least 99%.	-		
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement		
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement		
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples. It is worth noting that the accuracy is a global criterion which can be subdivided, to refine the analysis, into three other criteria: sensitivity, specificity and repeatability.	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)		

Table 2: Definition and calculation of performance characteristics

3.2 Performance characteristics already available

Performance characteristics obtained within this study were compared with performance characteristics already available for the respective tests. For the EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b) performance characteristics were already available in Appendix 1, paragraph 4 of the standard itself. For the other molecular tests and for the morphological test, performance characteristics were not available. In this latter case, the expected performance characteristics were considered equal to 100%, with the exclusion of the molecular analytical sensitivity, which consists in a measure of concentration expressed in ng/ μ l.

EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b), performance characteristics:

- Analytical sensitivity: DNA concentration (PCR amplicon) of 4 ng/μl sufficient for high quality amplicon sequencing HQ quality (Phred score >40) consensus sequence of at least 99%.
- **Analytical specificity:** The interspecific variation of the gene locus was determined to be sufficient for identification at species level.
 - <u>Inclusivity:</u> Summary list of identified arthropods in Appendix 1 (Table 1) of the standard and EPPO validation sheet (http://dc.eppo.int/tps.php).
 - Exclusivity: n.a.
- Diagnostic sensitivity: 98%-100%

Additional performance characteristics in literature: no additional information available.

3.3 Validation protocol

3.3.1 <u>Morphological test</u>

The set of 40 specimens was analyzed by three operators (for definition of staff roles, see 5 Time schedule and staff), belonging to the two different institutes (AGES and ANSES). The set composition was defined by the supervisors and known to the supervisors only.

Supervisors provided operators with the Check Lists and Summary Results sheet in Appendix 2, but did not provide operators with origin and host plants data. During the analysis, to be carried out at a stereomicroscope, operators have filled the Check List for each sample and record the identification results on the Summary Results sheet. For a better understanding of some morphological characters, especially concerning their colour, operators observed each specimens both in ethanol and dry. The results of the identification were expressed as:

- POSITIVE, if **all** the characters of the specimens matched with those of *B. dorsalis s.l.*;
- NEGATIVE, if **not all** the characters of the specimens matched with those of *B. dorsalis s.l.*;

- NOT DETERMINED (n.d.), if the matching of characters was ambiguous. In this case, operators were required to highlight which characters lead to the ambiguous results, i.e. the impossibility of identification (Notes column in the Summary Results sheet).

After the analysis, the Summary Results sheet were retrieved by the supervisors. In case of deviations of the results from the expected ones, the Check List allowed the supervisors to precisely identify any critical issues within the protocol.

Performance characteristics were assessed according to the following *a priori* established plan:

- <u>Diagnostic sensitivity and specificity</u> were assessed on the basis of the analysis of the whole sample set carried out by operator 3 (ANSES);
- <u>Repeatability</u> was assessed on the basis of the analysis of the whole sample set carried out by operator 3 (ANSES) (three repetitions of analysis).
- <u>Reproducibility</u> was assessed on the basis of the analysis of the whole sample set carried out by operator 1, 2 (AGES) and 3 (ANSES) (first of the three repetitions of analysis).

While performing the morphological analysis for the third and last repetition, operator 3 removed one leg from each specimen and placed it in an Eppendorf vial, in 70% ethanol, keeping the respective code. The leg samples were shipped to AGES for the DNA extraction and the molecular analysis. For some of the specimens, DNA extraction was repeated on the whole specimen due to the fact that DNA of unsufficient quality and quantity was purified from leg.

Figure 1 provides a scheme of the activity.

3.3.2 <u>Molecular tests</u>

DNA extraction

For DNA extraction of whole specimens (e.g. analytical sensitivity) the DNeasy Blood & Tissue Kit (Qiagen) was used. For the DNA extraction from single legs the QIAamp DNA Micro Kit (Qiagen) was used.

Analytical specificity

The same set of specimens used for the morphological analysis was used for the validation of the molecular tests - see 2.3 for further specifications. **Inclusivity:** 14 targets

Exclusivity: 26 non-targets, 16 of which belong to the Bactrocera dorsalis complex

The primer sets and PCR parameters are described in Appendix 3.

SANGER sequencing was outsourced to a certified sequencing service provider (EUROFINS Genomics).

Data-analysis: The software Geneious prime[®] 10.1.3 was used for the consensus sequence preparation. For sequence alignment following genetic databases were consulted: NCBI-GenBank, Bold and EPPO Q-Bank.

In silico testing of analytical specificity for molecular tests:

LCO1490/HCO2198 and LepF/LepR: Search for Tephritidae in silico by a database alignment (NCBI GenBank) (see App. 7).

ITS6/7 primer: Search for Bactrocera sp. in silico by a database alignment (NCBI GenBank) (see App. 7).

Analytical sensitivity

4 samples consisting of one adult, a leg, a larva and a pupa from *B. dorsalis* were prepared in different dilutions. Three experimental repetitions were performed with this sample set.

Sample set:

1 adult specimen of *B. dorsalis* (333/20) 1 leg of *B. dorsalis* (334/20) 1 larva of *B. dorsalis* (335/20) 1 pupa of *B. dorsalis* (336/20)

Dilutions (1:10, 1:100, 1:1.000; 1:10.000; 1: 100.000, 1:1.000.000). To define the limit of detection for DNA barcoding, the two highest dilutions which resulted in an amplicon were sequenced and analysed.

Repeatability

Four biological replicates of *B. dorsalis* (adult, leg, larva, pupa) in three different dilutions (last dilution near by the detection limit) were analysed with 3 technical replicates to determine the repeatability.

Reproducibility

Testing reproducibility of the PCR tests:

Three targets (adult, larva and pupa) and three non targets (adult, larva) were used to test the reproducibility of the PCR tests (Table 3). These tests were performed with three replicates and under different conditions (two operators at different days and using different thermocycler machines).

Target	Non target	Origin
B. dorsalis adult	/	Thailand /Saraburi
<i>B. dorsalis</i> larva	/	Thailand /Saraburi
B. dorsalis pupa	/	Thailand /Saraburi
	Bactrocera correcta larva	India
	Bactrocera carambolae adult	French Guyana
	Bactrocera latifrons larva	Thailand

Table 3: Sample set used to test reproducibility

Testing reproducibility of the SANGER sequence analysis:

The reproducibility of the SANGER sequence analysis was tested with the same sample set. The sequence analysis was performed by two operators on different days. The alignment of the consensus sequence was performed in three different databases (NCBI GenBank, Bold, Q-Bank).

Specifications and parameters for the molecular tests are provided in Appendix 3. Figure 1 provides a scheme of the activity.





4. Performance adequacy and validation

The performance values obtained by the diagnostic protocol/ test were compared with the predetermined, expected performance characteristics.

The adequate expected performance characteristics are shown in Table 4. They are also referred to as "limits of acceptability" of the test. If the obtained performance characteristics did not reach the expected values, a cause analysis was carried out to identify the critical steps in the test(s) that led to the unexpected results (i.e., false negatives, false positives, not determined). Some positive deviations were expected, as DNA barcoding is according to the IPPC standard insufficient to discriminate *B. carambolae* from *B. dorsalis*. Due to this, the lowest calculated value of expected performance characteristics with the current sampel panel is 77% (diagnostic specificity). This also influences the accuracy.

Table 4: Expected performance characteristics (limits of acceptability).

	Expected performance characteristics					
Performance criteria	IPPC 27 DD 20 Pastrosora dorsalis		IPPC 27 -DP 29			
	morphological identification	barcoding	Bactrocera dorsalis –			
	morphological identification	barcoung	ITS1 primer			
Diagnostic specificity	100%	77%	100%			
Analytical specificity			1000/			
(Inclusivity)	-	-	100%			
Diagnostic sensitivity	100%	100%	100%			
Analytical sensitivity	1 adult specimen	4 ng/μl	4 ng/μl			
Repeatability	100%	100%	100%			
Reproducibility	100%	100%	100%			
Accuracy	100%	85%	100%			

^a as from Appendix 1, paragraph 4 of EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b)

5. Time schedule and staff

The trial period was from May to August 2020 and involved staff from the EURL for Insects and Mites.

Participating staff:

- for morphological tests:
 - Experts/ Supervisors: Sylvia Blümel, Valérie Balmès, Raphaëlle Mouttet Role: definition, randomization and blind-codification of sample set, preparation of check-lists, collection and analyses of results, drafting of final report
 - Technical staff/ Operators: Christa Lethmayer, Alois Egartner, Andrea Taddei Role: performance of analyses, help to supervisor in the interpretation and analysis of results, drafting of final report
- for molecular tests:
 - Experts/ Supervisors: Richard Gottsberger, Helga Reisenzein
 - Role: definition, randomization and blind-codification of sample set, collection and analyses of results, drafting of final report
 - Technical staff/ Operators: Claudia Heiss, Christina Lippitz, Chiara Pohn Role: performance of analyses, help to supervisor in the interpretation and analysis of results, drafting of final report

6.1 Morphological test

Protocol: IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: Bactrocera dorsalis (IPPC, 2019)

The values obtained for diagnostic specificity, diagnostic sensitivity, accuracy and repeatability met the expected value of 100% (Table 5). The test was found to be inclusive for target specimens from Laos, Mali, Senegal, Taiwan, Thailand and Vietnam and exclusive for a range of non-target specimens belonging to the *B. dorsalis* complex (*B. carambolae, B. caryeae, B. kandiensis, B. occipitalis, B. pyrifoliae*), the *Bactrocera* genus (*B. oleae*) and non-*Bactrocera* Tephritidae (*Anastrepha obliqua, Anastrepha suspensa, Dacus ciliatus, Zeugodacus cucurbitae*).

The value obtained for reproducibility did not meet the expected value of 100%, but reached a value of 87.5%. The cause was found in the divergent results obtained for 9 specimens either by 1 (5 specimens) or 2 operators (4 specimens), as summarized in Table 6. The check lists compiled by operators during the performance of the analyses allowed to track back the critical steps in the protocol that led to the deviation from the expected results.

Appendix 4 of this document shows the results obtained by the three operators.

Appendix 5 shows the calculations for the performance characteristics.

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	100%	100%
	Inclusivity: The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	-	Laos Mali Senegal Taiwan Thailand Vietnam
Analytical specificity	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	-	B. carambolae B. caryeae B. kandiensis B. occipitalis B. pyrifoliae B. oleae Dacus ciliatus Zeugodacus cucurbitae Anastrepha obliqua Anastrepha suspensa
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	100%	100%
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	1 adult specimen	1 adult specimen
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	100%	100%
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	100%	87,5%
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	100%	100%

Table 5: Summary of the results obtained for the morphological protocol

6.1.1 <u>Analysis of critical steps in the protocol</u>

The morphological characters, as described in the protocol, which were recognized at the basis of divergent results are listed in Table 6. Among them, the descriptions of the following characters were particularly relevant:

- I. Character "Costal band", relevant for the divergent results for 7 specimens (5 *B. carambolae* and 2 *B. occipitalis*);
- II. Character "Transversal black band on tergite 3", relevant for the divergent results for 3 specimens (3 *B. carambolae*).

With reference to these diagnostic characters, it has been possible to detect that their description in the protocol (either in the diagnostic key or in the character table or both) is sometimes prone to uncertain interpretation, so that it can sometimes be misleading for the protocol user. An in-depth analysis is provided below.

Sample code Assigned value		Result by Operator 1	Result by Operator 2	Relevant character	Description in the protocol
4	Negative (B. carambolae)	Negative	Positive	Costal band confluent/ overlapping	Table 3 (4.2.3), page 11
	Negative			Costal band confluent/ overlapping	Table 3 (4.2.3), page 11
8	(B. carambolae)	Positive	Positive	Transverse band on abdominal tergite 3	Table 3 (4.2.3), page 9; Diagnostic key (4.2.4), page 12
18	Positive	Positive	Negative	Postpronotal lobe	Table 3 (4.2.3), page 10
22	Negative	Not determined	Positive	Transverse band on abdominal tergite 3	Table 3 (4.2.3), page 9; Diagnostic key (4.2.4), page 12
	(B. carambolae)			Costal band confluent/ overlapping	Table 3 (4.2.3), page 11
23	Negative (B. occipitalis)	Negative	Not determined	Costal band distinctly overlapping R2+3 and expanding broadly around apex of wing reaching mid- point between R2+3 and R4+5	Table 3 (4.2.3), page 11 Diagnostic key (4.2.4), page 12
	Negative	Negative		Scutum color	Table 3 (4.2.3), page 10
27	(B. carambolae)	Not determined	Positive	Costal band confluent/ overlapping	Table 3 (4.2.3), page 11
36	Positive	Not determined	Positive	-	-
39	Negative	Not determined	Positive	Transverse band on abdominal tergite 3	Table 3 (4.2.3), page 9; Diagnostic key (4.2.4), page 12
	(B. carambolae)			Costal band confluent/ overlapping	Table 3 (4.2.3), page 11
40	Negative (B. occipitalis)	Negative	Positive	Costal band distinctly overlapping R2+3 and expanding broadly around apex of wing reaching mid- point between R2+3 and R4+5	Table 3 (4.2.3), page 11 Diagnostic key (4.2.4), page 12

Table 6: Samples for which divergent results were obtained with respect to a priori assigned value

Character "Costal band" in the identification of B. occipitalis and discrimination with B. carambolae

In the Diagnostic key to six economically important species belonging to the *Bactrocera dorsalis* complex (paragraph 4.2.4, page 12), at point 3 the description states:

3. Costal band distinctly overlapping R₂₊₃ and expanding broadly around apex of wing <u>reaching mid-point between R₂₊₃ and R₄₊₅</u> (Figure 16(e)).....B. occipitalis,

This description can lead to misunderstanding, as one might be led to think that "mid-point" refers to a point along the edge of the wing and consider the only option of a point between R₄₊₅ and M as possible. This misunderstanding would consequently lead to the belief that the wing veins are misnamed in the description, which is not the case. As international Tephritidae experts Norman Barr and Camiel Doorenweerd explained, in fact "mid-point" refers to the imaginary line that divides the cell in half between R₂₊₃ and R₄₊₅ (Fig. 2) and it should be better considered as a "mid-line" rather than a "mid-point".

This misinterpretation of the term "mid-point" contributed to the divergent results for the two *B. occipitalis* specimens in the fact that the operators, when looking for a mid-point, failed to locate it between R₂₊₃ and R₄₊₅, refusing to choose *B. occipitalis*. Possible options for the costal band in the *Bactrocera* genus are as follows (Camiel Doorenweerd's communication):

- 1) Costal band confluent with R_{2+3}
- 2) Costal band faintly (slightly) crosses R₂₊₃
- 3) Costal band reaches midway (mid-point) between R2+3 and R4+5 [B. occipitalis]
- 4) Costal band reaches up to R₄₊₅
- 5) Costal band confluent with R₄₊₅

Figure 2 – *B. occipitalis* wing pattern (e), as from DP 29 (IPPC, 2019), modified by and courtesy of Camiel Doorenweerd: the dotted line indicates the mid-point between R_{2+3} and R_{4+5} ; Tephritidae wing venation (6), as from White & Elson-Harris (1992)





Figs 5-6. Adult morphology; 5, head; 6, wing. Abbreviations are listed on p. 31.

In addition, in the Diagnostic key (paragraph 4.2.4, page 12), the costal band of *B. occipitalis* and *B. carambolae* is described as follows:

3. Costal band <u>dist</u> (Figure 16(e))	<u>nctly overlapping R2+3</u> and	expandii	ng broadly arou	und apex	of wing re	aching ı	nid-p	oint bet	ween R2+3	and R ₄₊₅ cipitalis
5. Costal band	l <u>slightly overlapping</u>	<u>R2+3</u> ,	moderately	broad	around	apex	of	wing	(Figure B. card	' 16(a)); ambolae

The difference between the shape of costal band of *B. occipitalis* ("distinctly overlapping R_{2+3} ") and *B. carambolae* ("slightly overlapping R_{2+3} ") is not sufficiently clear from Figures 16 (a) and 16 (e) in the protocol (Figure 3 in this document). This unclear difference contributed to the divergent results obtained for the two *B. occipitalis* specimens included on the set. These descriptions are confirmed in Table 3 (paragraph 4.2.3, page 11).





Character "Costal band" in the discrimination between B. dorsalis s.l. and B. carambolae

In Table 3 (paragraph 4.2.3, page 11), the description of the costal band of *B. dorsalis s.l.* states: Narrow, <u>generally</u> confluent with R_{2+3} (inter- or intra-regionally variable), narrow to moderately broad around apex of wing (Figure 16(c)),

whereas in the Diagnostic key to six economically important species belonging to the *Bactrocera dorsalis* complex (paragraph 4.2.4, page 12), at point 5, costal band is *confluent with* R₂₊₃, *narrow to moderately broad around apex of wing*, the adverb "generally" is missing. In this way, the description in the Table 3 includes an element of uncertainty that is not present in the key, i.e. that generally the costal band is confluent with R₂₊₃, but in some cases may not be confluent (*Bactrocera carambolae* – like overlapping costal band?)

The adverb "generally" was relevant for the divergent results obtained for 5 *B. carambolae* specimens.

After consultation with Tephritidae experts Norman Barr and Luc Leblanc, the term "generally" would mean "typically" in the table. <u>A diagnosis of *B. dorsalis* requires confluence</u>. *B. dorsalis* populations which do not display a costal band confluent with R_{2+3} are not known. Although rare, some populations might have 'aberrant' specimens with costal band that crosses vein R_{2+3} , but more information regarding those specimens are needed and studies are currently ongoing (Norman Barr's communication).

Description of *B. dorsalis* costal band from Schutze et al. (2015a) is recalled here: *"Wing costal band width from vein subcostal to slightly below vein R*₄₊₅ at wing apex; confluent with vein R₂₊₃ in depth." and *"narrow fuscous costal band confluent with R*₂₊₃ and remaining very narrow or widening slightly if it overlaps this vein, to end just beyond apex of R₄₊₅ (in some specimens there is an expansion around extremity of R₄₊₅, which may be slight or expanding into a hook-like pattern)".

Character "Transversal black band on tergite 3"

In the Diagnostic key to six economically important species belonging to the *Bactrocera dorsalis* complex (paragraph 4.2.4, page 12, point 5) and in Table 3 (paragraph 4.2.3, page 9), the character tergite 3 of *B. carambolae* is described as follows:

with a narrow transverse black band across anterior margin (constituting a "T" pattern) widening to cover lateral margins.

However, this description does not take into account the variation of the T pattern that is described in FruitFly ID Australia (Plant Health Australia, 2021). This variation consists in a non-continuous transverse band on tergite 3 (Fig.4 (b)). The fact that this variation is not mentioned in the standard led the operator to refuse to identify some specimens displaying this variation as *B. carambolae* (Fig. 5 (c) and (d)).

Figure 4 – (a) *B. carambolae* abdomen as from DP 29 (IPPC, 2019); (b) *B. carambolae* abdomen variation as from FruitFly ID Australia (Plant Health Australia, 2021)



Bactrocera carambolae - Abdomen Variation CAR003

Figure 5 – Detail of abdomen in some *B. carambolae* specimens included in the sample set for this study; (a) Sample 6 and (b) sample 27 display a continuous band on tergite 3; (c) sample 8 and (d) sample 39 display the abdominal variation for this character.



6.2 Molecular tests

For the goal of species identification in animals and some protists the *cytochrome c oxidase* subunit 1 (*COI* or cox1) gene of the mitochondrial DNA has been introduced as standard marker. DNA sequencing of the *COI* DNA barcode can be applied to distinguish several *Bactrocera* species like *B. oleae* and non-*Bactrocera* Tephritidae (e.g. *Anastrepha obliqua, Anastrepha suspensa, Dacus ciliatus, Zeugodacus cucurbitae*). Nevertheless, it was described as not providing adequate resolution to identify many species in the *B. dorsalis* complex (*B. carambolae, B. caryeae, B. kandiensis, B. occipitalis, B. pyrifoliae*) (IPPC, 2019).

According to the recommendations in the IPPC protocol, the sequencing of the *ITS*1 to distinguish *B. dorsalis* from *B. carambolae* was applied. *B. carambolae* has a unique 44bp insert that is lacking in other *Bactrocera dorsalis* complex species. Furthermore in the standard it is stated, that if there is no insert in a sample, *B. carambolae* can be excluded, but it can not be attributed to another species in the *Bactrocera dorsalis* complex.

In contrast to the validation of specificity (sample set used from morphological validation) the samples for sensitivity, repeatability and reproducibility consisted of fresh specimens of different developmental stages (e.g. adults, larvae and pupae). Furthermore, samples consisting of DNA extracted from only one leg were also included to demonstrate the usual suitability of such kind of material. *Bactrocera pyrifoliae* was excluded from the analysis, as no amplicon could be obtained with sample 21 (the only sample of *B. pyrifoliae* in this study).

Protocol: EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021b)

Appendix 1 – DNA barcoding of arthropods (sequencing of *COI* locus, LCO1490/HCO2198 primer set and additionally LepF/LepR primer set.

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed with the DNA barcoding primer sets (LCO1490/HCO2198 and LepF/LepR). The search set was limited to Tephritidae. The results showed suitablity of both primer sets (see Appendix 6) for identification *of B. dorsalis*, although we have to state that barcoding is a generic test including targets and non-targets.

The values obtained for diagnostic specificity, diagnostic sensitivity, and accuracy met the expected values (Table 7).

The values for the diagnostic specificity were higher (96% and 95%) than the expected one (77%). It has to be mentioned that the expected value for the diagnostic specificity was calculated based on the current sample set and the assumed number of possible misidentifications between *B. dorsalis* and *B. carambolae* using DNA barcoding standard only.

The values of the performance characteristics showed the sequencing of the *COI* locus cannot fully discriminate all listed species. The test was found to be 100% inclusive for *B. dorsalis* from Laos, Mali, Senegal, Taiwan, Thailand and Vietnam.

For the exclusivity several non-targets were tested (including *B. dorsalis* complex species: *B. carambolae, B. caryeae, B. kandiensis, B. occipitalis, B. pyrifoliae,* and other non-targets: *B. oleae, Dacus ciliatus, Zeugodacus cucurbitae, Anastrepha obliqua, Anastrepha suspensa*). Only one *B. kandiensis* sample (16) was misidentified (false positive).

Contrary to the IPPC standard, in our study it was possible to discriminate *B. carambolae* from *B. dorsalis* and other species of *B. dorsalis* complex using sequence data on *COI* only.

The analytical sensitivity with both primer sets also easily met the expected value of 4 ng/µl. It is noteable, that the analytical sensitivity was higher (up to 100-fold) with LepF/LepR primers for certain matrices (legs and pupa).

For the reproducibility tests specimens of *B. correcta* and *B. latifrons* were included. The reproducibility of the PCR tests using two different primer sets and reproducibility of the SANGER sequence analysis were 100% in all cases.

The performance characteristics of the repeatability and the analytical sensitivity were different. Whereas the repeatability for the amplicon production of LepF/LepR primer set was 100%, the repeatability for the LCO1490/HCO2198 primer set was only 91.66%.

Appendix 7 of this document shows the results for diagnostic specificity. Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility. Appendix 9 shows the calculations for the performance characteristics.

	Table 7: Summary of the results obtained for the	molecular protocol – EPPO PM	7/129 (2), Appendix 1, COI gene locus.
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	iary of the results obtained for the molecul		0 1 1017 123 (2	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	<u>Beilie 1000051</u>
Performance criteria	Definition	Calculation	Expected performance characteristics (EPPO, 2021b)	Obtained performance characteristics for sequencing of <i>COI</i> (primer set LCO1490/HCO2198)	Obtained performance characteristics for sequencing of <i>COI</i> (primer set LepF/LepR)
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	77%	96%	95%
	Inclusivity: The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	-	Laos Mali Senegal Taiwan Thailand Vietnam	Laos Mali Senegal Taiwan Thailand Vietnam
Analytical specificity	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	-	B. carambolae B. caryeae B. kandiensis B. occipitalis B. pyrifoliae B. oleae Dacus ciliatus Zeugodacus cucurbitae Anastrepha obliqua Anastrepha suspensa B. latifrons B. correcta	B. carambolae B. caryeae B. kandiensis B. occipitalis B. pyrifoliae B. oleae Dacus ciliatus Zeugodacus cucurbitae Anastrepha obliqua Anastrepha suspensa B. latifrons B. correcta
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	100%	100%	100%
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	4ng/μl	0.325ng/µl	0.325/µl
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	100%	91.66%	100%
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	100%	100%	100%
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	85%	97%	97%

Protocol: IPPC ISPM 27 Diagnostic protocols for regulated pests DP 29: Bactrocera dorsalis

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed with the *ITS*1 sequencing primer set (*ITS*6/7). The search set was limited to Tephritidae. The results showed suitablity of the primer set (see App. 7) for identification of *B. dorsalis*.

Sequencing of ITS1 internal transcribed spacer (ITS6/7 primer set)

This test was not applied for samples which beforehand were unambiguously identified as *Anastrepha spp., B. oleae, Dacus ciliatus* or *Zeugodacus cucurbitae* by barcoding. *Bactrocera pyrifoliae* was excluded from the analysis, because no amplicon could be obtained with sample 21 (only sample of *B. pyrifoliae* in this study). We analysed all samples belonging to the *B. dorsalis* complex according to the *COI* barcoding results. This is not in line with the described procedure of the IPPC standard, as the *ITS6*/7 sequencing is only recommended as a follow up step after morphological identification of adult specimens. It is described as the tool for molecular discrimination of *B.dorsalis* and B. *carambolae*.

The values obtained for diagnostic sensitivity, analytical sensitivity, reproducibility and repeatability met the expected performance characteristics of 100% (Table 8). The analytical sensitivity was very high (amplification could be achieved with all samples at a 1: 100.000 dilution ($3.25pg/\mu$ l). The test was found to discriminate *B. dorsalis* from *B. carambolae*. However, in the case of sample 38 (assigned as *B. caryeae*) the sample could be discriminated from *B. carambolae* (no 44bp insert). Nevertheless, there was a match with *B. dorsalis* (NCBI GenBank query), resulting in a false positive result. This false positive result obtained for sample 38 (summarized in Table 8) also influenced the performance characteristics of the diagnostic specificity and accuracy. Therefore, the value obtained for diagnostic specificity and accuracy did not meet the expected value of 100%, but reached a value of 93% and 96% respectively.

In our study all samples of *B. kandiensis* could unambiguously identified with the *ITS*1 locus.

Appendix 7 of this document shows the results for diagnostic specificity. Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.

Appendix 9 shows the results for analytical sensitivity, repeatability and repro

Performance criteria	Definition	Calculation	Expected performance characteristics	Obtained performance characteristics
Diagnostic specificity	The proportion of non-target samples (true negatives) testing negative compared with results from an alternative test (or combination of tests)	Diagnostic specificity = true negatives/(true negatives + false positives)	100%	93%
Analytical	Inclusivity: The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	100%	100%
specificity	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	-	B. carambolae B. kandiensis B. occipitalis
Diagnostic sensitivity	The proportion of target samples (true positives) testing positive compared with results from an alternative test (or combination of tests)	Diagnostic sensitivity = true positives/(true positives + false negatives)	100%	100%
Analytical sensitivity	The smallest amount of target that can be detected reliably	-	4ng/μl	3.25pg/µl
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	100%	100%
Reproducibility	The ability of a test to provide consistent results when applied to aliquots of the same sample tested under different conditions (e.g. time, persons, equipment, location)	% level of agreement	100%	100%
Accuracy	The proportion of target samples (true positives) testing positive and non-target samples (true negatives) testing negative compared with the total number of samples	Accuracy = (true positives + true negatives)/(true positives + false negatives + true negatives + false positives)	100%	96%

Table 8: Summary of the results obtained for the molecular protocol – IPPC ISPM 27 DP29, ITS1

DNA barcoding and ITS sequencing

a. Quality of DNA and consensus sequence

Accurate comparison of sequences usually requires reliable consensus sequences. Due to the low phylogenetic resolution of some *Bactrocera* species, it was indispensable to work with high quality of DNA and consensus sequences (use sequence data from forward and reverse reaction for assembly, trimmed consensus sequences with correct orientation, length of the consensus sequences close to the expected value).

In this study we had to work on one leg per specimen (usually from a collection of long stored specimens), which resulted in low quality and/or fragmented DNA for some samples. In the case of sample 21, the DNA extraction failed in spite of several extraction repetitions (even using non destructive DNA extraction on the entire specimen), for other samples the quality of the DNA was poor (e.g. sample 33 and 38). Sample 40 was contaminated by human DNA.

This sometimes resulted in no amplification products (sample 21) or in bad or short consensus sequences (e.g. sample 16). For sample 16 the database alignment led to a false positive result (see chapter b). The database alignment of the *ITS1* consensus sequence was correct (*B. kandiensis*). This is an example of poor DNA quality masking the *COI* result. Only *ITS1* could resolve this sample originally assigned to *B. kandiensis*.

Two samples assigned as *B. caryeae* (sample 33 and 38) could not be resolved correctly although DNA extractions and PCR amplifications were repeated several times. DNA barcoding identified both samples as *B. carambolae*. This identification was wrong, but had no impact on the performance characteristics (true negative). Additionally, identification using *ITS* sequencing led to no consensus sequence (sample 33) and a false positive result (sample 38).

The contaminated sample 40 led to wrong results with barcoding. The more specific *ITS*1 sequencing allowed correct molecular identification of the sample to the assigned value.

In the light of these results it is important for the routine diagnosis to use adequate DNA extraction procedures especially for sequencing techniques (see EURL verification report: Verification of DNA Extraction Procedures for Insects 2021 (https://sitesv2.anses.fr/en/minisite/insects-and-mites/approved-reagents).

b. Availability and reliability of sequence data in NCBI GenBank, Bold and Q-Bank

During our validation study the number of correct hits and ability to exclude incorrect hits were a critical issue for identification of *Bactrocera* species.

COI gene sequences

At the time of our query NCBI GenBank and Bold have the highest number of deposited sequence data for the selected *Bactrocera* species). Contrary to the IPPC standard mentioning zero entries for *B. pyrifoliae*, one entry of the *COI* gene has meanwhile been made available for this species.

The EPPO Q-Bank currently lacks *COI* gene sequences of relevant species for *B. dorsalis* identification (e.g. no *B. kandiensis* sequences are available). Nevertheless, the sequence data included are obtained from properly documented and identified specimen (see Table 9). The Bold database has a comprehensive number of *COI* sequences and questionable results are indicated (see Fig. 6). The query in the NCBI GenBank also revealed a high number of sequences, but there is no information on the search result.

Therefore and due to quality assurance reasons, the database alignment of this study was performed in three different databases (NCBI GenBank, Bold and Q-Bank). In addition, reference alignment was performed using Geneious prime[®] 10.1.3.

<u>Table 9</u>: Numbers of *COI* gene sequences of relevant *Bactrocera* species represented in different databases – (Query from 22 February 2021).

Species	NCBI GenBank	Bold	EPPO Q-Bank
B. dorsalis	5384	7314	412
B. kandiensis	34	132	0
B. carambolae	219	408	6
B. pyrifoliae	1	0	0
B. occipitalis	82	239	0
B. caryeae	14	15	0

Figure 6 - Inconclusive results indicated by Bold database

```
Search Result:
```

A species level match could not be made, the queried specimen is likely to be one of the following:

- Bactrocera kandiensis
- Bactrocera invadens
- Bactrocera dorsalis
- Bactrocera tryoni Bactrocera caryeae

For a hierarchical placement - a neighbor-joining tree is provided:

Identification Summary

Taxonomic Level	Taxon Assignment	Probability of Placement (%)
Phylum	Arthropoda	100
Class	Insecta	100
Order	Diptera	100
Family	Tephritidae	100
Genus	Bactrocera	100

Similarity Scores of Top 100 Matches

TREE BASED IDENTIFICATION



An example for possible misidentification due to lack of sequence can be shown with barcoding results of sample 16. The *COI* consensus sequence of sample 16 was aligned in all three databases. NCBI GenBank gave three equal hits of *B. dorsalis* and *B. kandiensis* (equal scores, query covers, E-values and percentage identities). Since the *B. dorsalis* accession comprises a sequence of 676bp instead of 658bp, it is ordered above the *B. kandiensis* accessions. The database alignment in Bold revealed *B. kandiensis* only, but it was indicated that a species level match could not be made (see Fig. 6). In EPPO Q-Bank the query resulted in *B. dorsalis*. Because, two out of three database alignments resulted in *B. dorsalis*, the final judgment for this sample was *B. dorsalis*. Our false positive assignment can mainly be ascribed to the result of the BLAST search in NCBI GenBank and to the false result in EPPO Q-Bank at the date of query. At this time there were no sequences for *B. kandiensis* deposited in EPPO Q-Bank, which might be the reason for the false assignment.

|--|

Database	Result	Documen	itation				_							
NCBI GenBank	Organism: Bactrocera		Description Scientific Name S									E value	Per. Ident	
	dorsalis	Bactrocera	Bactrocera dorsalis isolate Ugb5 cytochrome c oxidase subunit 1 (COI), gene, partial cds; Bactrocera d 1029										100.00%	
	MK314052 1	Bactrocera I	Bactrocera kandiensis mitochondrial COI gene for cytochrome oxidase subunit 1, partial c Bactrocera k 1029 1029 100% 0.0 10											
	%identiy: 100%	Bactrocera I	kandiensis vouch	er Bk4 cytochrome oxid	lase subunit	1 (COI) gene, pa	artial cdsBac	ctrocera k	1029	1029	100%	0.0	100.00%	
	e-value: 0.0	Bactrocera I	kandiensis vouch	er Bd1590 cytochrome	oxidase subu	unit I (COI) gene	. partial Bac	strocera k	1024	1024	100%	0.0	99.82%	
	Score:1029	Bactrocera i	nvadens vouche	r BiSLT4.1 cytochrome	oxidase subu	init 1 (COI) gene	partial Bad	ctrocera d	1024	1024	100%	0.0	99.82%	
		Bactrocera kandiensis voucher Bk7 cytochrome oxidase subunit 1 (COI) gene, partial cds Bactrocera k 1024 1024 100% 0.0 99.82												
Bold	Organism	Phylum	Class	Order Family	/ G	ienus	Species	Subspe	cies	Sim	ilarity (%)	Status	
	Bactrocera kandiensis	Arthropoda	Insecta	Diptera Tephri	tidae <i>B</i>	lactrocera	kandiensis			100			Published	
	Accession Nb.:		Insecta	ecta Diptera Tephritic		lactrocera	kandiensis			100			Published	
	GBMIN62999-17	Arthropoda	Insecta	Diptera Tephri	tidae <i>B</i>	lactrocera	kandiensis			100			Published	
	%identiy: 100%	Arthropoda	Insecta	Diptera Tephri	tidae <i>B</i>	lactrocera	kandiensis			100			Published	
		Arthropoda	Insecta	Diptera Tephri	tidae <i>B</i>	lactrocera	kandiensis			100			Published	
		Arthropoda	Insecta	Diptera Tephri	tidae <i>B</i>	lactrocera	invadens			100			Published	
EDDO	Organism								_					
Q-Bank	Bactrocera	#≎	Reference	-	Score \$	Similarity %	overlap %	Direction	n ¢					
	dorsalis		Search		Search	Search	Search	Search.						
	Accession Nb.: CO1/VBAL 1001	+ 1	CO1/VBAL_100 dorsalis	01036_COI - Bactroce	a 884.409	100	100	+/+						
	036 %identiv: 100%	+ 2	CO1/VBAL_100 dorsalis	0481_COI - Bactroce	a 881.239	99.82	100	+/+						
	%identiy: 100%	+ 3	CO1/CCOC097 Bactrocera dor	07_0101_COI - salis	881.239	99.82	100	+/+						
		+ 4	CO1/VBAL_100 dorsalis	CO1/VBAL_1000295_COI - Bactrocera dorsalis		99.82	100	+/+						
		+ 5	CO1/VBAL_110 dorsalis	01045_COI - Bactroce	a 878.069	99.641	100	+/+						
		+ 6	CO1/VBAL_110 dorsalis	01524_COI - Bactroce	a 878.069	99.641	100	+/+						

ITS sequences

The IPPC standard mentions that *B. carambolae* is distinguishable from *B. dorsalis* on the *ITS*1 due to the presence of a unique 44bp insert in *B. carambolae*. However, this was based on the *ITS*1 comparison of only four species in the *Bactrocera dorsalis* complex: *B. dorsalis s.l., B. occipitalis, B. opiliae* and *B. cacuminata.* Guidance is given in the IPPC standard for reference alignments including reference sequences (NCBI accession Nb. KC446737.1 for *B. carambolae* and KC446776.1 for *B. dorsalis*).

Figure 8 - *ITS1* reference alignment: *B. dorsalis* samples of this study aligned to the recommended reference sequence (KC446776.1) according to the IPPC standard.

Consensus Coverage	12 0	-	ų	100	190	200	250	300	210	400	40 45
KC646776 Bactrocera dorsalis CM_RR0 Sample 32 CM_RR0 Sample 32 CM_RR0 Sample 34 DM_RR0 Sample 15 DM_RR0 Sample 15 DM_RR0 Sample 17 DM_RR0 Sample 25 DM_RR0 Sample 24			ų	ų.	ų.	391	211	24	201	Ni	

To elucidate the quality and reliability of the *ITS*1 sequencing, a comprehensive search of available sequences in databases was performed. Sequences on the *ITS*1 region are only available in the NCBI GenBank, but some entries are questionable. It has to be noted that at the date of the query no *ITS*1 sequences for *B. caryeae* and *B. pyrifoliae* (only one *ITS*2) were available.

An example for unreliable entries is the NCBI blast result for sample 38 (assigned value is *B. caryeae*):

First hit with this sample was *Bactrocera carambolae* "voucher" (accession number KJ544953.1 Guangzhou, P.R. China), followed by two hits for *B. dorsalis* with equal percent identity and score values (see Fig. 9). However, after a reference alignment with the specific *B. carambolae* insert, the sequence deposited lacked the specific insert (see Fig. 10). Therefore, the sample was determined as *B. dorsalis*, which finally was false positive.

No reference alignment could be performed for *B. caryeae* due to unavailability of *ITS* data.

Figure 9 - BLAST result in NCBIGenbank for *ITS*1 sequencing of sample 38. Three equal hits *B. carambolae* (1) and *B. dorsalis* (2) were obtained.

Se	quences producing significant alignments Download 🗡	Manaj	ge colu	imns	≺ sh	ow 10	• • •
	select all 100 sequences selected	Gen	Bank	Graph	ics D	listance tr	ee of results
	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
	Bactrocera carambolae voucher CMSJ1 internal transcribed spacer 1_partial sequence	850	850	100%	0.0	100.00%	KJ544953_1
	Dactrocera dorsalis 183 ribosomal RNA gene_partial sequence_internal transcribed spacer 1, 5,83 ribosomal RNA gene_internal transcribed s	850	850	100%	0.0	100.00%	AF276516.1
	Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene_complete sequence; and internal tra	850	850	100%	0.0	100.00%	AF121145.1
	Bactrocera dorsalis isolate BX171226-082 internal transcribed spacer 1, partial sequence	845	845	99%	0.0	100.00%	MK184685.1
	Bactrocera so ms8729 isolate BX171226-050 internal transcribed spacer 1, partial sequence	845	845	99%	0.0	100.00%	MK184669.1
	Bactrocera sp. ms8723 isolate BX171226-053 internal transcribed spacer 1_partial sequence	845	845	99%	0.0	100.00%	MK184662.1
	Bactrocera dorsalis isolate BX171226-028 internal transcribed spacer 1, partial sequence	845	845	99%	0.0	100.00%	MK184640_1
	Bactrocera dorsalis voucher FF01_BD internal transcribed spacer 1. partial sequence	845	845	99%	0.0	100.00%	KY558400.1
	Bactrocera dorsalis voucher Bd1540 internal transcribed spacer 1_partial sequence	845	845	99%	0.0	100.00%	KM453349.1

Figure 10 - *ITS*1 reference alignment: One each unambiguously identified *B. carambolae* (sample 11) and *B. dorsalis* (sample 08) from this study and sample 38 (ambiguous sample), and the NCBI accession numbers KJ544953.1 (deposited as *Bactrocera carambolae* "voucher") aligned to the reference sequence for *B. carambolae* KC446737.1 (IPPC standard). Sample 38 lacks the specific insert and was therefore assigned as *B. dorsalis* (false positive). NCBI accession number KJ544953.1 (deposited as *Bactrocera carambolae* "voucher") lacks the specific insert and misidentification is highly probable.

c. Specific deviations and issues in ITS1 sequencing

For the reliable discrimination of *B. carambolae* from *B. dorsalis* it is necessary to analyse the presence or absence of a 44bp insert near the *ITS7* primer binding site. This insert is only present in *B. carambolae* and therefore specific for this species (IPPC 2019). However, there is an editing mistake in the sequence insert for *B. carambolae* displayed in the IPPC protocol (chapter 4.3.4). Here the insert consists only of 41bp (3 bases at the 5' end are missing) and should be corrected (Fig. 11).

Figure 11 - *ITS*1 Reference alignment: Several *B. carambolae* samples from this study and sequence of insert of *B. carambolae* according to the IPPC standard aligned to the reference sequence (accession Nb. KC446737.1). All of the aligned sequences contain the characteristic *B. carambolae* insert, except for sample 38 (assigned value *B. caryeae*). The red box indicates three base-pairs missing from the aligned insert in comparison to the other sequences.

Consensus Coverage 0	50 • TA GCT	GTACTTAT	TATTTATGAAA	80 ΑΑΤΤΑΑΤΑΑΑ	™ AAGTTÅAATGA	100 ATCTTTTTAT	AAAAAATATG	120 TAAATGATAA	130 GTTAATT
	1	8	18	28	38	48	58	68	78
C KC446737_Bactrocera_carambolae	GCT	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAATATO	TAAATGATAA	GTTAATT
🕩 Sample 39	N-TAGCT	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
C+ Sample 27	A-TAGCT	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
C• Sample 04	AATAGCI	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
C Sample 23	A-TAGCT	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
C Sample 22	A-TAGCT	GTACTTAT	TATTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
C+ Sample 38	A-TAGCT	GTACTTAT	TATT				ATA	AAAATGATAA	GTTAATT
C Sample 08	A-TAGCT	GTACTTAT	TATTTATGAAA	AATTAATAAA	AAGTTAAATGA	TCTTTTTAT	AAAAAATATO	TAAATGATAA	GTTAATT
🖙 Insert IPPC Standard (41bp)			GAAA	ΑΑΤΤΑΑΤΑΑΑ	AAGTTAAATGA	ТСТТТТТАТ	AAAAAT		

Uncertainties in original assignment of specimens used in the validation sample set

In the case of one sample of the set (sample 23), the morphological and molecular identification results obtained did not allow to confirm the a priori assigned value of the sample. Sample 23 represents a very interesting and controversial case. The specimen, originally from the Philippines, was given to AGES as *Bactrocera occipitalis* and as such was included in the sample set of this validation study. However, molecular analyses indicate that the specimen is a *Bactrocera carambolae* (Fig. 12 and 13), possessing the 44bp insert that is unique and characteristic of this species (Fig. 13). In addition, identification by the three operators gave conflicting results (2 'negative results' and one 'not determined', see Appendix 4). To try to shed light on the case, a new morphological analysis of the specimen was conducted (Appendix 10). On the basis of this analysis, given the non-concordance of the results of the three operators, the specimen cannot be assigned with certainty to *B. occipitalis* on a morphological level. In cases like this it is necessary to identify the specimen as generically belonging to the *B. dorsalis* complex. To our knowledge, there are no data to date on the possible introgression of mitochondrial DNA from *B. carambolae* into specimens of *B. occipitalis*, which has been hypothesised in the case of intogression of *B. kandiensis* mitochondrial DNA into specimens of *B. dorsalis* (Schutze et al., 2015b).

For the purpose of this validation study, specimen 23 remains negative with respect to *B. dorsalis s.l.* and the uncertainty associated with its assigned value does not affect the performance characteristics.

Figure 12 - *COI* reference alignment: *COI* sequence of *B. carambolae* reference specimen (accession Nb. KC446059.1) aligned with sample 39 (unambiguous B. carambolae from this study) and sample 23. It is visible that they are identical on the *COI* gene locus.

Figure 13 - *COI* reference alignment: Alignment of a *B. occipitalis* sequence mined from NCBI GenBank (accession Nb. KM023416.1) with sample 23. Sample 23 is different to *B. occipitalis* on the *COI* gene locus.

Figure 14 - *ITS* reference alignment: Sequences of sample 8, 39 and 23 aligned to the *ITS* sequence with the accession Nb. KC446737.1 (mined from NCBI GenBank according to the IPPC Standard) and the characteristic *B. carambolae* insert (IPPC Standard). It is visible that the sequences are identical to each other and do not differ from the described insert.

	40	50	60	70	80	90	100	110	120	130
Consensus	AACCTTTAAACATA	TATAGETGT	ACTTATTATT	TATGAAAAAT	FAATAAAAG	TTAAATGATC	TTTTATAAA	AAATATGTAA	ATGATAAGTT	AATTTGTTCAC/
Coverage 0										
🖙 KC446737 Bactrocera carambolae		GETIGT			31			61 AAATATGTAA	71	81
De Sample 39 De Sample 23 De Insert IPPC Standard (41bp)	AACCTTTAAACATA AACCTTTAAACATA	ATATAGETGT/ ATATAGETGT/	АСТТАТТАТТ АСТТАТТАТТ	TATGAAAAAT TATGAAAAAT GAAAAAT	FAATAAAAAG FAATAAAAAG FAATAAAAAG	TTAAATGATC TTAAATGATC TTAAATGATC	ΓΤΤΤΤΑΤΑΑΑ ΓΤΤΤΤΑΤΑΑΑ ΓΤΤΤΤΑΤΑΑΑ	AAATATGTAA AAATATGTAA AAAT	ATGATAAGTT/ ATGATAAGTT/	AATTTGTTCAC/ AATTTGTTCAC/

7. Discussion and conclusions

This study aimed at the validation of IPPC and EPPO diagnostic protocols for the morphological and molecular identification of *Bactrocera dorsalis s.l.*. The study has involved staff of the EURL for Insects and Mites from ANSES and AGES and the analytical activities have been carried out from May to August 2020. A main sample set of 40 Tephritidae specimens, including target and non-target species, has been used. Additionally, smaller sample sets have been prepared for validating the molecular tests. The drafting of the final report has taken a longer time due to necessary consultation with IPPC DP 29 authors and international Tephritidae experts.

Morphological diagnostic protocol

The morphological identification according to the diagnostic protocol IPPC DP 29 (IPPC, 2019) achieved the expected value of 100% for the validation criteria diagnostic specificity, diagnostic sensitivity, accuracy and repeatability. However, reproducibility obtained a value of 87,5% due to divergent results between the three operators performing the identification. After an in-depth cause analyses, it was assessed that these divergent results were originated from the description of some diagnostic characters in the protocol (key and/or table of characters) that may lead the user to misinterpretation and consequently to a wrong identification. To summarize,

- the term "mid-point" in the description of *Bactrocera occipitalis* costal band could be misleading as it refers to a point; it should be interpreted as a "mid-line" between wing venation R₂₊₃ and R₄₊₅ (clarification by Norman Barr and Camiel Doorenweerd);
- difference between the shape of costal band of *Bactrocera occipitalis* ("distinctly overlapping R₂₊₃") and *Bactrocera carambolae* ("slightly overlapping R₂₊₃") is not sufficiently clear from Figures 16 (a) and 16 (e);
- adverb "generally" referred to character "confluent costal band" in *Bactrocera dorsalis s.l.* is ambiguous and should be rather interpreted as "typically" confluent (clarification by Norman Barr and Camiel Doorenweerd);
- description of *Bactrocera carambolae* character "tergite 3" does not take into account possible variation ("noncontinuous transverse band"), which on the contrary is documented in FruitFly ID Australia (Plant Health Australia, 2021).

Based on these results, the EURL recommends the use of the IPPC DP 29 (IPPC, 2019) to EU National Reference Laboratories for the morphological identification of *Batrocera dorsalis s.l.*. with some advice for the correct use of the diagnostic protocol:

- "mid-point" in the description of *Bactrocera occipitalis* costal band should be interpreted as a "mid-line" between wing venation R₂₊₃ and R₄₊₅ (see Fig. 2). <u>Position in the document</u>: page 11, Table 3 (4.2.3); page 12, Diagnostic key (4.2.4), couplet 3;
- in the Diagnostic key, couplet 3, decision between *B. occipitalis* and *B. carambolae* should be taken on the basis of all the diagnostic characters included in Table 3, not only on the basis of the shape of costal band. <u>Position in the document</u>: Diagnostic key (4.2.4), couplet 3, page 12;
- adverb "generally" referred to character "confluent costal band" in *Bactrocera dorsalis s.l.* should be rather interpreted as "typically" confluent. "Generally" may be misinterpreted as that a differently-shaped (e.g. overplapping) costal band is sometimes present in *B. dorsalis s.l.*. The diagnosis of *B. dorsalis* requires confluence of costal band. <u>Position in the document</u>: page 11, Table 3 (4.2.3);
- variation of the character "tergite 3 with a narrow transverse black band across anterior margin (constituting a "T" pattern)" for *Bactrocera carambolae* should be considered, even if not mentioned in the document; a non-continuous transverse band on tergite 3 can sometimes be found (see Fig. 4 and 5).

In addition, it is very important to remind that the Diagnostic key serves as a first screening tool and final decision about the identification should rely on the careful examination of all the characters in Table 3 (possibly with the only exception of genitalia, see 4.2.3 in the DP 29). This is stated in the diagnostic protocol itself: "An identification to one of the six species in the protocol requires the adult specimen to be examined for the characters provided in Table 3. This can be accomplished using the key in section 4.2.4 to screen specimens and then identification can be confirmed by comparing fly morphology to information in Table 3." "If one or more characters are inconsistent between the specimen and the descriptions provided in Table 3, then the specimen cannot be diagnosed as one of these species" and identification should be limited to **Bactrocera dorsalis complex**.

The present validation study has generated useful elements to improve the morphological part of the diagnostic protocol DP29. Therefore the authors of this report suggest the following points for improvement of the DP 29 to the IPPC bodies and the authors involved:

• a figure (i.e. Fig. 2) could be very useful to correctly interprete "mid-point" in the description of *Bactrocera occipitalis* costal band and showing that it is actually a "mid-line";

- if possible, a second diagnostic character could be very useful to distinguish between *Bactrocera occipitalis* and *Bactrocera carambolae* in couplet 3 of the Diagnostic key (e.g. dark markings on the abdomen?) as the character "costal band" alone hardly allows the discrimination between the two species (Fig. 16 (a) and (e) do not allow a certain interpretation of "distinctly" and "slightly" overlapping); however, this might be challenging since the alternative choice to *B. occipitalis* in couplet 3 leads to further couplets which consider three other species of the complex showing different features (*B. pyrifoliae, B. carambolae, B. dorsalis* s. l.);
- the adverb "generally" as referred to character "confluent costal band" in *Bactrocera dorsalis s.l.* should be preferably replaced by "typically";
- variation of the character "tergite 3" for *Bactrocera carambolae* should be mentioned in the protocol (non-continuous transverse band on tergite 3), as from FruitFly ID Australia (Plant Health Australia, 2021).

Molecular diagnostic protocols

This validation study aimed to generate the performance characteristics including molecular identification of *B. dorsalis* using DNA barcoding (*COI*) as well as *ITS*1 sequencing.

According to the IPPC standard (2019) **molecular methods alone are not recommended** for the identification of the six economically most relevant *B. dorsalis* complex species. However, the IPPC protocol recommends molecular tools for the discrimination of *B. dorsalis* and *B. carambolae* specimens after morphological determination (*ITS*1 sequencing).

In routine diagnosis, especially when dealing with larvae e.g. in the frame of import control, molecular identification is sometimes the only available method and therefore the EPPO DNA barcoding standard (EPPO, 2021b) was also validated. Hence, *COI* barcoding was applied as first line identification.

The experience gathered in this study was that for samples from which reasonable DNA quality could be extracted, the identification was quite straightforward for all samples not belonging to the *B. dorsalis* complex. All these samples could be identified at least at genus level using EPPO barcoding standard only. All samples belonging to the *B. dorsalis* complex were subsequently analysed with *ITS*1-sequencing.

It has to be considered that molecular identification via sequence analysis is a multistep process (DNA extraction, PCR, sequencing and sequence analysis). Performance characteristics were elaborated for PCR and sequence analysis steps.

COI sequencing: The performance characteristics of the diagnostic specificity and accuracy displayed that sequencing of the *COI* locus cannot fully discriminate all listed species. Nevertheless, the obtained values (96% for the LCO1490/HCO2198 primer set and 95% for the LepF/LepR primer set) were higher than the expected values, which had been calculated as 77%. Samples where no amplicons could be generated at all were excluded, whereas lacking amplicons in one of the two primer sets were assigned as negative deviations. All *B. dorsalis* assigned samples from different geographic origins could be correctly identified. In regards to the exclusivity cross-reactions could be observed with one sample (sample 16), which was assigned to *B. kandiensis (ITS1*) and misidentified as *B. dorsalis* on the *COI* locus.

The results of this study also showed that the analytical sensitivity of both primer sets $(0.325 \text{ ng}/\mu)$ was below the expected value (4ng/ μ). However, the value for the repeatability of LCO1490/HCO2198 primer set was lower than the expected 100%.

ITS1 sequencing was proven to be a valid confirmatory tool for *B. dorsalis* and *B. carambolae*. *B. carambolae* could be identified and clearly distinguished from *B. dorsalis* complex.

Most of the performance characteristics met the expected values except the values for the diagnostic specificity and accuracy which were below 100%. One sample (sample 38) lacked the *ITS1* insert and was misidentified as *B. dorsalis s.l.*. According to the IPPC standard the lack of the insertion and a match to *B. dorsalis s.l.* cannot exclude other species in the *B. dorsalis* complex. However, in our study, barcoding in combination with *ITS1* sequencing could accurately identify *B. dorsalis s.l.* in all cases, if the results on both loci (*COI* and *ITS1*) were congruent. If the results deviated between the loci (sample 38 and 16), *ITS1* sequencing was more reliable. Contrary to the IPPC protocol, this study shows that molecular identification of *B. kandiensis* with *COI* can be confirmed by *ITS1* sequencing. We assume that this could also be the case for *B. occipitalis*, but due to the lack of further specimens of this species, this could not be confirmed in this study. The possible suitability of the species identification of *B. caryeae* and *B. pyrifoliae* based on *ITS1* could not be evaluated since no *ITS1* sequences are available in the databases.

Several **critical issues** during this validation, which need to be addressed.

Firstly, DNA quality is important for the success of subsequent sequence analysis. This is highly dependent on the quality and the yield of the sample tissue. In some cases DNA quality was not suitable for a successful molecular identification process, even upon repeated extractions (singular legs and/or non-destructive DNA extractions from specimens from collections).

According to the EPPO DNA barcoding standard, when identifying unknown samples via barcoding, the choice of sequence database has a great impact on the results. Different databases utilize a different combination of nucleotide similarity, tree clustering et cetera, with varying focus on the similarity, query cover and the like.

Availability of sequence data in NCBI GenBank, Bold and EPPO Q-Bank differed greatly and affected the results. This has to be taken into account when using these databases in routine diagnosis. If for example no sequence data are available in the database this can lead to a false result. In the case of sample 16, the lack of sequences for *B. kandiensis* in EPPO Q-Bank at the time of query led to an incorrect barcoding result, in addition to the poor DNA quality of this sample. The different ways to display the results is also noteable: Bold database provides a preliminary result and indicates inconclusive results, contrary to NCBI GenBank and EPPO Q-Bank databases, which depict only hits.

Furthermore, the reliability of the deposited accessions is not always given (e.g. *B. carambolae* accessions like Nb. KJ544953.1 shown in this study or Nb. KF998794.1 according to Manger *et al.* 2017). This might be due to the reason that voucher specimens for generating the barcodes have been wrongly identified (Manger *et al.* 2017).

In the case of one sample of the set (sample 23), the morphological and molecular identification results diverged. This sample originated in the Philippines. The original assignment was *Bactrocera occipitalis*, nevertheless, in this validation study some uncertainties occurred during the morphological identification process. The assigned value remains unclear. This does not influence the performance characteristics of this validation study, as the true negative stands correct.

During the molecular validation for sample 23, a clear deviation from the assigned value (B. *occipitalis*) could be recorded. DNA barcoding (*COI*) as well as sequencing of the *ITS1* unambiguously resulted in *B. carambolae*. This included the presence of the 44bp insert near the *ITS7* primer binding site that is described in the IPPC standard and could be observed in this study for *B. carambolae* only.

Recommendation / Conclusion

The choice of the DNA extraction procedure is a very important first step when applying sequence-based molecular methods (https://eurl-insects-mites.anses.fr/en/minisite/insects-and-mites/approved-reagents).

If only molecular methods are used for identification, it is recommended to perform the diagnosis stepwise. In a first step, DNA barcoding should be used for discrimination of species not included in the *B. dorsalis* complex. *ITS*1 sequencing can be applied as a confirmatory step, to discriminate *B. carambolae* from all other species of the *B. dorsalis* complex and to possibly increase the resolution within the complex.

Sanger sequence analysis requires adequately proficient operators and the employment of multiple online resources. Caution is necessary when evaluating the hits achieved in various databases, as single sequences might be questionable (e.g. so-called voucher sequences) and the lack of sequences for some species leads to false hits altogether. In addition to database alignment, we therefore recommend to perform a reference sequence alignment.

A follow up study, including newly generated *ITS1* sequences of *B. caryeae*, *B. occipitalis* and *B. pyrifoliae* is planned. The aim is to generate approved sequence data (*COI* and *ITS1*) on to date underrepresented economically relevant *Bactrocera* species from the complex and make them available, e.g. via EPPO Q-Bank.

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Appendix 1 – Composition of the sample set and codification

Sample codification	New codification	Country of collection	Identification	Notes
0700634_4	1	Sri Lanka	Bactrocera kandiensis	ANSES extra samples
F20049	2	Thailand (Saraburi)	Bactrocera dorsalis	AGES
F20050	3	USA/Florida	Anastrepha suspensa	AGES
0502118_2	4	French Guyana	Bactrocera carambolae	ANSES sample set
1800896	5	Mali	Bactrocera dorsalis	ANSES sample set; only 1 specimens
F20043	6	Malaysia (Selangor)	Bactrocera carambolae	AGES
F20051	7	USA/Florida	Anastrepha suspensa	AGES
1002478_1	8	French Guyana	Bactrocera carambolae	ANSES sample set
1800894_1	9	Laos	Bactrocera dorsalis	ANSES sample set
0700634_3	10	Sri Lanka	Bactrocera kandiensis	ANSES extra samples
1800897	11	Mali	Bactrocera dorsalis	ANSES sample set; only 1 specimens
1800894_2	12	Laos	Bactrocera dorsalis	ANSES sample set
0700634_5	13	Sri Lanka	Bactrocera kandiensis	ANSES extra samples
1901279_1	14	Vietnam	Bactrocera dorsalis	ANSES sample set
1901277_2	15	Sri Lanka	Zeugodacus cucurbitae	ANSES sample set
0700634_2	16	Sri Lanka	Bactrocera kandiensis	ANSES extra samples
1301340_2	17	Senegal	Bactrocera dorsalis	ANSES sample set
F20048	18	Thailand (Saraburi)	Bactrocera dorsalis	AGES
1901277_1	19	Sri Lanka	Zeugodacus cucurbitae	ANSES sample set
1901064	20	France	Bactrocera oleae	ANSES sample set
F20047	21	Vietnam	Bactrocera pyrifoliae	AGES
1002478_2	22	French Guyana	Bactrocera carambolae	ANSES sample set
F20042	23	Philippines	Bactrocera occipitalis	AGES
1901279_4	24	Vietnam	Bactrocera dorsalis	ANSES sample set
F20053	25	Mexico/Tapachula /Chiapas	Anastrepha obliqua	AGES
1500326_1	26	Reunion	Dacus ciliatus	ANSES sample set
0502118_1	27	French Guyana	Bactrocera carambolae	ANSES sample set
1401020_1	28	Sri Lanka	Dacus ciliatus	ANSES sample set
2000042	29	Taiwan	Bactrocera dorsalis	ANSES sample set; only 1 specimens
1301340_1	30	Senegal	Bactrocera dorsalis	ANSES sample set
0700634_1	31	Sri Lanka	Bactrocera kandiensis	ANSES extra samples
1901279_2	32	Vietnam	Bactrocera dorsalis	ANSES sample set
F20046	33	India (Kerala)	Bactrocera caryeae	AGES
1800894_3	34	Laos	Bactrocera dorsalis	ANSES sample set
F20052	35	Mexico/Tapachula /Chiapas	Anastrepha obliqua	AGES
1901279_3	36	Vietnam	Bactrocera dorsalis	ANSES sample set
1901549	37	France	Bactrocera oleae	ANSES sample set
F20045	38	India (Kerala)	Bactrocera caryeae	AGES
1002478_3	39	French Guyana	Bactrocera carambolae	ANSES sample set
F20041	40	Philippines	Bactrocera occipitalis	AGES

Appendix 2 - Check lists for the morphological analysis

Operator

Date

A combination of characters to diagnose the Bactrocera dorsalis complex (modified from Table 3, IPPC ISPM 27 DP29: Bactrocera dorsalis)

Morphologic	al character	Sample code										
Head	Face yellow with distinct facial spots present (Figures 9(a), 9(b), 12)											
	Colour mostly black to mostly red-brown (inter-regionally variable) (Figure 13)											
Scutum	Lateral vittae present (Figure 11) and yellowish (Figures 13 and 14)											
	Medial vittae absent (Figure 11)											
	Yellowish colour (Figures 1 and 13)											
Scutellum	With a dark basal band (Figures 11 and 1)											
	Never with other dark patterns (Figure 13)											
Femora	Entirely or mostly fulvous (reddish-yellow or tawny) colour but may possess dark patterns particularly on and around apices (Figure 15)											
	Cells bc and c hyaline (colourless) or, at most, with an extremely pale tint (Figures 10 and 16)											
14/100	Without dense microtrichia covering cells bc and c (Figure 10)											
wing	Costal band narrow (never confluent with R4+5) (Figure 10)											
	Narrow anal streak present (diagonal marking that is above anal lobe) (Figures 10 and 16)											
Abdomen	With a "T" pattern on tergites 3–5 (Figures 7(a) and 17)											

Comments / Results					
B. dorsalis complex confirmed?					
Y / N					

Diagnostic key to six economically important species belonging to the Bactrocera dorsalis complex (adult) (modified from key 4.2.4, IPPC ISPM 27 DP29: Bactrocera dorsalis)

Key for 6 speci	es from the Bactrocera dorsalis complex (adult)	go to (mark the decision; note any comments)										
			Sample code									
worphological												
1	Postpronotal lobe yellow with dark anteromedial corner (Figures 19(b) and (d))											2
1	Postpronotal lobe entirely yellow (Figures 19(a), (c), (e), (f))											3
	Scutum <u>entirely</u> black (Figure 13(b)), abdominal tergites 3–5 with <u>broad black dorsolateral markings</u> (Figures 17(b) & 18(b)); lateral vittae <u>very</u> narrow (Figure 4(b))	 	 	 	 	 	 	 	 	 	 	B. caryeae
2	Scutum <u>mostly</u> black (Figure 13(d)), abdominal tergites 3–5 with <u>"T" pattern</u> and tergites 4–5 with very <u>narrow anterolateral black marking</u> (Figures 17(d) and 18(d)); lateral vittae narrow (Figure 4(d))	 	 	 	 	 	 	 	 	 	 	B. kandiensis
3	Costal band <u>distinctly overlapping R2+3</u> and <u>expanding</u> broadly around <u>apex of wing</u> reaching mid-point between <u>R2+3 & R4+5</u> (Figure 16(e))											B. occipitalis
	Costal band widening <u>slightly</u> (Figure 16(c)) to moderately (Figure 16(a)) <u>around apex of</u> wing											4
4	Abdominal tergites 3–5 <u>with</u> broad black dorsolateral markings (Figures 17(f) and 18(f))											B. pyrifoliae
	Abdominal tergites 3–5 without broad black dorsolateral markings											5

5	Costal band <u>slightly overlapping</u> R2+3, <u>moderately broad</u> around apex of wing (Figure 16(a)); abdominal tergite 3 with a narrow transverse black band across anterior margin (constituting a "T"pattern), widening to cover lateral margins; tergite 4 with <u>rectangular (occasionally triangular</u>) anterolateral or narrow lateral black markings; tergites 3–5 with medium-width medial longitudinal black stripe (Figures 17(a) and 18(a))	 	 	 ··· ···	 ··· ···	 	B. carambolae
	Costal band <u>confluent</u> with R2+3, <u>narrow to moderately broad</u> around apex of wing (Figure 16(c)); abdominal tergite 3 <u>exhibits</u> variations from <u>black band across anterior margin</u> (<u>constituting a "T" pattern</u>) to broad lateral bands, tergite 4 <u>without</u> markings or with anterolateral or narrow lateral black margins (occasionally rectangular), tergite 5 <u>without markings</u> or with anterolateral black markings (Figures 17(c) and 18(c))	 	 	 ··· ···	 	 	B. dorsalis s.l.
	Comments / Results						

Diagnostic morphological characters of adult fruit flies of two economically important species of the Bactrocera dorsalis complex (modified from Table 3, IPPC ISPM 27 DP29: Bactrocera dorsalis)

		Samp	ample code				Sample co	ode							
Structure	B. dorsalis s.l.								B. carambolae						
Facial spot	Medium to large, circular to oval (interregionally variable)								Medium-sized, oval						
Tergites III-V	With narrow to medium width medial longitudinal black stripe								With medium-width medial longitudinal black stripe						
тш	Exhibits variations from transverse black band across anterior margin (constituting a "T" pattern) to broad lateral bands								With a narrow transverse black band across anterior margin (constituting a "T" pattern) widening to cover lateral margins						
T IV	Without any markings or with anterolateral black markings (occasionally rectangular in shape)								With rectangular anterolateral (occasionally triangular) black markings						
τv	Without any markings or with anterolateral black markings								With anterolateral black markings						
Scutum colour	Black to red–brown (inter or intra- regionally variable)								Dull black						

Postpronotal lobe	Entirely yellow						Entirely yellow					
Anterior margin of anepisternal stripe	Reaching midway between anterior margin of notopleuron and anterior npl. bristle; straight to convex (anterior margin)						Reaching midway between anterior margin of notopleuron and anterior npl. bristle; convex (anterior margin)					
Basal band of scutellum	Narrow						Narrow					
Lateral vittae	Narrow to broad (inter-regionally variable), parallel- sided, ending at or just behind ia. Bristles						Broad, parallel-sided, ending at or behind ia. Bristles					
Costal band	Narrow, generally confluent with R2+3 (inter- or intra- regionally variable), narrow to moderately broad around apex of wing						Narrow, slightly overlapping R2+3, moderately broad around apex of wing					
Femora	Generally fulvous, occasionally with a small dark marking on outer surface of fore femora (inter- regionally variable)						Fulvous, generally with a large elongate oval black marking on outer surface of fore femora					

Summary Results sheet for the morphological test IPPC ISPM 27 DP29: Bactrocera dorsalis

Operator	
Stereomicroscope	

Sample code	Identification result	Date of analysis	Notes
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Specification of the PCR Assay 1 (COI)

Name of the primer incl. sequence, literature reference, fragment length in bp:

LepF: 5'- ATTCAACCAATCATAAAGATATTGG-3' LepR: 5'- TAAACTTCTGGATGTCCAAAAAAAATCA-3'

Literature: Hajibabaei et al., 2006: DNA barcodes distinguish species of tropical Lepidoptera, PNAS _ January 24, 2006 _ vol. 103 _ no. 4, 968-971

Fragment length: 658bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol[®] Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per	
	reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5µM
Primer2:	0,5	0,5µM
Σ	9	
DNA	1	

PCR conditions:

	°C	Duration	Nr. of
		(min., sec.)	Cycles
Start	95	15 min	1
Denaturation	95	45 sec	5
Annealing	44	45 sec	
Extension	72	45 sec	
Denaturation	95	45 sec	35
Annealing	49	45 sec	
Extension	72	45 sec	
Final extension	72	7 min	1
Cooling	15	8	

Specification of the PCR Assay 2 (COI)

Name of the primer incl. sequence, literature reference, fragment length in bp:

LCO1490: 5'- GGTCAACAAATCATAAAGATATTGG-3' HCO2198: 5'- TAAACTTCAGGGTGACCAAAAAATCA-3'

Literature: Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine, Biology and Biotechnology 3, 294–299.

Fragment length: 709bp

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol[®] Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per	
	reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5μM
Primer2:	0,5	0,5µM
Σ	9	
DNA	1	

PCR conditions:

	°C	Duration	Nr. of
		(min., sec.)	Cycles
Start	95	15 min	1
Denaturation	95	30 sec	5
Annealing	45	30 sec	
Extension	72	1 min	
Denaturation	95	30 sec	35
Annealing	51	1 min	
Extension	72	1 min	
Final extension	72	10 min	1
Cooling	15	∞	

Specification of the PCR Assay 3 (ITS1)

*ITS*6: 5'- AGC CGA GTG ATC CAC CGC T-3' *ITS*7: 5'- GAA TTT CGC ATA CAT TGT AT-3'

Boykin et al., (2014); Armstrong and Cameron, (2000)

Fragment length: 499–543bp (the amplicon size varies for species and individuals) *B. carambolae* seem to have an additional insert of 44bp compared to *B. dorsalis*

PCR - Parameters:

Thermocyler used: Biometra T3000 Thermal cycler

Mastermix: 5x HOT FIREPol® Master Mix, Solis Biodyne:

Composition:		Final concentration:
	Volume per	
	reaction µl	
Water	6	
Mastermix	2	1x
Primer1:	0,5	0,5μM
Primer2:	0,5	0,5μM
Σ	9	
DNA	1	

PCR conditions:

	°C	Duration	Nr. of
		(min., sec.)	Cycles
Start	95	15 min	1
Denaturation	95	30 sec	40
Annealing	55	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1
Cooling	15	8	

Appendix 4 – Summary Results sheets with the results from the three operators

Operator 1	
Instrument	ZEISS Stemi 2000-C
Date of analysis/identification	29/05/20 – 03/06/20

Sample number	Analysis/Identification	Notes	Expected result	Assigned value	
1	Negative	B. kandiensis	Negative	Bactrocera kandiensis	
2	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
3	Negative	Anastrepha sp.	Negative	Anastrepha suspensa	
4	Negative	B. carambolae ?	Negative	Bactrocera carambolae	
5	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
6	Negative	B. carambolae	Negative	Bactrocera carambolae	
7	Negative	Anastrepha sp.	Negative	Anastrepha suspensa	
8	Positive	B. dorsalis	Negative	Bactrocera carambolae	
9	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
10	Negative	B. kandiensis	Negative	Bactrocera kandiensis	
11	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
12	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
13	Negative	B. kandiensis	Negative	Bactrocera kandiensis	
14	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
15	Negative	Dacus sp. ?	Negative	Zeugodacus cucurbitae	
16	Negative	B. kandiensis ?	Negative	Bactrocera kandiensis	
17	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
18	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
19	Negative	Dacus sp. ?	Negative	Zeugodacus cucurbitae	
20	Negative	Not B. dorsalis complex	Negative	Bactrocera oleae	
21	Negative	B. pyrifoliae ?	Negative	Bactrocera pyrifoliae	
22	Not determined	B. dorsalis or B. carambolae ? Negative		Bactrocera carambolae	
23	Negative	Bactrocera occipitalis ?	Negative	Bactrocera occipitalis	
24	Positive	B. dorsalis Positive		Bactrocera dorsalis	
25	Negative	Anastrepha sp.	Negative	Anastrepha obliqua	
26	Negative	Dacus sp. ?	Negative	Dacus ciliatus	
27	Not determined	B. dorsalis or B. carambolae?	Negative	Bactrocera carambolae	
28	Negative	Dacus sp.	Negative	Dacus ciliatus	
29	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
30	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
31	Negative	B. kandiensis	Negative	Bactrocera kandiensis	
32	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
33	Negative	B. caryeae	Negative	Bactrocera caryeae	
34	Positive	B. dorsalis	Positive	Bactrocera dorsalis	
35	Negative	Anastrepha sp.	Negative	Anastrepha obliqua	
36	Not determined	B. dorsalis or B. carambolae?	Positive	Bactrocera dorsalis	
37	Negative	Not B. dorsalis complex	Negative	Bactrocera oleae	
38	Negative	B. caryeae	Negative	Bactrocera caryeae	
39	Not determined	B. dorsalis or B. carambolae?	Negative	Bactrocera carambolae	
40	Negative	B. carambolae	Negative	Bactrocera occipitalis	

Operator 2	
Instrument	ZEISS Stemi 508
Date of analysis/identification	08/06/20 – 18/06/20

Sample number	Analysis/Identification	Notes	Expected result	Assigned value	
1	Negative	/	Negative	Bactrocera kandiensis	
2	Positive	/	Positive	Bactrocera dorsalis	
3	Negative	/	Negative	Anastrepha suspensa	
4	Positive	Specimen characters match also with <i>B. carambolae</i>	Negative	Bactrocera carambolae	
5	Positive	/	Positive	Bactrocera dorsalis	
6	Negative	/	Negative	Bactrocera carambolae	
7	Negative	/	Negative	Anastrepha suspensa	
8	Positive	T pattern on T3: transverse band not continuous	Negative	Bactrocera carambolae	
9	Positive	/	Positive	Bactrocera dorsalis	
10	Negative	/	Negative	Bactrocera kandiensis	
11	Positive	/	Positive	Bactrocera dorsalis	
12	Positive	/	Positive	Bactrocera dorsalis	
13	Negative	/	Negative	Bactrocera kandiensis	
14	Positive	/	Positive	Bactrocera dorsalis	
15	Negative	/	Negative	Zeugodacus cucurbitae	
16	Negative	Several characters ambiguous, however NEGATIVE because of anepisternal stripe and markings on femora	Negative	Bactrocera kandiensis	
17	Positive	/	Positive	Bactrocera dorsalis	
18	Negative	Postpronotal lobe is NOT entirely yellow (T3 transversal band not continuous)	Positive	Bactrocera dorsalis	
19	Negative	/	Negative	Zeugodacus cucurbitae	
20	Negative	/	Negative	Bactrocera oleae	
21	Negative	/	Negative	Bactrocera pyrifoliae	
22	Positive	T pattern on T3: transverse band not continuous	Negative	Bactrocera carambolae	
23	Not determined	Thorax is partly covered by a layer. Medial longitudinal stripe ambiguous	Negative	Bactrocera occipitalis	
24	Positive	/	Positive	Bactrocera dorsalis	
25	Negative	/	Negative	Anastrepha obliqua	
26	Negative	/	Negative	Dacus ciliatus	
27	Positive	/	Negative	Bactrocera carambolae	
28	Negative	/	Negative	Dacus ciliatus	
29	Positive	/	Positive	Bactrocera dorsalis	
30	Positive	T pattern on T3: transverse band not continuous	Positive	Bactrocera dorsalis	
31	Negative	Specimen not in good condition	Negative	Bactrocera kandiensis	
32	Positive	Absence of medial vittae not visible	Positive	Bactrocera dorsalis	
33	Negative	/	Negative	Bactrocera caryeae	
34	Positive	/	Positive	Bactrocera dorsalis	
35	Negative	/	/ Negative Anastrepha		

36	Positive	/	Positive	Bactrocera dorsalis
37	Negative	/	Negative	Bactrocera oleae
38	Negative	/	Negative	Bactrocera caryeae
39	Positive	T pattern on T3: transverse band not continuous	Negative	Bactrocera carambolae
40	Positive	/	Negative	Bactrocera occipitalis

Operator 3	
Instrument	LEICA M205 c
Date of analysis/identificat ion	20/07/20 - 23/07/20 n_1 28/07/20 - 30/07/20 n_2 04/08/20 n_3

Sample number	Analysis/Identificatio n_1	Analysis/Identificatio n_2	Analysis/Identificatio n_3	Notes	Expected result	Assigned value
1	Negative	Negative	Negative	B. kandiensis	Negative	Bactrocera kandiensis
2	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
3	Negative	Negative	Negative	Anastrepha sp.	Negative	Anastrepha suspensa
4	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
5	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
6	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
7	Negative	Negative	Negative	Anastrepha sp.	Negative	Anastrepha suspensa
8	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
9	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
10	Negative	Negative	Negative	B. kandiensis	Negative	Bactrocera kandiensis
11	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
12	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
13	Negative	Negative	Negative	B. kandiensis	Negative	Bactrocera kandiensis
14	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
15	Negative	Negative	Negative	/	Negative	Zeugodacus cucurbitae
16	Negative	Negative	Negative	B. kandiensis	Negative	Bactrocera kandiensis
17	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
18	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
19	Negative	Negative	Negative	/	Negative	Zeugodacus cucurbitae
20	Negative	Negative	Negative	/	Negative	Bactrocera oleae
21	Negative	Negative	Negative	B. pyrifoliae	Negative	Bactrocera pyrifoliae
22	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
23	Negative	Negative	Negative	B. occipitalis	Negative	Bactrocera occipitalis
24	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
25	Negative	Negative	Negative	Anastrepha sp.	Negative	Anastrepha obliqua
26	Negative	Negative	Negative	Dacus ?	Negative	Dacus ciliatus
27	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
28	Negative	Negative	Negative	/	Negative	Dacus ciliatus
29	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
30	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
31	Negative	Negative	Negative	B. kandiensis	Negative	Bactrocera kandiensis
32	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis

33	Negative	Negative	Negative	B. caryeae	Negative	Bactrocera caryeae
34	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
35	Negative	Negative	Negative	Anastrepha sp.	Negative	Anastrepha obliqua
36	Positive	Positive	Positive	Bactrocera dorsalis s.l.	Positive	Bactrocera dorsalis
37	Negative	Negative	Negative	/	Negative	Bactrocera oleae
38	Negative	Negative	Negative	B. caryeae	Negative	Bactrocera caryeae
39	Negative	Negative	Negative	B. carambolae	Negative	Bactrocera carambolae
40	Negative	Negative	Negative	B. occipitalis	Negative	Bactrocera occipitalis

Sensitivity, specificity, accuracy :

Diagnostic sensitivity, specificity and accuracy is assessed on the basis of the analysis of the whole set carried out by operator 3 (ANSES)

Operator_3_R1

Diagnostic sensitivity = true positives/(true positives + false negatives) Diagnostic specificity = true negatives/(true negatives + false positives)

		Expected res	sult	
		positive		negative
Operator	positive		14	0
result	negative		0	26

Sensitivity	100
Specificity	100
Accuracy	100

Repeatability : Operator_3_R1, Operator_3_R2, Operator_3_R3

Repeatability is assessed on the basis of the analysis of the whole set carried out by operator 3 (ANSES) (three repetitions of analysis).

Operator_3_R1, Operator_3_R2, Operator_3_R3

Expressed as % level of agreement among repetitions by Operator 3

Sample code	Repetitions	Operator3_ R1	Operator3_ R2	Operator3_ R3	Agreement	Disagreement	Level of agreement
1	3	Negative	Negative	Negative	3	0	100
2	3	Positive	Positive	Positive	3	0	100
3	3	Negative	Negative	Negative	3	0	100
4	3	Negative	Negative	Negative	3	0	100
5	3	Positive	Positive	Positive	3	0	100
6	3	Negative	Negative	Negative	3	0	100
7	3	Negative	Negative	Negative	3	0	100
8	3	Negative	Negative	Negative	3	0	100
9	3	Positive	Positive	Positive	3	0	100
10	3	Negative	Negative	Negative	3	0	100
11	3	Positive	Positive	Positive	3	0	100
12	3	Positive	Positive	Positive	3	0	100
13	3	Negative	Negative	Negative	3	0	100
14	3	Positive	Positive	Positive	3	0	100
15	3	Negative	Negative	Negative	3	0	100
16	3	Negative	Negative	Negative	3	0	100
17	3	Positive	Positive	Positive	3	0	100
18	3	Positive	Positive	Positive	3	0	100
19	3	Negative	Negative	Negative	3	0	100
20	3	Negative	Negative	Negative	3	0	100
21	3	Negative	Negative	Negative	3	0	100
22	3	Negative	Negative	Negative	3	0	100
23	3	Negative	Negative	Negative	3	0	100
24	3	Positive	Positive	Positive	3	0	100
25	3	Negative	Negative	Negative	3	0	100
26	3	Negative	Negative	Negative	3	0	100
27	3	Negative	Negative	Negative	3	0	100
28	3	Negative	Negative	Negative	3	0	100
29	3	Positive	Positive	Positive	3	0	100
30	3	Positive	Positive	Positive	3	0	100
31	3	Negative	Negative	Negative	3	0	100
32	3	Positive	Positive	Positive	3	0	100
33	3	Negative	Negative	Negative	3	0	100
34	3	Positive	Positive	Positive	3	0	100
35	3	Negative	Negative	Negative	3	0	100
36	3	Positive	Positive	Positive	3	0	100
37	3	Negative	Negative	Negative	3	0	100
38	3	Negative	Negative	Negative	3	0	100
39	3	Negative	Negative	Negative	3	0	100
40	3	Negative	Negative	Negative	3	0	100
	120				120	0	100

Repeatability

100

Reproducibility : Operator_1, Operator_2, Operator_3_R1

Reproducibility is assessed on the basis of the analysis of the whole set carried out by operator 1, 2 (AGES) and 3 (ANSES) (first of the three repetitions of analysis).

Operator_1, Operator_2, Operator_3_R1

Expressed as % level of agreement among repetitions by the three Operators

Sample code	Operator3_R1	Operator1	Operator2	Repetitions	Agreement	Disagreement	Level of agreement
1	Negative	Negative	Negative	3	3	0	100,0
2	Positive	Positive	Positive	3	3	0	100,0
3	Negative	Negative	Negative	3	3	0	100,0
4	Negative	Negative	Positive	3	2	1	66,7
5	Positive	Positive	Positive	3	3	0	100,0
6	Negative	Negative	Negative	3	3	0	100,0
7	Negative	Negative	Negative	3	3	0	100,0
8	Negative	Positive	Positive	3	2	1	66,7
9	Positive	Positive	Positive	3	3	0	100,0
10	Negative	Negative	Negative	3	3	0	100,0
11	Positive	Positive	Positive	3	3	0	100,0
12	Positive	Positive	Positive	3	3	0	100,0
13	Negative	Negative	Negative	3	3	0	100,0
14	Positive	Positive	Positive	3	3	0	100,0
15	Negative	Negative	Negative	3	3	0	100,0
16	Negative	Negative	Negative	3	3	0	100,0
17	Positive	Positive	Positive	3	3	0	100,0
18	Positive	Positive	Negative	3	2	1	66,7
19	Negative	Negative	Negative	3	3	0	100,0
20	Negative	Negative	Negative	3	3	0	100,0
21	Negative	Negative	Negative	3	3	0	100,0
22	Negative	Not determined	Positive	3	1	2	0,0
23	Negative	Negative	Not determined	3	2	1	66,7
24	Positive	Positive	Positive	3	3	0	100,0
25	Negative	Negative	Negative	3	3	0	100,0
26	Negative	Negative	Negative	3	3	0	100,0
27	Negative	Not determined	Positive	3	1	2	0,0
28	Negative	Negative	Negative	3	3	0	100,0
29	Positive	Positive	Positive	3	3	0	100,0
30	Positive	Positive	Positive	3	3	0	100,0
31	Negative	Negative	Negative	3	3	0	100,0
32	Positive	Positive	Positive	3	3	0	100,0
33	Negative	Negative	Negative	3	3	0	100,0
34	Positive	Positive	Positive	3	3	0	100,0
35	Negative	Negative	Negative	3	3	0	100,0
36	Positive	Not determined	Positive	3	2	1	66,7
37	Negative	Negative	Negative	3	3	0	100,0
38	Negative	Negative	Negative	3	3	0	100,0
39	Negative	Not determined	Positive	3	1	2	0,0
40	Negative	Negative	Positive	3	2	1	66,7
				120	105	15	87 5

Reproducibility

87,5

Appendix 6 – In silico testing of analytical specificity with DNA barcoding primer sets

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed with the DNA barcoding primer sets (LCO1490/HCO2198 and LepF/LepR). The search set was limited to Tephritidae. The *ITS*1 primer set (*ITS*7/*ITS*6) was aligned in the same manner with the search being limited to *Bactrocera*. The results showed suitability of both primer sets (see Fig. 15-20) for identification of several *Bactrocera* spp., although we have to state that both barcoding and *ITS*1 sequencing are generic tests including targets and non-targets.

Distance trees of results from BLAST search were created with organism search set to Tephritidae with single primers (LepF, LepR, LCO1490, HCO2198, *ITST* and *ITSG*)

Figure 15 - Distance tree of results from BLAST search for LepF primer

<u>Constitutes p. 5 MV-2016 secular ADR/1379E2 conductment indiana abultat 1 (COI) gans, partial ode mitochondrial Diffactorisen domilia colos 15, 4 cyclechrona ondana antheni 1 (COI) gans, partial ode, mitochondrial <u>Constitutes p. 5 MV-2016 seculare ADR/157070 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana subarti 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana seculare 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana seculare 1 (COI) gans, partial det mitochondrial</u> <u>Constitutes p. 5 MV-2016 seculare ADR/157076 cyclechrone ondana seculare 1 (COI) gans, partial det</u></u>

Figure 16 - Distance tree of results from BLAST search for LepR primer

Figure 17 - Distance tree of results from BLAST search for LCO1490 primer

era taberculata mitochoodrice, partial gaocere Bactrosers railisense mitochoisdrioe, complete get lactroom ratiging mitochondrion, complete genome us bivitata mitochosdrios, complete genom Occus hivittatas subschoodring, complete genome ets ratiging mitochondrive, complete ge Bettroars teasensis mitrobostrion, complete genome Campiglosia aponet washer YSL/W140201110 cytochrone coldase subunit 1 (COI) game, partial ofic mitochondria Campiglosae sommala noucher YSUW13090(145 cytechrone omdass substit) (COI) gene, partial cde, mitochoudrial Campiglose annuals reacher YSUW130001003 cytechrome onidae submit 1 (COI) gate, partial eds, mitechendrial Campiglosse destatane voucher YSU/W130901200 sytischrone oralase schutzt 1 (COI) gene, jurtial ode, mitochondrial Campiglossa shanakana yoschar YSU/W00015042 sytechronie oradaas aduant 1 (COI) gana, partial ede, reitschondrial Campigloust shanaasa voacher YSUW090915041 sytechrones oxidaas advant 1 (COI) gens, partial ode, neinschondrial Campiglouss quadrigertats voacher YSUW090915000 cytechrones oxidaas advant 1 (COI) gens, partial ode, neinschondria Campiglossa qualifigurata voacher YNUWI90915089 cytochrone orcidae schenit I (COI) gene, partial ode, mittechoadrial Campiglouse products voucher YSUW130901194 cytochrome oxidase subesit 1 (COI) gene, partial cdc; mitschoodrial Campiglossa personalasma worden VSUW999(5068 extechnome couldase submit 1 (CON) gans, partial cde mitochoodrial Campiglone peransisere voscher YSUW140201106 cytochmese oxidee solumi 1 (COX) gans, partial ode, mitochoodrial Campiglone peransisere voscher YSUW090915019 cytochmese oxidee solumi 1 (COX) gans, partial ode, mitochoodrial Campiglosa paramilaana vonkhe YRI/W090915044 oytochrone oxidaaa nebanii 1 (COR) gena, partai odi; minchondrial O campiglosa ne gatsila HYH-2019 voncher YRI/W140201012 oytochrona omdaaa anhanii 1 (COR) gena, partai odi; mitochondrial O campiglosa ne gatsila HYH-2019 voncher YRI/W140201077 oytochrona oxidaaa anhanii 1 (COR) gena, partai odi; mitochondrial Campighous melaans wascher YM7W140201106 cytichrome oxidese sahanit 1 (CO3) gans, partial ode mitochondrial ampigione melama washer YSUW140201105 sytochrone oxidae schenit I (COI) gene, partial odi; mitochoudrial Campiglosa missile washer YHOW140201042 sytochrone resides schedit I (CO3) geos, partial eds, mitochoedrial Campigicae minile vocher YRDW140201041 sylochrone ordans minuit 1 (COI) gene, partal ode, mitochembrai Campigicae manife vocher YRDW130000215 sylochrone ordans silvanit 1 (COI) gene, partal ode, mitochembrai Campigicae minile vocher YRDW180203 sylochrones ordans advant 1 (COI) gene, partal ode, mitochembrai Campignose manaliza vozdar YSUWWWW SUB cytechnose ordane admit ((CO) gane, partal ob; minchondrid Campignose manaliza vozdar YSUWWWW SUF cytechnose ordane admit ((CO) gane, partal ob; minchondrid Campiginus mesuline vostler YSUW0000034 cytochrone oxidese saharit 1 (COI) gene, partial circ, mitochondrial Campiginus mesuline voscher YSUW0000133 cytochrone oxidese saharit 1 (COI) gene, partial circ, mitochondrial Campiglous longipunts wather VSUW00015062 cytochrons oxidae schutt 1 (COI) gaps, partial odg mitochoodrial ampiglosa loswiana weacher YEUW14001001 sytechnimu suidaus subout 1 (COI) gens, partial ode, mitechondrial Campiglous loswians voncher YSIIW140000076 sytochrone retidate atheast 1 (CDI) gene, partial eds: mitochondrial Campigliose lowers water for water (2001) (2001) and ampigious Innyame voncher VSUW00010014 cytochrone oxidae tabanit 1 (COI) gene, partial ode, mitochondrial Campiglosa, guttalla vosober YSUW140201039 evtochmete onidase subanit I (COI) gene, partial ode, mitochondrial Campiglousi difficilis voucher YSUW14000018 cytochrome midase submit 1 (COI) gene, partial ode minochondrial Campiglousi deserts voischer YSLIWIR100130 cytochronse oridaas scherit 1 (COI) gene, partial ede, mitochoudrial Campiglosas deserts voucher YSUW00100129 cytochronie middase subunit 1 (COI) gene, partial ade, mitochondrial Campignoss defautate vostlar YSUW140201103 cytochrone roddass sibust 1 (COI) gens, partial ode; minohondrial Campignose orei voscher YSUW140201035 cytochrone orddass submit 1 (COI) gens, partial ode; minohondrial Campiglosa, osi vuscher YSUW140201034 oynothrons ordates schnitt ((OD) gene, partial ode, nitochondrial Campiglosa, and vuscher YSUW130901059 cytochrone ordates schnitt ((OD) gene, partial ode, mitochondrial Campighous unei wurcher YSUW130001058 cytochrome midass suburit I (COI) gene, partial ode mitochoadrial Campiglowa advoskyi voucher VSI/W94900511 cytochrines ondass auhunt I (COI) gans, parial odi; mitochondrial Campiglowa albisepi visucher VSI/W99915005 cytochrines oridase auhuni 1 (COI) gans, parial odi; mitochondrial Campigloose achytophori vosaber YBLW140200007 cytochrome oxidase submit 1 (COI) gene, pertial ods; mitochord/tal Campiglous, abstrabil woacher YSUW140201302 sylochrome exidase subspir J (COF) gave, partial ods, mitochondrial liectroom cannibolas mitochondrion, partial annous actrocers carecteriae tarilate \$2 rotochondrion, complete genore Dacas concessidas indate DC2 mitochoadrine, complete general Dactrioers donalis mitochondrics, complete genome Nactrioers ritainal isolate IRT mitochondrice, complete ge Bactroom limbifum isolate BL1 subschendrion, complete ganome upatrophe atrata voscher RX130130-076 cytochrone oxidane adminit 1 (COI) gane, partial ode; mitoch Assertingha serpentica voacher EX130130-023 cytochrome orddesa asbanit 1 (COI) gane, partial ode; mitochondrial Assertingha serpentica voacher X110427-03 cytochrome orddesa asbanit 1 (COI) gane, partial ode; mitochondrial nphe serpentine voucher XI 10427-014 cytochrome oxidase nehemit 1 (COI) gene, partial cdo; netrochondrial mattripha obliqua weather RX130130-013 cytochrome ortidae schuett I (COI) gees, partial edge mitochondrial Ausampha fraiendau voiather X110427-002 oytochrone oxidasa subuuti 1 (COI) gans, pertial ode; mitochoafrial Ausampha fraiendau voiather X110427-004 oytochrone oxidaan subunti 1 (COI) gans, pertial ode; mitochoafrial Agestrepha frequenzias vospher X110427-005 evtochrone oxidase returnit 1 (COI) gans, pertial ode mitrochondrial mpha fraternalus voncher X110427-006 cytochrome condans subunit I (COII) game, partial ode; mitochondrial quatruphs finiseculus voucher X110427-007 cytochrome coldans subunit 1 (COI) gane, partial cdr, mitochoodrial Apatrophe (meurodau voucher X110427-008 cytochrone oxidaus suburit 1 (XXII) gans, partial ode; mitochostrial Apatrophe (meurodau voucher UTOL, DEUV_2 mitochostiton, complete genome actroom dereves mitschoodtion, complete sessione Decas longicornia mitochendrice, complete genomi Electropets invadent netschoodtion, complete genotes neigo bellus voscher YSUW97011111 cytochrone oxidaas achenit 1 (COX1) gans, period oh; nunchoodriel oantrapha serpeotaa voscher YSUW94062009 cytochrone oxidaas anbanit 1 (COX1) gans, period afs; mitocho Anastrophe Intercular weather 110 PRU25 extectiones oxidate subtant L(CD) core, nartial eds. mitochoodital nphe fraemulas voucher 100 700015 cytochrone orticles submit 1 (COR) gans, partial cds, mitochoodrial Anastrophe distincts vescher 093 190005 cytochrone ontdass substiti (000) gene, perial sde, natiochondrial mantropha celliqua voacher 078. JHUI-4 cytochrone ooidaas mhunti I (COI) gane, partial odi; teitschondrial warmpha celliqua voacher 077. JHUI-6 cytochrone ooidaas sehanii I (COI) gane, partial odi; minshondrial warmpha celliqua voacher 074. JHUI-6 cytochrone ooidaas sehanii I (COI) gane, partial odi; minshondrial repta obligua voucher 067. MXID cytochrome oxidase subanit i (COX) gene, partial cdc, mitochoodrial repta obligua soucher 066. MXOF cytochrome oxidase subanit i (COX) gene, partial cdc, mitochoodrial Anastracha obligua veseber 063. MOOS extechrones oxidasa subgait 1 (COI) gena, restal ode reitschondrial autrophe oblique woucher 028 (M200 cytochrome oxidese schenir I (COR) game, partial odiç mitschoodrial Apartrapha obligas toucher 047, COL07 synchrones residant schustel (COI) gene, partial eds: referchondrial mpha obligna vousher OH. COLOI vytochrone otslase subatii 1 (COI) gene, partai odc, teitochondrial mpha obligna vousher OH. PRUM sytochrone otslase sabasti 1 (COI) gene, partial odc, teitochondrial sastruphs obligns washer 637 BB synchrone exides advant I (COI) gess, period adv, mitochooddal rupha obliqua voucher 506 MX03 cytochrome oxidase achunit I (COR) gaus, partial ode; mitochoodrial Ametropha oblique voucher 031, MX00 cytochrome coldese submit I (COI) gene, partial odg, mitochoodrial unitupha malanian voucher (10) MX01 eyeschrone opidase solumi 1 (COF) gane, partiel ede, mitochondra antrophe malanian voucher (12) MX02 eyeschrone opidase advanti 1 (COF) gane, partiel ede, mitochondrial quetrophy melaniae voucher (28 M001) cytochrinee condase adment I (COI) gene, pertial ade, n/tochondrial nphe superie vouder 127 PE cytochrone codese schutt I (COI) gene, partial cdr, estochoodrie Anastropha Jadama rouchar 025 MEO02 cytochrome oreidase subanti I (COR) gene, partial edie mitochrodrial uphe ludens woucher 023_OM cytochrome oridase suffanti I (COI) gave, pe nial ob; mitschentrial Sectorers canapholas ratiochondrion, complete genotes attroars donalis netochondrios, complete genome siele heraciel mitodoodrion, partial ganone (Query, 14120)

Figure 18 - Distance tree of results from BLAST search for HCO2198 primer

actroam deradia isolata B.dor. 1.180 cytochroma oraidasa subanit I (COO) gane, partial ode mitochondrial Bactroore accesses vouder (UIIM mat239 cycolinum coidea mheiri 1 (COT) guns, pertai ole, mirochrodrai Bactroore accesses vouder UIIM melifel cycolinum coidear scheiri 1 (COT) guns, pertai ole, mirochrodria Sectocers consects vescher CUDM.mail196 cylochrone oxidase submit I (COF) gens, partial ods, mitochrodital iactocens visenda voucher UHUM mol485 cytochrome oxidam subant 1 (COI) gene, partial ode; evit Sectocers visende voeder UHBM mel486 cytochrome ondere achurit I (COI) gene, pertai ede, mitochondrial Decroces donalis isolas IID Ja cynchrose c suidae sabani i gene, partal ody, mitochoadrial Decroces donalis isolas IID 1e cynchrose c ondae schuri i gene, partal ody, mitochoadrial Zeogodacon cucurbitae isolate KD70R cytrohmete ortidase subunit I (COR) gape, partial ode, mitochondrial dama sucurbitas isolain K13707 cytochronis oxidase scheniil 1 (COI) gare, partial ode, mitochoodrial Varigedance occurringe invites KD700 cytochrone orcidate subunit 1 (COI) gape, partial edg. mitochoodrial . Zaugodame ourarbeau nolasi KUNRS cytochrone oridaes mihanisi ((CR) pete, partial ode mitochoodrial Mangodame sucarbiau isolasi 71.303 cytochrone oridaes mihanis ((CR) gene, partial ode minochoodrial Vaugedama cucurbites indata TLNC cytochrome coldese mbanit I (COI) gane, yettal ode; mitochondrial esgedatas sucarteitas isolats TL300 cytochrone ordiaes subant I (COR) geos, petial cds, rainchondris Vaugodame cucurbitas isolats 71,200 extochrome oredass subanit i (COII) gaus, partial cde, mitochondrial Vergedane monthe inlate 11.204 synchrone oxides robenit I (COI) gens, petial ode, mitochoodrial Vargodama memblasi aolasi 11.200 cytochrome ordose subspit I (COR) gana, partial ede, minochoddial Zenandaine coostribue indete TI 202 ovinchrome coldare subscript ((CO)) ages, methal our minorhooddial Vaugodame escuristias indem 11.200 optochrime reidese nahmit I (COI) gans, partial oli; mitochoodda Vaugodame escuristias indem PT205 optochrime reidese nahmit I (COI) gans, partial oli; mitochoodda Vaugodacce succettitae isolata PT204 cytochrome oxidase miterit I (COF) gene, partial ode; mitochondrad dana cutatita iolas P1202 cristinose otidas athait1(COR) gene, pertal ode; reinchoodra Zaugodatus sucurititas isolata MCI04 cytochronas ortidase subunit L(COI) gene, partial ede mitochrodital Magodame recentritas nolais (MCR03 oytochrona onidase subunit) (CCR) pena, partial ode mitochoadrial Magodame excentritas inolais (MCR02 cytochrona oriclase subunit) (CCR) pena, partial ode mitochoadrial Zaugodama sucurbites isolata MCR01 cytochrome onidase subunit1(COX) gone, partial ode, mitochendrial Varigodaius cusarbiter indete MA205 sytochrone onidese suburiti (COI) gene, partial sile, mitochoodria Vaugedance ourarbites inclute MA204 cytochrome outdate subarit I (COI) game, partial odg mitochrodrial . Vlaugodacne ouzarhitse itolaa MA203 cytochtone ondase subsci11 (COI) gene, perial ode mitochosdrial Vlaugodacne escarbitse itolaa MA202 cytochtone ondase subsci11 (COI) gene, perial ode mitochosdrial Vacandatos caractetas indata MA201 evicebrona ortidas admiti 11000 acos, nartial ede mitrobradrial os cucartitas isolats TL2815 cytochrones condase subsett ((COR) gene, perial cde; mitochondria Zaugodaine cacerbine incluie 17,2514 cytochrome ordides substiti I (COI) gene, partial cdr, mittechondrial Margedama sucurities index TL283 cytochrons coules suburit (COR) gass, partial cdc, mondonodrial Margedama cacarities index TL283 cytochrons coulese suburit (COR) gass, partial cdc mitochoodrial Zeugodame curarbina isolata 71.2011 cytochrome oxidate subunit I (COI) gane, perial ode, mitochondrial os sucarhites index TI 2010 cytochrone codese acharal I (COI) gane, petial ode mitoch Vaugedams cucurtities invian TL289 evtochrome coldese mbunit I (COI) gans, patial ode mitochyndrial odazos sucarbitas indats 11.200 cytochrono oxidans subanti I (COR) gens, partial cde, minochoodnal odazos sucarbitas indats 11.201 cytochrono oxidaes subanti I (COR) gens, partial cde, minochoodnal Vaugodame cucurbitas isolats 71.200 octochrome orodass subanit i (COI) gaus, partial cde, mitochondrial Vergedana nacarbita isolais 17,203 sytectmens midaes submit (CON) gass, partial cds, mitochoodrial Vargedama sucretites isolate TL2M cytochrome ordines subspit I (CON) gana, partial ads: minochrodital Varigodaine constitue invista TL20 sytochrome coldase subspit I (COR) gene, partial ode, mitochoodrial dana oscarbitas indata 71,212 oytochrone coldase substiti (COI) gans, partial ofe; mitochoodra Vaugodacus oucarhites isolata TL201 eytochrime oxidase subspiri [(CO0) gans, partial cde minohoodrial Vaugedame cucarbina indea 11.125 sytochrone codian mbash ((COI) gees, patial edit, mitocloodial Vaugodatos sucarbibar indem 11.124 sytochrome ordinas achanit I (COR) gaus, partial ode; minohoodrial Vacgodame cucurbites indets 71.123 extechnome optime mbanit i (COI) gaue, partial dde mitochoddial na cucarbitee isolais 12.122 sytochrome oxidase subunit ((COI) gans, partial ode, mitoch Vargodama sucurfities inden 11.121 extodurums ordines subspit I (COI) gans, partial ofer mitochondrial edante cucurfithe indata BA304 cytechrome exident schenit I (COR) gene, partial ade mitechendria Zaugodame succerbites itolate BAND cytochrome oxidese subanit I (COI) gene, partial ody, mitochoodrial Vaugodacus sucurhitae indem BA301 cytochrome oxidaes subsuit I (OOI) gane, partial sdir, untochondrial na susarhitse isolaa DK103 sytochrone oicidase subarit1(COI)gene, partial udi; mitochoodria Zeogodacos cacarbitas ieviata DK1/C cytechness ortidass submit1 (COI) gene, partial cdg mitechoadrial odaine cucarbitae isolate IW.101 cytochrone oxidase submit1 (COI) gene, partial odq mitochoodrial Zaugodame meartrine isolate TASIII cytochrome oxidase schenit I (COI) gene, partial eds, netochondrial pdana nucerhite solan TASSI sytochrone oxidae substitl (COI) gene, perial olq, mitochendrial pdana nucerhite isolan TASSI sytochrone oxidae substitl (COI) gene, perial odq, mitochendrial Zaugodaine caractelus index 13.3230 cytochrone codese schwitt (COI) gans, partial ole, mitochoodrial Wargodame ourarbene inden TL329 sytochrome ordene nabuni i (COI) gane, perial circ, minokondrial Wargodame sucarbine inden TL328 sytochrome ordene nabuni i (COR) gane, perial circ minokondrial Vaugedame cucurbites invites 11.127 extechrome coddae mbunit ((COI) gave, yettal edg mitochondrial odaine sucarteina iedata TLUB cytochrone coddae achunit I (COI) gaus, partial cdg, minchoodrial Vaugodame constribue isolate 71.325 cytochrome coldase subunit I (COI) gene, partial dde, mitochondrial Ungedame exception induit TL334 sytechnine oxidas submit (CON) gans, patial ofe, mitochooddal Ungedame exception induit TL321 sytechnine oxidas submit (CON) gans, patial ofe mitochooddal Varigodaine cucorfulae invlata 11.322 cytochrome coldase substiti I (COR) gene, rastial ode, mitochoodrial dame sucurity in isolati TL327 sylochrome oxidaes subunit ((COI) gans, partial ode; mitochoodda Vaugodacce succertities index PK505 sytochrone oxidaes submit I (COI) gene, partial odg mitochondrial Vergedarus cucartina: indas (9534 cynchrone oridaus solvaris I (CO) gene, periol nér, mitochendria) Vergedarus sucartina: indas (9530 cynchrone oridaus solvaris I (CO) gene, periol cór, mitochendria) Vaugodame cucurbitae indets PKSU cytochrone orders submit I (COI) gene, partial ode mitochondrial dame mearbine isolate PC501 synchrone oxidate submit I (COR) gene, partial ode, mitochondrial Varagodama sucurfities incluis MA103 cytochrome onidate subsoit1 (COI) gone, partial ode mitochinadrial Plangodance cacartrine indais MA102 cytechrone ondase minusi 1 (COI) gene, partial ode mitochosdrial Vlangodance cucartrine indais MA101 cytechrone ondase admini 1 (COI) gene, partial ode mitochosdrial Varigodacce circurtulae isolate TA305 cytochronic oxidaes suborit I (COF) game, partial edg. mitochondrial odacus sucurititae isolaat TA304 cytochtones oxidaas sultaosi I (COR) gene, partial ole, mitochondrai odama sucurititae isolaat TA305 cytochtones oxidaas subsoit I (COR) gene, partial ole, mitochondrai Vaugodatos sucarbitas isolatis TA302 evitodorume ortidase aubunti I (COF) gene, merital tide, netrochondrial Augodams cucarbina isolata TANII cytochrone onidaa subanti I (COF) gene, partial ada, mitochondrial Geogradams monthing incluin 13.182 synchronic coldaes submit ((COI) gans, partial ode, mitochoodnal pdama monthibe isolati 13.187 cytochrone ordine schemit I (COR) gave, partial ode, minichondrial ana cascarfeitae ierdata TLAID sytochrome croblese aubezoit I (CCR) gene, petital odo; mitocho Vacgodame ourarbites incluin TL/90 oviochrome oxidase subspiri I (COI) gane, pertial cdir mitochondrial odacos sucarteitas isolata 71.601 cytochreme oxidase nebazir! (COI) gane, partial cde; minchondria Vaugedama susarhitas isolata KD140 sytechnose oxidase subanit1 (COI) gane, partial sdig mitochoodrial Zaugodatus sucarbitas isolata KDI-G sytochronis orcidaes milausti l (COI) gene, partial cdi; mitochoadrial Zaugodame cucarbitae isolate KIDI 41 cytochrome oxidase subanit I (COR) gene, partial cds; mitochoodrial Zaugodama sucurbitae isolate TGEOD extochrome oxidase subsoit L(COF) gene, partial odg, mitochoadrial Vargedama mentitiw isolan 70302 cytochrone exidan substit! (COI) gass, partial ada; mitochendrial Zaugedatos cassefular indata TOIOI cytoobrupe exidant subanti I (COI) gane, partial ode, mitoshoodrial Vaugodaene energibise, indam 17105 symothemise verdaas submitti (COI) gene, partial olis, nimchendital Vaugodaene energibise indam 17105 symothemise verdaas submitti (COI) gene, partial olis, enimchendital Variandame circurbities indust THO cytochrome ortidate subant L(COI) gene, mettal ode, mitochondrial dains susarbitas voucher 25 cytochrones o coldans subaux I (COXI) gens, partial olic raise Acidopantha gallhanda voucher AO cytochrome c ortidate subunt 1 (COUI) game, partial ode, mitochondnal Others 74

Figure 19 - Distance tree of results from BLAST search for ITS7 primer

Bactrocera philippinensis Bph871 from Philippines internal transcribed spacer 1, partial sequence

Bactrocera fuscitibia Bfu851 from Indonesia internal transcribed spacer 1, partial sequence

Bactrocera endiandrae Ben783 from Australia internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera carambolae internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, p.

Bactrocera cf. cognata 'Cue-lure' Bcg872 from Philippines internal transcribed spacer 1, partial sequence

Bactrocera cognata Bcg846 from Philippines internal transcribed spacer 1, partial sequence

Bactrocera cacuminata Bca779 from Australia internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera arecae Bar762 from Thailand internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera dorsalis 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, an...
 Bactrocera xanthodes internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, part...
 Bactrocera trilineola internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, part...
 Bactrocera neohumeralis strain Bn213 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, part...
 Bactrocera psidii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial...
 Bactrocera psidii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera psidii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera facialis internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera facialis internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera neohumeralis strain Bn179 internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera musae internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera jarvisi strain Bj177 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ...
 Bactrocera frauenfeldi internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa...
 Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa...
 Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa...
 Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa...
 Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed sp...
 Icl/Query_185633

Figure 20 - Distance tree of results from BLAST search for ITS6 primer

PREDICTED: Bactrocera oleae 5.8S ribosomal RNA (LOC118682848), rRNA

PREDICTED: Bactrocera oleae 5.8S ribosomal RNA (LOC118682317), rRNA

Bactrocera verbascifoliae Bvb845 from Sri Lanka internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera endiandrae Ben783 from Australia internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera carambolae internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, p...

Bactrocera cacuminata Bca779 from Australia internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera arecae Bar762 from Thailand internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera affinidorsalis Baff847 from Indonesia internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

Bactrocera dorsalis 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, an... Bactrocera xanthodes internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, par..

Bactrocera trilineola internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, part...

Bactrocera neohumeralis strain Bn213 internal transcribed spacer 1, partial sequence; 5.85 ribosomal RNA gene, complete sequence; and internal transcri.

Bactrocera psidii internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ... Bactrocera facialis internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partia...

Bactrocera musae internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial ... Bactrocera jarvisi strain Bj177 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 3.8

Bactrocera frauenfeldi internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, pa.. Bactrocera dorsalis strain Bd80 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed sp... Bactrocera aquilonis internal transcribed spacer 1, and 5.8S ribosomal RNA gene, partial sequence

lcl|Query 188421

Appendix 7 – Results diagnosic specificity with DNA barcoding primer sets and *ITS*1 sequencing

	E (LCO	PPO PM7/129 91490/HCO2198)	EPPO PI	M7/129 (LepF/LepR)	IPPC 27:DG29 (<i>ITS6/ITS</i> 7)		Final	
Sample Nb.	Result	Note	Result	Note	Result Note		Expected result	Assigned value
1	Negative	B. kandiensis	Negative	B. kandiensis	Negative	B. kandiensis	Negative	Bactrocera kandiensis
2	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
3	Negative	Anastrepha fraterculus	Negative	Anastrepha sp.	Negative	Not tested	Negative	Anastrepha suspensa
4	Negative	B. carambolae	Negative	B. carambolae	Negative	B. carambolae	Negative	Bactrocera carambolae
5	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
6	Negative	B. carambolae		No amplicon	Negative	B. carambolae	Negative	Bactrocera carambolae
7	Negative	Anastrepha suspensa	Negative	Anastrepha sp.	Negative	Not tested.	Negative	Anastrepha suspensa
8	Negative	B. carambolae	Negative	B. carambolae	Negative	B. carambolae	Negative	Bactrocera carambolae
9	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
10	Negative	B. kandiensis	Negative	B. kandiensis	Negative	B. kandiensis	Negative	Bactrocera kandiensis
11	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
12	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
13	Negative	B. kandiensis	Negative	B. kandiensis	Negative	B. kandiensis	Negative	Bactrocera kandiensis
14	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
15	Negative	Zeuaodacus cucurbitae	Negative	Zeuaodacus cucurbitae	Negative	Not tested	Negative	Zeugodacus cucurbitae
16	Positive	B. dorsalis	Positive	B. dorsalis	Negative	B. kandiensis	Negative	Bactrocera kandiensis
17	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
18	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
19	Negative	Zeuaodacus cucurbitae	Negative	Zeuaodacus cucurbitae	Negative	Not tested	Negative	Zeugodacus cucurbitae
20	Negative	B oleae	Negative	B oleae	Negative	Not tested	Negative	Bactrocera oleae
21		No amplicon	riegutire	No amplicon		No amplicon	Negative	Bactrocera pyrifoliae
21	Negative	B carambolae	Negative	B carambolae	Negative	B carambolae	Negative	Bactrocera carambolae
23	Negative	B. carambolae	Negative	B. carambolae	Negative	B. carambolae	Negative	Bactrocera occipitalis
24	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
24	Negative	Anastrenha sn	Negative	Anastrenha sn	Negative	Not tested	Negative	Anastrepha obliqua
25	Negative	Dacus ciliatus	Negative	No amplicon	Negative	Not tested	Negative	Dacus ciliatus
20	Negative	B carambolae	Negative	B carambolae	Negative	B carambolae	Negative	Bactrocera carambolae
27	Negative	Dacus ciliatus	Negative	Dacus ciliatus	Negative	Not tested	Negative	Dacus ciliatus
20	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
30	Positive	B. dorsalis	Positive	B. dorsalis	Positive	B. dorsalis	Positive	Bactrocera dorsalis
21	Nogativo	P. kandionsis	Nogativo	P. kandionsis	Nogativo	P. kandionsis	Negative	Bactrocera kandiensis
27	Positivo	B. dorsalis	Rositivo	B. dorsalis	Positivo	B. dorsalis	Positive	Bactrocera dorsalis
22	Nogativo	P. carambolao	Nogativo	P. carambolao	POSITIVE	No consonsus	Negative	Bactrocera carveae
33	Docitivo	B. dorsalis	Docitivo	B. dorsalis	Docitivo	B. dorsalis	Positive	Bactrocera dorsalis
34	Negative	Anastropha sp	Nogativo	Anastropha sp	Nogotivo	Nottostad	Negative	Anastrenha obligua
35	Desitive	B. dorsalis	Desitivo	Anustrephu sp.	Desitivo	B. dorsalis	Positive	Bactrocera dorsalis
30 27	Nogative	P. ologo	Nogative	D. UUISUIIS	Nogative	Nottestad	Negative	Bactrocera oleae
3/	Negative	D. UIEQE	Negative	B. UIEQE	Regative	B. dorsalis	Negative	Bactrocera carveae
38	Negative	B. carambolae	Negative	в. carambolae	POSITIVE	D. constant	Negative	Bactrocera carambolae
39	Negative	B. carambolae	Negative	B. carambolae	Negative	B. carambolae	Negativo	Bactrocera occinitalio
40		No amplicon		No amplicon	Negative	B. occipitalis	Negative	Buchocera Occipicalis

Table 10: Results diagnostic specificity with DNA barcoding primer sets and ITS1 sequencing

Appendix 8 – Summary Results sheets for analytical sensitivity, repeatability and reproducibility – molecular tests

Sample panel:

Sample 333/20: 1 adult specimen of *B. dorsalis* Sample 334/20: 1 leg of *B. dorsalis* Sample 335/20: 1 larvae of *B. dorsalis* Sample 336/20: 1 pupa of *B. dorsalis*

Three experimental replicates were performed with this sample panel.

Measurement of DNA concentration

Quantity of DNA was determined using the Thermo Scientific Nanodrop 2000 Spectrophotometer, samples were measured three times (technical replicates), the mean and the standard deviation were calculated (Table 11)

Results for analytical sensitivity (DNA barcoding and ITS1 sequencing)

4 samples with one adult or part of the individuals were prepared in different dilutions (1:10, 1:100, 1:1000; 1:10.000; 1:100.000, 1:1.000.000). Specifications and parameters for the molecular tests are provided in Appendix 3. Amplicons at the detection limit and the last dilution step before the detection limit were sent for SANGER sequencing (Table 11). The quality of sequences was assessed by the length of the consensus sequences and % of high quality bases (%HQ), see Table 12.

			DNA Concer	ntration [ng/	μ]	EPPO P	M7/129	IPPC ISPM 27:DP 29
Sample Nb. & Developmental stage of <i>B.</i> <i>dorsalis</i>	Dilution	Repetition 1	Repetition 2	Repetition 3	Mean ± SD	Amplicon (LCO1490/H CO2198)	Amplicon (LepF/lepR)	Amplicon (ITS6/ITS7)
	Undiluted	202.9	203.4	206.6	204.3 ±2 .00			
	1:10	21.2	21.7	21.3	21.4 ± 0.24	Strong	Strong	Strong
333/20 (adult)	1:100	1.9	1.9	1.7	1.8 ± 0.12	Strong*	Strong	Strong
000,20 (dddir)	1:1.000	N/A	N/A	N/A		Weak*	Strong*	Strong
	1:10.000	N/A	N/A	N/A		Negative	Negative	Strong
	1:100.000	N/A	N/A	N/A		Negative	Weak	Strong*
	Undiluted	141.4	142.1	141.9	141.8 ± 0.36			
	1:10	15.1	15.3	15.1	15.2 ± 0.12	Strong	Strong	Strong
224/20 (log)	1:100	1.1	1.7	1.1	1.3 ± 0.35	Strong*	Strong	Strong
554/20 (leg)	1:1.000	N/A	N/A	N/A		Weak*	Strong	Strong
	1:10.000	N/A	N/A	N/A		Negative	Strong	Strong
	1:100.000	N/A	N/A	N/A		Negative	Strong*	Strong*
	Undiluted	387.9	390.7	387.2	388.6 ± 1.85			
	1:10	41.3	41.8	41.7	41.6 ± 0.26	Strong	Strong	Strong
225/20 (lanva)	1:100	4.3	3.9	4.5	4.2 ± 0.31	Strong*	Strong	Strong
555/20 (lai va)	1:1.000	N/A	N/A	N/A		Strong*	Strong	Strong
	1:10.000	N/A	N/A	N/A		Negative	Strong	Strong
	1:100.000	N/A	N/A	N/A		Negative	Strong*	Strong*
	Undiluted	500.4	500.4	501.7	500.8 ± 0.75			
	1:10	53.4	54.4	54.0	53.9 ± 0.50	Strong	Strong	Strong
226/20 (2002)	1:100	5.9	5.5	5.7	5.7 ± 0.20	Strong*	Strong	Strong
550/20 (pupa)	1:1.000	N/A	N/A	N/A		Strong*	Strong	Strong
	1:10.000	N/A	N/A	N/A		Negative	Strong*	Strong
	1:100.000	N/A	N/A	N/A		Negative	Weak*	Strong*

N/A: not validly measurable

*Sequenced amplicons

Table 12: Sequence quality criteria for *B. dorsalis* sample panel used for sensitivity testing

Test	Sample Nb. & Developmental stage of <i>B.</i> <i>dorsalis</i>	Dilution	Approx. Consensus Length (bp)	High Quality (HQ%) of Consensus	Calculated DNA Concentration [ng/µl]		
EPPO	1 (adult)	1:1.000	562	100	0.18	Mean	0.325
PM7/129 (LCO1490/	8 (leg)	1:1.000	562	100	0.13		
HCO2198)	15 (larvae)	1:1.000	573	100	0.42		
	22 (pupa)	1:1.000	567	100	0.57		
EPPO	1 (adult)	1:1.000	582	100	0.18	Mean	0.325
PM7/129 (LepF/LepR)	8 (leg)	1:1.000	579	100	0.13		
(15 (larvae)	1:1.000	581		0.42		
	22 (pupa)	1:1.000	584	100	0.057		
IPPC27:DG26	1 (adult)	1:100.000	406	100	0.0018	Mean	0.00325
(<i>ITS6/ITS</i> 7)	8 (leg)	1:100.000	426	100	0.0013		
	15 (larvae)	1:100.000	411	100	0.0042		
	22 (pupa)	1:100.000	405	100	0.0057		

Results for repeatability (DNA barcoding and ITS1 sequencing)

Three replicates of *B. dorsalis* (adult – dilutions) were analysed with 3 technical repetitions.

The sample panel was analysed with three dilution steps and each with three technical repetitions. The results were summarized in table 13.

 Table 13: Amplicon generation for DNA barcoding and ITS1 sequencing PCR repeatability test

			Amplicon production		
Test	Sample Nb. & Developmental stage of B. dorsalis	Dilution	Repetition 1	Repetition 2	Repetition 3
EPPO PM7/129	1 (adult)	1:100	Strong	Strong	Strong
(LCO1490/HCO2198)		1:1.000	Strong	Strong	Weak
		1:10.000	Negative	Negative	Negative
	8 (leg)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Weak	Weak	Strong
	15 (larvae)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Weak	Strong	Strong
	22 (pupa)	1:100	Strong	Strong	Negative
		1:1.000	Negative	Negative	Strong
		1:10.000	Strong	Strong	Strong
EPPO PM7/129	1 (adult)	1:100	Strong	Strong	Strong
(LepF/LepR)		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	8 (leg)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	15 (larvae)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	22 (pupa)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
IPPC 27:DG29	1 (adult)	1:100	Strong	Strong	Strong
(<i>ITS</i> 6/7)		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	8 (leg)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	15 (larvae)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
	22 (pupa)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong

Results for reproducibility (DNA barcoding and ITS1 sequencing)

Table 14: Sample panel

Target	Non target	Origin
B. dorsalis adult	/	Thailand /Saraburi
B. dorsalis larva	/	Thailand /Saraburi
B. dorsalis pupa	/	Thailand /Saraburi
	Bactrocera correcta larva	India
	Bactrocera carambolae adult	French Guyana
	Bactrocera latifrons larva	Thailand

Testing reproducibility of the PCR tests:

The tests were performed with three technical replicates and under different conditions (two operators on different days and using different thermocycler machines). The results are shown in tables 15 and 16.

 Table 15: Reproducibility of the PCR tests operator 1

Operator:	Pohn
Date of performance:	22.02.2021
Thermocycler machine:	BiometraT3000 (I)

	EF (LCO	EPPO PM 7/129 EPPO PM 7/129 (LepF/LepR) IPPC 27:DG29 (ITS6, (LCO1490/HCO2198) EPPO PM 7/129 (LepF/LepR) IPPC 27:DG29 (ITS6,			EPPO PM 7/129 (LepF/LepR)			5/ITS7)	
Species & Sample Nb.	Repetitio n 1	Repetitio n 2	Repetitio n 3	Repetitio n 1	Repetitio n 2	Repetitio n 3	Repetitio n 1	Repetitio n 2	Repetitio n 3
Bactrocera dorsalis (adult), 333/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera dorsalis (adult), 335/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera dorsalis (adult), 336/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera correcta (larva), 158/21	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera carambola e (adult), 8	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera latifrons (larva), 867/20c	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon

*Sequenced

Table 16: reproducibility of the PCR tests operator 2

Operator:	Heiss
Date of performance:	21.02.2021
Thermocycler machine:	BiometraT3000 (II)

	EI (LCO	EPPO PM7/129 (LCO1490/HCO2198) EPPO PM7/129 (LepF/LepR) IPPC 27:DG29 (<i>ITS6/ITS7</i>)				EPPO PM7/129 (LepF/LepR)			5/ITS7)
Species & Sample Nb.	Repetitio n 1	Repetitio n 2	Repetitio n 3	Repetitio n 1	Repetitio n 2	Repetitio n 3	Repetitio n 1	Repetitio n 2	Repetitio n 3
Bactrocera dorsalis (adult), 333/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera dorsalis (adult), 335/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera dorsalis (adult), 336/20	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera correcta (larva), 158/21	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera carambola e (adult),8	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera latifrons (larva), 867/20c	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon

*Sequenced

Testing reproducibility of the SANGER sequence analysis:

The reproducibility of the SANGER sequence analysis was tested with the same sample panel. The sequence analysis was performed by two operators on different days. The alignment of the consensus sequence will be performed in three different data bases (NCBI GenBank, Bold, Q-Bank). Tables 17 and 18 depict the results of reproducibility.

Table 17: Reproducibility of the SANGER sequence analysis operator 1

Operator:	Pohn
Date of performance:	23.02.2021
Software:	Geneious prime [®] 10.1.3

	EPPO PM7/	129 (LCO1490	/HCO2198)) EPPO PM7/129 (LepF/LepR) IPPC 27:DG29 (<i>ITS6/ITS7</i>)			
Species & Sample Nb.	NCBI GenBank	Bold	Q-Bank	Species & Sample Nb.	NCBI GenBank	Bold	Q-Bank
Bactrocera dorsalis (adult), 333/20	B. dorsalis	B. dorsalis	B. dorsalis	Bactrocera dorsalis (adult), 333/20	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera dorsalis (adult), 335/20	B. dorsalis	B. dorsalis	B. dorsalis	Bactrocera dorsalis (adult), 335/20	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera dorsalis (adult), 336/20	B. dorsalis	B. dorsalis	B. dorsalis	Bactrocera dorsalis (adult), 336/20	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera correcta (larva), 158/21	B. correcta	B. correcta	B. correcta	Bactrocera correcta (larva), 158/21	B. correcta	B. correcta	B. correcta
Bactrocera carambolae (adult),8	B. carambolae	B. carambolae	B. carambolae	Bactrocera carambolae (adult),8	B. carambolae	B. carambolae	B. carambolae
Bactrocera latifrons (larva), 867/20c	B. latifrons	B. latifrons	B. latifrons	Bactrocera latifrons (larva), 867/20c	B. latifrons	B. latifrons	No sequence in database

Table 18: Reproducibility of the SANGER sequence analysis operator 2

Operator:	Gottsberger
Date of performance:	26.02.2021
Software:	Geneious prime [®] 10.1.3

	EPPO PM7/129 (LCO1490/HCO2198)			EPPO PM7/129 (LepF/LepR)			IPPC 27:DG29 (ITS6/ITS7)
Species & Sample Nb.	NCBI GenBank	Bold	Q-Bank	NCBI GenBank	Bold	Q-Bank	NCBI GenBank
Bactrocera dorsalis (adult), 333/20	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera dorsalis (adult), 335/20	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera dorsalis (adult), 336/20	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis	B. dorsalis
Bactrocera correcta (larva), 158/21	B. correcta	B. correcta	B. correcta	B. correcta	B. correcta	B. correcta	B. correcta
Bactrocera carambolae (adult),8	B. carambolae	B. carambolae	B. carambolae	B. carambolae	B. carambolae	B. carambolae	B. carambolae
Bactrocera latifrons (larva), 867/20c	B. latifrons	B. latifrons	B. latifrons	B. latifrons	B. latifrons	B. latifrons	No sequence in database

Appendix 9 – Calculations for the performance characteristics - molecular tests

Appendix 9 shows the calculations for the performance characteristics.

Table 19: Calculations of the applicable performance characteristics (sensitivity, specificity and accuracy) for all three primer sets.

Target Species	Criteria	EPPO PM7/129 (LCO1490/HCO2198)	EPPO PM7/129 (LepF/LepR)	IPPC 27:DG29 (<i>ITS</i> 7/ <i>ITS</i> 6)
Bactrocera dorsalis	Number of Positive Agreements	14	14	14
	Number of Negative Agreements	23	21	13
	Number of Negative Deviations	0	0	0
	Number of Positive Deviations	1	1	1
	Sensitivity	100	100	100
	Specificity	96	95	93
	Accuracy	97	97	96

Due to the conflicting results obtained from the molecular analysis with respect to the assigned value of sample 23 (*Bactrocera occipitalis*), an in-depth morphological analysis was conducted by the three operators involved in the morphological validation study. High resolution pictures of sample 23 and *B. occipitalis* and *B. carambolae* specimens included in the sample set were taken to support conclusions (see Fig. 21). Pictures from FruitFly ID Australia were also checked (<u>https://fruitflyidentification.org.au/</u>), in addition to figures from DP 29 (IPPC, 2019).

Sample 23 was donated to the AGES collection (recodification F20042). Data of sample 23 are the following.

- origin: Philippines, UPLB campus, rainforest area (mixed vegetation, trap catch)
- sampling date: 08.01.2000
- leg. and/or det. (no indication of the name's role available): G. Quimio

Characters that allow the discrimination of *B. occipitalis* and *B. carambolae* (IPPC, 2019) are resumed in table 20, together with the comments and the general final opinion of operators.

Structure	B. carambolae	B. occipitalis	Operator 1	Operator 2	Operator 3	General opinion of operators
Tergites III-V	With medium- width medial longitudinal black stripe	With very broad medial longitudinal black stripe	There is a (very?) broad medial longitudinal black stripe on tergites 3–5	The broadness of the medial longitudinal stripe on T3-5 was not fully clear to me	Medial longitudinal black stripe looks broad/very broad	Not definitively clear if it is broad or very broad (see also Fruit Fly ID Australia - abdomen variation CAR002 in <i>B.</i> <i>carambolae</i>)
т III	With a narrow transverse black band across anterior margin (constituting a "T" pattern) widening to cover lateral margins	With a narrow transverse black band across anterior margin widening to cover lateral margins	Figures match higher with B. occipitalis, but no difference to B. carambolae in description	Figures match higher with B. occipitalis, but no difference to B. carambolae in description	Considering Fig. 17 and 18, higher matching with (e), B. occipitalis. However variations are possible, see FruitFly ID Australia	Even if a higher match with <i>B. occipitalis</i> Figures in DP 29, no clear differences arise from the description in Table 3. See also Fruit Fly ID Australia - abdomen variation CAR002 in <i>B.</i> <i>carambolae</i> and abdomen variation
TIV	With rectangular anterolateral (occasionally triangular) black markings	Exhibits variations from anterolateral black markings to broad lateral bands	Pictures match higher with B. occipitalis, but no difference to B. carambolae in description	Pictures match higher with B. occipitalis, but no difference to B. carambolae in description	Considering Fig. 17 and 18 in IPPC, 2019, higher match with (e), B. occipitalis. However variations are possible, see FruitFly ID Australia	
τv	With anterolateral black markings	With broad lateral black bands that cover lateral margins	Pictures match higher with B. occipitalis, but no difference to B. carambolae in description	Pictures match higher with B. occipitalis, but no difference to B. carambolae in description	Considering Fig. 17 and 18, higher matching with (e), B. occipitalis. However variations are possible, see FruitFly ID Australia	occipitalis

Table 20: When not otherwise indicated, referring to figures and table 3 means in the DP29 (IPPC, 2019)

Lateral vittae	Broad, parallel- sided, ending at or behind ia. Bristles	Broad, parallel- or subparallel- sided; either ending at ia. bristles or (in some specimens) ending behind ia. Bristles	Hard to see after DNA extraction; vittae look subparallel sided; Considering Fig. 13, higher matching with (e), B. occipitalis	Hard to see after DNA extraction; vittae look subparallel sided; Considering Fig. 13, higher matching with (e), B. occipitalis	Hard to see after DNA extraction; vittae look subparallel sided; Considering Fig. 13, higher matching with (e), B. occipitalis	Hard to see after DNA extraction; vittae look subparallel sided; Considering Fig. 13, higher matching with (e), <i>B. occipitalis</i>
Costal band	Narrow, slightly overlapping R2+3, moderately broad around apex of wing	Narrow, distinctly overlapping R2+3, broad around apex of wing extending to mid-point between R ₂₊₃ and R ₄₊₅	The costal band overlaps only "slightly" but not until the "mid- point between R ₂₊₃ and R ₄₊₅ ".	I crossed out the costal band character at B. occipitalis ('distinctly') and noted: ~slightly overlapping, moderately broad around the apex	Considering Fig. 16, higher matching with (e), B. occipitalis.	Costal band doesn't seem to be "distinctly" overlapping R ₂₊₃ , but rather "slightly" overlapping. It is noted that the difference between "slightly" and "distinctly" overlapping is not clear from Fig. 16 (a) and (e)
Operators independent conclusion		B. occipitalis - but with uncertainty.	Not determined. Excluded B. occipitalis ('distinctly') and B. dorsalis ('confluent') in the key	Bactrocera occipitalis with a certain degree of uncertainty	<i>Bactrocera dorsalis</i> complex	

The general conclusion after the comments of the operators is that sample 23 cannot be morphologically identified as *Bactrocera occipitalis* with a sufficient degree of certainty. Even if the shape of lateral vitte looks subparallel-sided, other key characters to distinguish between *B. occipitalis* and *B. carambolae* do not lead to a clear, undoubtful identification. In addition, figures from FruitFly ID Australia website about variations from the typical abdomen appearance for the two species add a further level of uncertainty with respect to DP 29.

In the light of this morphological examination and the results from the molecular analysis (*Bactrocera carambolae*), sample 23 should be only identified as belonging to the *Bactrocera dorsalis* complex. It should be recalled that for regulamentary purposes, the identification to the level of "complex" is already largely sufficient to EU National Plant Protection Organisations to trigger adequate phytosanitary measures, like, for example, the destruction of an infested lot at an EU entry point.

Figure 21

Lateral vittae

Sample 23 Sample 27 (B. carambolae) Image: sample 23 Image: sample 27 (B. carambolae) Image: sample 40 (B. occipitalis) Sampe 4 (B. carambolae) Image: sample 40 (B. occipitalis) Sampe 4 (B. carambolae)