EURL
European Union Reference Laboratory for
INSECTS AND MITES

FINAL REPORT

Validation of the morphological and molecular identification protocols for
Bactrocera zonata (Saunders, 1842)

EPPO PM 7/114 (1) *Bactrocera zonata*
and
EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests

Version N. 01 – 07 Avril 2022

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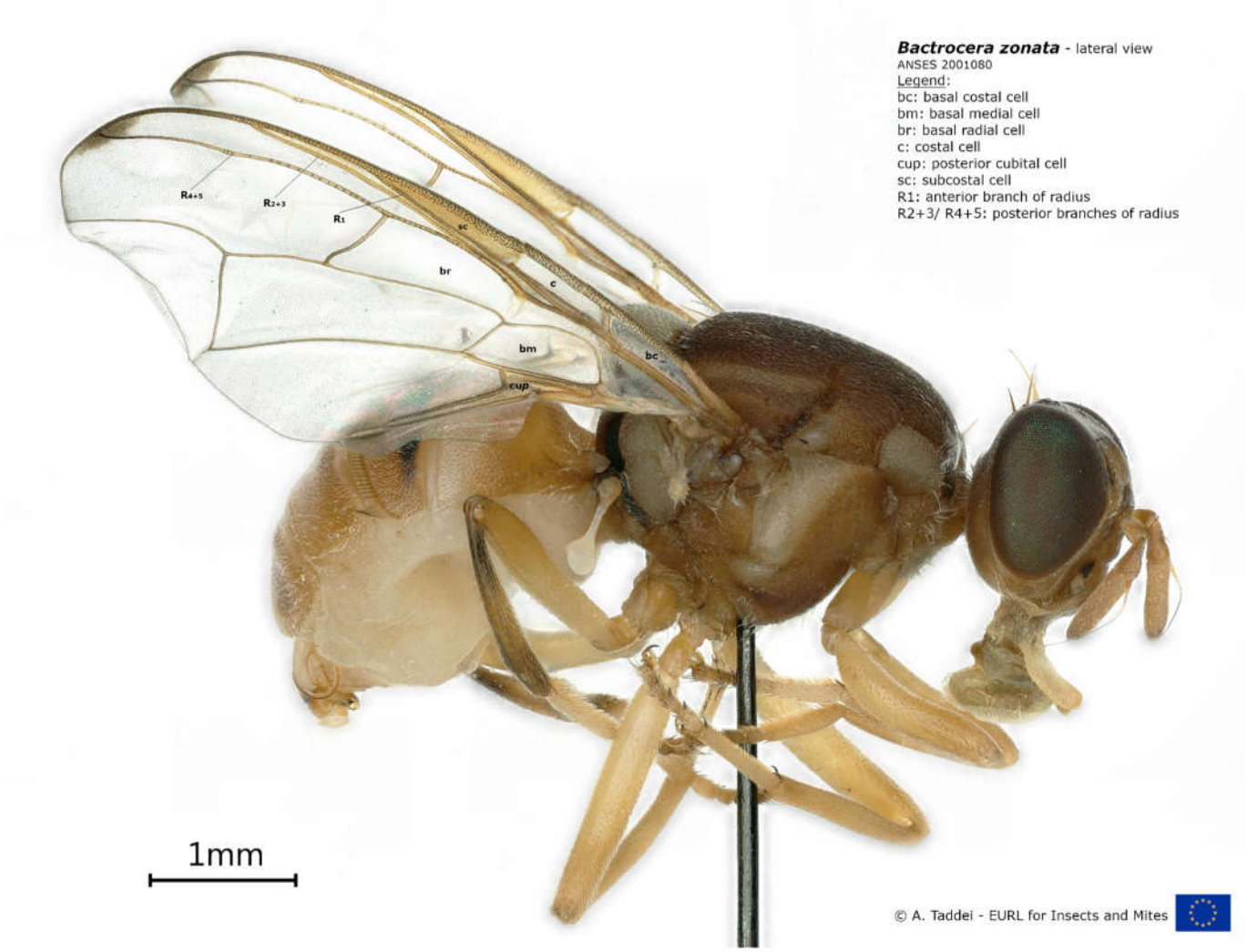


1. Introduction

The European Reference Laboratory for Insects and Mites has to select, adapt or develop reliable identification protocols for European Union regulated insect and mite species (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 diagnostic protocols before recommending their use to the National Reference Laboratories of the European Union.

The Entomology and Invasive Plants Unit of Anses Plant Health Laboratory (Montpellier, France) and the Institute for Sustainable Plant Production of AGES (Vienna, Austria) are in charge of the activities of the EURL for Insects and Mites. The consortium performs validation studies for morphological and molecular identification tests.

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 tests are qualitative, meaning that they 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 zonata (Saunders, 1842) (Diptera: Tephritidae) is native to South and South-East Asia (CABI, 2021; EFSA, 2021). Due to its high reproductive and biotic potential, strong flying ability and broad host range, it is considered a species with a high invasive capacity (EFSA, 2021). In recent history, *B. zonata* has expanded its geographical range to the drier climate regions of the Middle East and northern Africa and it is now found in more than 20 countries in Asia and Africa (CABI, 2021; EFSA, 2021). Outside its native range, *B. zonata* occurs in northern Africa (Egypt and Libya) (CABI, 2021), in some of the islands in the Indian Ocean (Mauritius and Réunion) (Permalloo *et al.*, 1998), in Sudan (Mahmoud *et al.*, 2020) and in several Middle East countries like Oman (Azam *et al.*, 2004), Iran (Koohkanzade *et al.*, 2019), Iraq (Abdulrazak *et al.*, 2016), Saudi Arabia, Lebanon, Yemen and the United Arab Emirates (EFSA, 2021). In America, specimens were trapped in California (1988) and Florida (2010, 2018) but no establishment has been accomplished (Carey and Dowell, 1989; FDACS, 2018; CABI, 2021). In Europe, the recent interceptions in Austria (2012-2018) keep the European Plant Protection Organisations on alert (Egartner *et al.*, 2019; EPPO, 2021a). At present, no established populations have been reported in the EU territory.

Bactrocera zonata (Diptera: Tephritidae) is a European Union regulated species, listed among the EU quarantine pests (Annex II of the Commission Implementing Regulation (EU) 2021/2285) and among the EU priority pests (Commission Delegated Regulation (EU) 2019/1702).

2. Scope of validation and tests

2.1 Scope

The scope of this validation study was to provide objective evidence that the selected protocols are suitable to perform routine identification of *Bactrocera zonata* (Saunders, 1842) by the staff of the EU National Reference Laboratories.

2.2 Description of the tests under validation

The tests under validation are based on two diagnostic protocols for the morphological and molecular identification of *Bactrocera zonata*, in addition to a published pest-specific real-time PCR, i.e.:

- EPPO PM 7/114 (1) *Bactrocera zonata* (EPPO, 2013);
- 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.
- Real-time PCR according to Koohkanzade *et al.* 2018

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 Morphological identification of adults

Protocol: EPPO PM 7/114 (1) *Bactrocera zonata* (EPPO, 2013)

The identification at species level for *Bactrocera zonata* requires morphological examination of adult flies. The identification is possible both on male and female specimens. The use of a stereomicroscope is needed (≥ 20 magnification).
The protocol provides guidance for the identification at species level of *Bactrocera zonata* adults:

- **Appendix 1 – Key for identification of adult *B. zonata***: a simplified key is given for the morphological identification of adults of *B. zonata*
- **Description of adult** morphology (head, thorax, abdomen, legs, wings).

The **description of larvae** is also provided. However, the authors state in the text of the standard itself that "A reliable identification can only be performed on an adult specimen and although larvae are described below, identification based on this stage is not recommended".

The validation planned in this document took into account the list of characters for the identification **of adult *B. zonata* included in the key in Appendix 1**. However, **the observation of the aculeus was not subject of this study**, due to the following practical reason:

- The dissection of genitalia must be performed in advance by supervisor and, if the whole abdomen has to be removed, that means that the characters of the abdomen are no more available for the operators to be checked.

2.2.2 Molecular identification of adults, larvae and pupae

Molecular tests can support morphological identifications of adults. Furthermore, these tests can especially be used when dealing with other developmental stages (e.g. larvae, pupae). One barcoding protocol was validated, as well as a pest-specific real-time PCR.

- Protocol: EPPO PM 7/129 (2) DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021), Appendix 1 – DNA barcoding of arthropods. DNA barcoding is used to identify the 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.
- Pest-specific real-time PCR according to Koohkanzade *et al.* 2018.

2.3 Composition of the sample set

A sample set of 30 Tephritidae specimens was used. It consisted of 30 adult specimens belonging to target and 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 originated from 4 different countries (Egypt, India, Réunion Island, Pakistan). Non-target specimens all belonged to the Tephritidae family and were selected primarily based on the close similarity to the target species ("look-alikes") and the availability in the partner laboratories reference collections. The origin of the non-target specimens was variable, including Asian, African and European countries. After randomization, each sample was re-labelled (coded) with numbers from 1 to 30 by supervisors. Original codification of samples was available only to supervisors. For uniformity, 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 some performance characteristics with the molecular tests additional, adapted sample sets were prepared (see 3.3.2 – Molecular tests)

Table 1: Summary of the composition of the sample set

Species	**Total Number**	Country of collection
Bactrocera albistrigata	**1**	Thailand
Bactrocera correcta	**4**	Laos, Thailand
Bactrocera dorsalis	**4**	Benin, Sri Lanka
Bactrocera latifrons	**3**	Cambodia, India, Laos
Bactrocera oleae	**3**	France

Bactrocera zonata	**7**	Egypt, India, Pakistan, Réunion Island
Dacus bivittatus	**2**	Ivory Coast
Dacus ciliatus	**3**	Réunion Island, Sri Lanka
Dacus etiennellus	**1**	Mayotte
Dacus punctatifrons	**1**	Congo
Zeugodacus cucurbitae	**1**	India
	30	

3. Validation of the tests

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, 2018), 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 in this study.

Table 2: Definition and calculation of performance characteristics

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) *Comments: 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 specificity	**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 reliably be detected. 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 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.	Accuracy = (true positives + true negatives)/(true positives + false

Performance criteria	Definition	Calculation
	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.	negatives + true negatives + false positives)

3.2 Performance characteristics already available

Performance characteristics obtained within this study were compared with performance characteristics already available for the respective tests. In the EPPO PM 7/129(2) DNA barcoding standard, performance characteristics were already available in Appendix 1. Performance characteristics for analytical sensitivity and specificity for the pest-specific real-time PCR could be retrieved from the original publication (Koohkanzade *et al.* 2018). For the morphological test performance characteristics were not available. In the case of the molecular tests, the expected performance characteristics were considered equal to 100%, with the exclusion of the analytical sensitivity, which consists in a measure of concentration expressed in ng/µl.

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 4ng/µl sufficient for high quality amplicon sequencing
- **Analytical specificity:** The interspecific variation of the gene locus was determined to be sufficient for identification at species level.
    - Inclusivity: Summary list of identified arthropods in Appendix 1 (Table 1) of the standard
- **Diagnostic sensitivity:** 98%-100%

Performance characteristics for pest-specific real-time PCR (Koohkanzade *et al.* 2018):

- **Analytical sensitivity:** The limit of detection (LOD) of the assay was 1.4pg of target DNA extracted from one entire specimen.
- **Analytical specificity:** The interspecific variation was determined to be sufficient for identification at species level.
    - Inclusivity: 16 target specimens from Iran and India
    - Exclusivity: 19 non-target species from 13 different African and Asian countries

3.3 Validation protocol

3.3.1 Morphological test

The set of 30 specimens was analysed by three operators, 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. zonata*;
- NEGATIVE, if **not all** the characters of the specimens matched with those of *B. zonata*;
If the matching of characters was ambiguous, operators were required to highlight which characters lead to the ambiguity and which parts in the protocol are weak (Notes column in the Summary Results sheet).
After the analysis, the Summary Results sheet has been 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 plan:

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

While performing the morphological analysis, operator 3 removed one leg from each specimen and placed it in an Eppendorf vial, in 95% ethanol, keeping the respective code. The leg samples were used for DNA extraction and molecular analysis.

3.3.2 Molecular tests

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 QIAamp DNA Micro Kit (Qiagen) was applied.

Analytical specificity

Sample set: The same set of specimens as for the morphological analysis was considered for the validation of the molecular tests - see 2.3 for further specifications.
Inclusivity: 7 targets
Exclusivity: 23 non-targets

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, the following genetic databases were consulted: NCBI-GenBank, Bold and EPPO Q-bank.

In silico testing: The analytical specificity for the barcoding primer sets (LCO1490/HCO2198 and LepF/LepR) and the primer set for real-time PCR (BzonF/BzonR/BzonP) were tested *in silico* by a database alignment (NCBI- GenBank).

Analytical sensitivity
5 samples (single specimens in different live stages and one leg, respectively) obtained from a rearing at the IAEA were prepared in different dilutions. Three experiments were performed with this sample set.

Sample set:
1 adult specimen of *B. zonata* (female)
1 adult specimen of *B. zonata* (male)
1 larva of *B. zonata*
1 pupa of *B. zonata*
1 leg of *B. zonata*

Dilutions (1:100, 1:1.000; 1:10.000; 1: 100.000, 1: 1.000.000, 1: 10.000.000).
To define the limit of detection for DNA barcoding, the two highest dilutions from which amplicons could be generated, were sequenced and analysed.

Repeatability
Three biological replicates of *B. zonata* (dilution near by the detection limit) were analysed with 3 technical repetitions to determine the repeatability.

Reproducibility

Sample set for testing reproducibility of the PCR tests:
Three targets and three non-targets were used to test the reproducibility of the PCR tests (Table 3). These tests were performed with three technical replicates and under different conditions (two operators on different days and using different thermocycler machines).

Table 3–Sample set for the evaluation of the reproducibility of the molecular *B. zonata* identification

Target	**Non target**	**Origin**
B. zonata – adult		Rearing IAEA (2020)
B. zonata – larva		Rearing IAEA (2020)
B. zonata – pupa		Rearing IAEA (2020)
	Bactrocera correcta – adult	Laos
	Bactrocera latifrons – adult	Laos
	Dacus bivittatus – adult	Ivory Coast

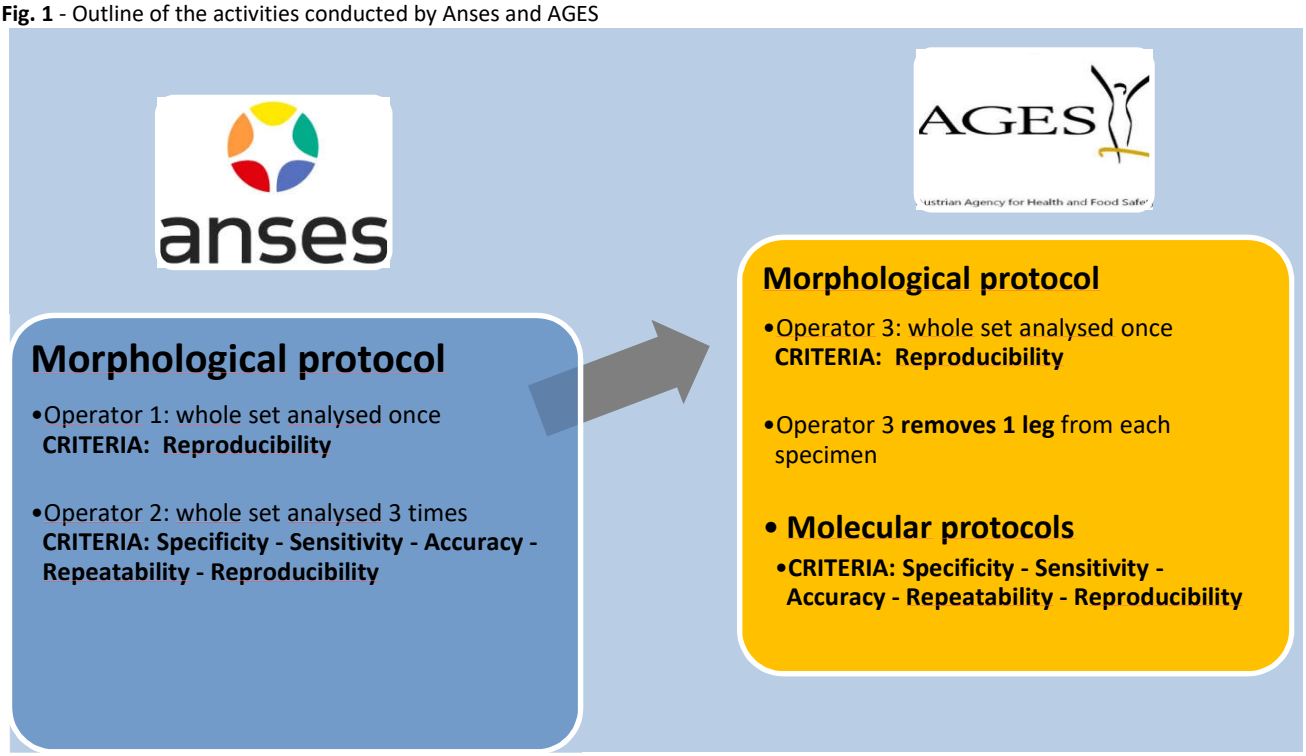
Sample set for testing reproducibility of the SANGER sequence analysis:

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

Specifications and parameters for the molecular tests are provided in Appendix 3.

Figure 1 provides a scheme of the overall activity of validation of the *B. zonata* identification.

Fig. 1 - Outline of the activities conducted by Anses and AGES



4. Performance adequacy and validation

The performance values obtained by the method 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).

Table 4: Expected performance characteristics (limits of acceptability) for morphological and molecular validation.

Performance criteria	Expected performance characteristics		
	EPPO PM7/114(1) *Bactrocera zonata* – morphological identification	EPPO PM 7/129 DNA barcoding	Koohkanzade *et al.* 2018 Real-time PCR
Analytical sensitivity	1 adult specimen	4ng/µl	1.4pg/µl*
Analytical specificity (Inclusivity)	100%	100%	100%
(Exclusivity)	100%	100%	100%
Diagnostic specificity	100%	100%	100%
Diagnostic sensitivity	100%	98-100%	100%
Repeatability	100%	100%	100%
Reproducibility	100%	100%	100%
Accuracy	100%	100%	100%

*expected for the analytical sensitivity in the case of DNA extracted from one entire specimen.

5. Time schedule and staff

The trial period was from November to December 2020 for the morphological analysis and from May to August 2021 for the molecular analysis and involved staff from the EURL for Insects and Mites.

Participating staff:

- for morphological tests:
  Experts/ Supervisors: Valérie Balmès, Sylvia Blümel
  Technical staff/ Operators: Christa Lethmayer, Raphaëlle Mouttet, Andrea Taddei

- for molecular tests:
  Experts/ Supervisors: Richard Gottsberger, Helga Reisenzein
  Technical staff/ Operators: Claudia Heiss, Chiara Pohn

6. Results of the validation analysis

6.1 Morphological test

Protocol: EPPO PM 7/114 (1) *Bactrocera zonata* (EPPO, 2013)

The values obtained for diagnostic specificity, diagnostic sensitivity, accuracy, repeatability and reproducibility met the expected value of 100% (Table 5). The test was found to be inclusive for target specimens from Egypt, India, Pakistan and Réunion Island and exclusive for a range of non-target specimens belonging to the *Bactrocera genus* (*B. albistrigata, B. correcta, B. dorsalis s.l., B. latifrons, B. oleae*) and other Dacini (*Dacus bivittatus, Dacus ciliatus, Dacus etiennellus, Dacus punctatifrons, Zeugodacus cucurbitae*).

Appendix 4 of this document shows the results obtained by the three operators.
Appendix 5 shows the calculations for the performance characteristics.

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

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%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100% (Egypt India Pakistan Réunion Island)**
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**100% (*Bactrocera albistrigata Bactrocera correcta Bactrocera dorsalis s.l. Bactrocera latifrons Bactrocera oleae Dacus bivittatus Dacus ciliatus Dacus etiennellus Dacus punctatifrons Zeugodacus cucurbitae*)**
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%**	**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%**	**100%**

6.1.1 *Analysis of critical steps*

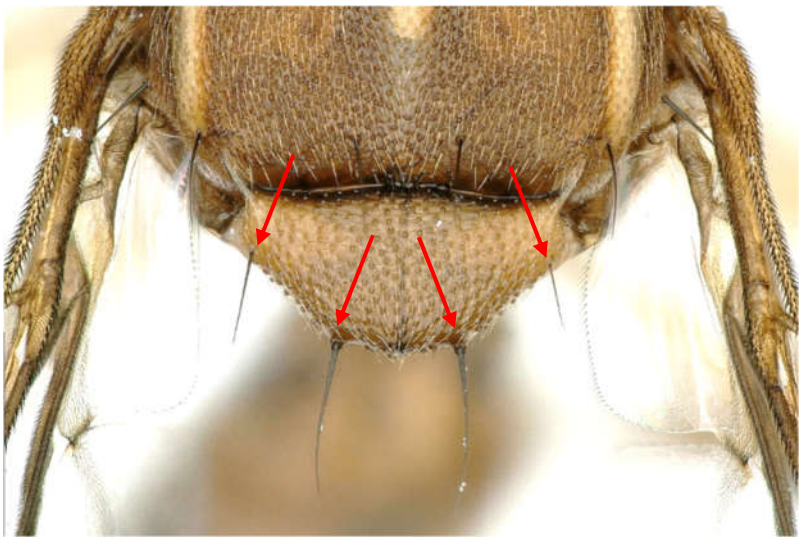
When performing the analyses, the operators did not identify any weaknesses in the protocol that could lead to a risk of misidentification of the target species. However, the operators did identify the need for minor corrections and improvements of the protocol key (page 416). It has to be noted that the way in which those characters are currently described did not affect the correct identification (expressed in its qualitative form as positive/negative) of all samples in the sample set. These suggestions for improvement are listed below:

- at couplet 2, character "abdominal segment fused/not fused": it is not always clear to distinguish, adding figure comparing fused and not fused abdominal segments could be useful (Fig. 2);

Fig. 2 – Abdominal segments, ventrolateral view: (A) *Bactrocera zonata*, not fused, red arrows show the separation and partial overlap of abdominal segments, better seen in ventrolateral view; (B) *Dacus bivittatus*, fused, the green arrow shows the fusion of abdominal segments (© A. Taddei, Anses)

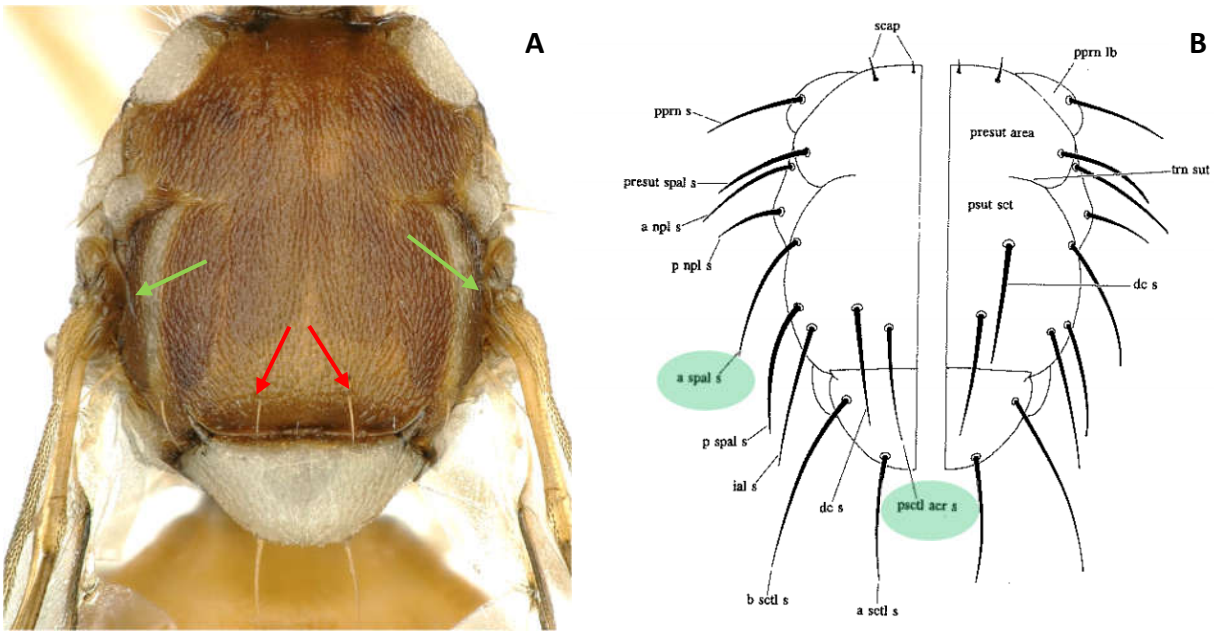
- at couplet 3, character "scutellum not bilobed and with 2 marginal setae/scutellum bilobed": none of them works for sample 22 (*Zeugodacus cucurbitae*, that displays scutellum not bilobed and with 4 marginal setae, Fig. 3);

Fig. 3 – Sample 22, *Zeugodacus cucurbitae*, particular of scutellum with 4 marginal setae (red arrows), dorsal view (© A. Taddei, Anses)



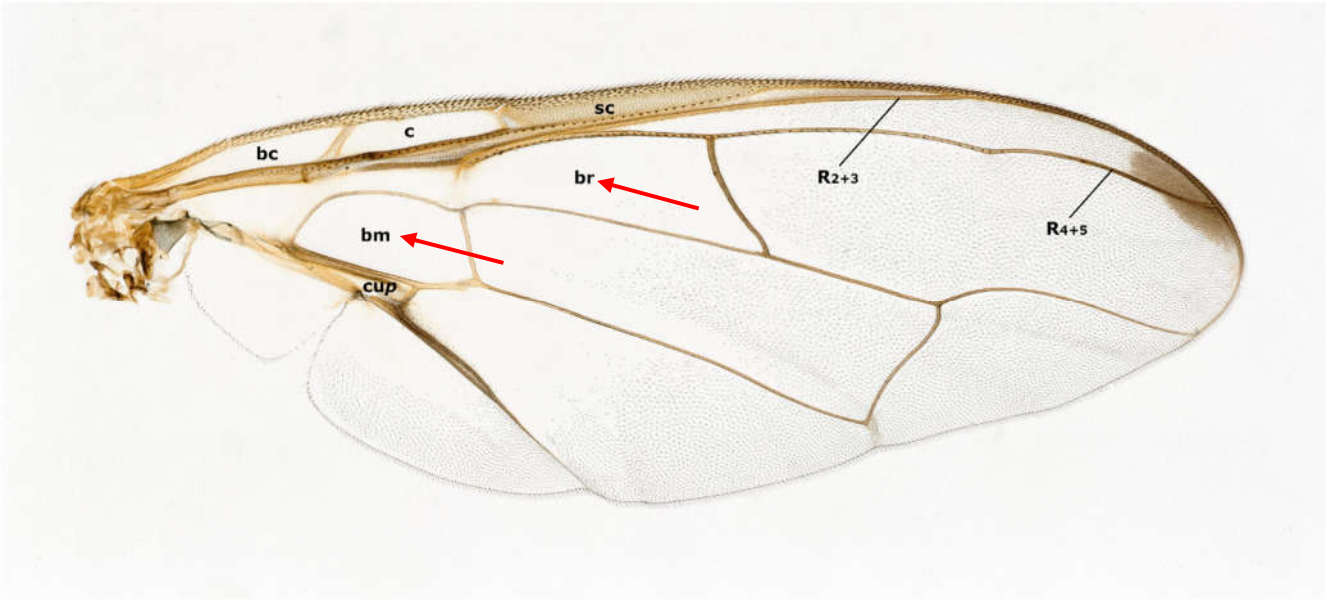
- at couplet 4, character “prescutellar acrostichal setae and anterior supra-alar setae”: adding figures to remind their position on the thorax could be useful (Fig. 4);

Fig. 4 – (A) *Bactrocera zonata*, thorax in dorsal view: red arrows showing prescutellar acrostichal setae and green arrows showing anterior supra-alar setae (Photo: © A. Taddei, Anses); (B) schematic representation of Tephritidae thorax, dorsal view (from White & Elson-Harris, 1992): anterior supra-alar seta (a spal s) and prescutellar acrostichal seta (psctl acr s) are highlighted in green



- at couplet 7, character “wing cells br and bm”: adding figures could be useful (Fig. 5);

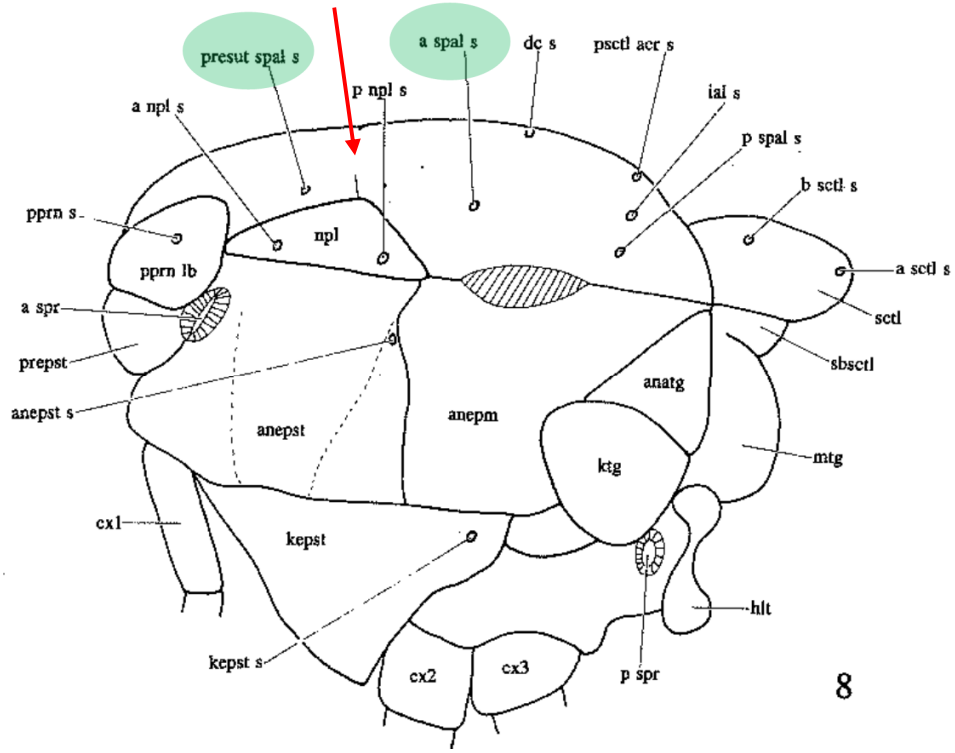
Fig. 5 - *Bactrocera zonata*, wing: cells bm and br are highlighted because of diagnostic importance (© A. Taddei, Anses)



- at couplet 7, the word “spot” is missing at the end of the first description and should be added.

In addition, operators identified that in the description of the thorax (page 413), the same pair of bristles is included twice and named with two synonyms (anterior supra-alar bristles and postsutural supra-alar bristles) (Fig. 6).

Fig. 6 - Schematic representation of Tephritidae thorax, lateral view (from White & Elson-Harris, 1992): presutural supra-alar seta (presut spal s), anterior supra-alar seta (a spal s) are highlighted in green; the red arrow shows the thoracic suture. Anterior supra-alar bristles and postsutural supra-alar bristles are synonyms



6.2 Molecular tests

For the goal of species identification in animals and some protists the cytochrome c oxidase subunit 1 (*COI*) 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 and non-*Bactrocera* Tephritidae e.g. of the *Dacus* genus.

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.

Sufficient amount and quality of sample DNA is crucial when performing molecular tests. Unfortunately, it was not possible to attain this in the case of sample 4 of the specificity sample set, which was therefore excluded from the molecular study.

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

Appendix 1 – DNA barcoding of arthropods (sequencing of *COI* locus, LCO1490/HCO2198 and LepF/LepR 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 "*Bactrocera zonata* species complex" (taxid:317241). The results showed suitability of both primer sets (see Appendix 6) for identification of *B. zonata*, although we have to state that barcoding is a generic test including targets and non-targets.

The values obtained for **analytical specificity** met the expected values (Table 6).
Sequencing of the *COI* locus was able to fully discriminate all listed species. The test was found to be 100% inclusive for *B. zonata* from Egypt, India, Pakistan and Réunion Island. For the exclusivity, several non-targets (including *Bactrocera* species: *B. albistrigata*, *B. correcta*, *B. dorsalis s.l.*, *B. latifrons*, *B. oleae*, and Dacus species: *D. bivittatus*, *D. ciliatus*, *D. etiennellus*, *D. punctatifrons*, *Zeugodacus cucurbitae*) could be distinguished (see Appendix 7).

The **analytical sensitivity** with both primer sets also easily met the expected value of 4 ng/µl.
The **reproducibility of the PCR** tests using two different primer sets and **reproducibility of the SANGER sequence analysis** were 100% in all cases. The same is true for the **repeatability**, reaching 100% (Table 6).

The **diagnostic sensitivity and specificity** as well as **accuracy** were 100% for both validated tests (Table 6).

Appendix 6 displays the results of the *in silico* testing of analytical specificity.
Appendix 7 of this document shows the detailed results for analytical specificity.
Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.
Appendix 9 shows the calculations for the diagnostic sensitivity, specificity and accuracy.

Table 6: Summary of the results obtained for the molecular protocol – EPPO PM7/129 (2), Appendix 1, *COI* gene locus.

Performance criteria	Definition	Calculation	Expected performance characteristics PM7/129 (EPPO, 2021)	Obtained performance characteristics for sequencing of *COI* (primer set LCO1490/HCO2198)	Obtained performance characteristics for sequencing of *COI* (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)	**100%**	**100%**	**100%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100% (Egypt India Pakistan Réunion Island)**	**100% (Egypt India Pakistan Réunion Island)**
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	**100% *(Bactrocera albistrigata Bactrocera correcta Bactrocera dorsalis s.l. Bactrocera latifrons Bactrocera oleae Dacus bivittatus Dacus ciliatus Dacus etiennellus Dacus punctatifrons Zeugodacus cucurbitae)***	**100% *(Bactrocera albistrigata Bactrocera correcta Bactrocera dorsalis s.l. Bactrocera latifrons Bactrocera oleae Dacus bivittatus Dacus ciliatus Dacus etiennellus Dacus punctatifrons Zeugodacus cucurbitae)***
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**	**0.1ng/µl**	**0.1ng/µl**
Repeatability	The level of agreement between replicates of a sample tested under the same conditions	% level of agreement	**100%**	**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%**	**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%**	**100%**	**100%**

Pest-specific real-time PCR according to Koohkanzade *et al.* 2018.

The *Bactrocera zonata*-specific real-time PCR according to Koohkanzade *et al.* (2018) was included in this validation study and performed according to the parameters given in Appendix 3.

***In silico* testing of analytical specificity** by a database alignment (NCBI GenBank) was performed with the primer/probe set (BzonF/BzonR/BzonP). The primers and probe for the real-time PCR (BzonF/BzonR/BzonP) were aligned without restricted search set. *In silico* specificity could be shown (see Appendix 6).

Analytical specificity: The test was found to be 100% inclusive for *B. zonata* from Egypt, India, Pakistan and Réunion Island. For the exclusivity several non-targets were tested (including Bactrocera species: *B. albistrigata*, *B. correcta*, *B. dorsalis s.l.*, *B. latifrons*, *B. oleae*, and Dacus species: *D. bivittatus*, *D. ciliatus*, *D. etiennellus*, *D. punctatifrons*, *Zeugodacus cucurbitae*). As expected all non-targets did not result in any signal (Table 7).

The **analytical sensitivity** in the original paper was given as 1.4pg/µl, with the results of this study being in the same order of magnitude (Ø 1.69pg/µl). It has to be mentioned, that for the calculation of the analytical sensitivity for the real-time PCR, the sample consisting of one leg only (EURL_Pool) was not considered. This single leg could however still be detected up to a $1:10^3$ dilution (corresponding to approx. 16pg DNA).
Reproducibility and repeatability of the real-time PCR both met the expected 100% (Table 7).

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

Appendix 6 displays the results of the *in silico* testing of analytical specificity.
Appendix 7 of this document shows the detailed results for analytical specificity.
Appendix 8 shows the results for analytical sensitivity, repeatability and reproducibility.
Appendix 9 shows the calculations for the diagnostic sensitivity, specificity and accuracy.

Table 7: Summary of the results obtained for the molecular test real-time PCR according to Koohkanzade *et al.* (2018)

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%**
Analytical specificity	**Inclusivity:** The performance of a test with a range of target organisms covering genetic diversity, different geographical origin and hosts	-	**100%**	**100% (Egypt India Pakistan Réunion Island)**
	Exclusivity: The performance of a test with regards to cross-reaction with a range of non-targets (e.g. closely related organisms)	-	**100%**	***100% Bactrocera albistrigata Bactrocera correcta Bactrocera dorsalis s.l. Bactrocera latifrons Bactrocera oleae Dacus bivittatus Dacus ciliatus Dacus etiennellus Dacus punctatifrons Zeugodacus cucurbitae***
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	-	**1.4pg/µl**	**1.69pg/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%**	**100%**

7. Discussion and conclusions

This study aimed at the validation of the EPPO diagnostic protocol for the morphological identification of *Bactrocera zonata*. For the molecular identification of *Bactrocera zonata*, the EPPO PM 7/129(2) DNA barcoding as an identification tool for a number of regulated pests, and the pest-specific real-time PCR according to Koohkanzade *et al.* (2018) were validated. 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 November to December 2020 and from May to August 2021 for the morphological and molecular parts respectively. A main sample set of 30 Tephritidae specimens, including target and non-target species, has been used. Additionally, smaller sample sets have been prepared to validate the molecular tests.

Morphological diagnostic test

The morphological identification of adult specimens according to the diagnostic protocol EPPO PM7/ 114 (1) *Bactrocera zonata* achieved the expected value of 100% for all validation criteria diagnostic specificity, diagnostic sensitivity, accuracy, repeatability and repeatability. The test was inclusive for *B. zonata* specimens originated from different countries (Egypt, India, Pakistan and Réunion Island) and exclusion for a number of non-target specimens belonging to the genera *Bactrocera*, *Zeugodacus* and *Dacus*. Therefore, no critical points in the dichotomous key (page 416) were identified that may be prone to misinterpretation and consequently that may lead the user to a wrong identification. However, the need for minor corrections and improvements of the protocol key was identified. To summarize,

- figures for important diagnostic characters used in the key should be added. Those characters are
  - the fusion of the abdominal segments (fused and not fused) at couplet 2;
  - the location of thoracic setae at couplet 4;
  - location of wing cells at couplet 7;
- the possibility of 4 marginal setae should be included in couplet 3. If not, no suitable option is available for the user in the case of 4 marginal setae, as it might be the case for *Zeugodacus cucurbitae*. Possible modifications (*in italics*) of the couplet might be the following

  "3 Scutellum not bilobed and with 2 marginal *(apical)* setae
  3* Scutellum bilobed *or with 4 marginal setae*";

- the word "spot" is missing at the end of sentence in couplet 7 and should be added;
- "postsutural supra-alar bristles present" should be removed from thorax description (page 413) as these bristles are already mentioned and named as anterior supra-alar seta (according to nomenclature given in White & Elson-Harris, 1992)
- either "bristles" or "setae" should be chosen and adopted throughout the document for consistency, as both identify the same morphological structures in this context.

Based on these results, the EURL recommends the use of the EPPO PM7/ 114 (1) (EPPO, 2013) to EU National Reference Laboratories for the morphological identification of *Batrocera zonata* adult specimens. Nevertheless, the diagnostic protocol may be improved for a better, stand-alone usability.

Molecular diagnostic tests

In routine diagnosis, especially when dealing with larvae e.g. in the frame of import control, molecular tests are sometimes a suitable method for rapid identification. Therefore, the EPPO PM7/129 DNA barcoding standard (EPPO, 2021) was validated, as well as a *Bactrocera zonata*-specific real-time PCR (Koohkanzade *et al.* 2018).

Both molecular tests validated using the defined sample sets showed to be 100% specific for *B. zonata*. The validated barcoding primer sets and the real-time PCR proved to be sufficient sensitive to identify adults, pupae, larvae and even one leg of a specimen.
Nevertheless, the limit of detection of the real-time PCR did not meet the expected value, but was in the same order of magnitude. For comparability in the actual study, the sample consisting of one leg only was excluded from the analytical sensitivity calculation. It has to be mentioned, that the sample with lowest DNA yield (single leg) could be detected to $1:10^3$ dilution (corresponding to approx. 16pg DNA), readily enabling this test to be used on one Tephritidae leg in routine analysis.

Although the specific real-time PCR was shown to be very sensitive, subsequent testing revealed late unspecific amplification in some cases as observed with undiluted DNA extracts of entire larvae of *B. dorsalis* and *C. capitata*. Therefore, it is recommended

to use at least 1:10 dilutions (DNA) of suspicious Tephritidae for real-time PCR according to Koohkanzade *et al.* 2018, and to eventually implement cut-off values.

The level of agreement for repeatability, reproducibility and accuracy was 100% for both molecular tests.

The suitability of correct identification of important Tephritidae with barcoding could be shown under our conditions for *B. zonata,* and for other Tephritidae tested. The validated real-time PCR can be recommended for the pest-specific identification of *B. zonata* only.

Date:

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References

Abdulrazak A.S., Hadwan H.A., Hassan S.A., Aydan N.I., Mohammed A.K., Haider K.M. and Hussein S.A. (2016). New record of peach fruit fly *Bactrocera zonata* (Saunders) (Tephritidae: Diptera) in Iraq. Arab and Near East Plant Protection Newsletter,69, 4.

Azam K.M., Al-Ansari M.S.A. and Al-Raeesi A.A. (2004). Fruit flies of Oman with a new record of *Carpomya vasuviana* Costa (Diptera: Tephritidae). Research on Crops, 5, 274–277.

CABI (last consultation November 2021) Invasive Species compendium: *Bactrocera zonata* https://www.cabi.org/isc/datasheet/17694#B608AE06-2E46-4CA2-B492-EE434F02A602

Carey J.R. and Dowell R.V. (1989). Exotic fruit pests and California agriculture. California Agriculture, 43(3):38-40

EFSA (European Food Safety Authority), Schenk M., Mertens J., Delbianco A., Graziosi I. and Vos S. (2021). Pest survey card on *Bactrocera zonata*. EFSA supporting publication 2021:EN-1999. 28pp.

Egartner, A., Lethmayer, C., Gottsberger, R. A., & Blümel, S. (2019). Survey on *Bactrocera* spp. (Tephritidae, Diptera) in Austria. EPPO Bulletin, 49(3), 578-584.

EPPO (2013). PM 7/114(1) Diagnostics. *Bactrocera zonata*. *Bulletin OEPP/EPPO Bulletin*, *44*(3), 390-399.

EPPO (2014). PM 7/122(1) Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. *Bulletin OEPP/EPPO Bulletin*, *44*(3), 390-399.

EPPO (2018) EPPO standards PM 7/76 (5) Diagnostics. Use of EPPO Diagnostic Standards. EPPO Bulletin 48 (3), 373–377.

EPPO (2019) EPPO standards PM 7/98 (4) Diagnostics. Specifics requirements for laboratories preparing accreditation for plant pest diagnostic activity. EPPO Bulletin 49 (3), 530–563.

EPPO (2021a) Global Database: *Bactrocera zonata* (last consultation November 2021) https://gd.eppo.int/taxon/DACUZO

EPPO (2021b). PM 7/129(2) DNA barcoding as an identification tool for a number of regulated pests. EPPO Bulletin, 46, 501-537.

FDACS (Florida Department of Agriculture and Consumer Services) (2018). Pest Alert–A peach fruit fly, *Bactrocera zonata* (Saunders) (Tephritidae). Division of Plant Industry.
Available online: https://www.fdacs.gov/content/download/80866/file/PEST_ALERT_-_Peach_Fruit_Fly_Bactrocera_zonata.pdf

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.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences*, *103*(4), 968-971.

Koohkanzade, M., Zakiaghl, M., Dhami, M. K., Fekrat, L., Sadeghi Namaghi, H. (2018). Rapid identification of *Bactrocera zonata* (Dip.: Tephritidae) using TaqMan real-time PCR assay. PLoS ONE 13(10): e0205136.

Mahmoud M.E.E., Mohamed S.A., Ndlela S., Azrag A.G.A., Khamis F.M. and Ekesi S. (2020). Distribution, relative abundance, and level of infestation of the invasive peach fruit fly *Bactrocera zonata* (Saunders) (Diptera: Tephritidae) and its associated natural enemies in Sudan. Phytoparasitica, 48, 589–605.

Permalloo S., Seewooruthun S.I., Joomaye A., Soonnoo A.R., Gungah B., Unmole L., Boodram R. (1998). In: Proceedings of the 2nd annual meeting of Agricultural scientists, 1997. Food and Agricultural Research Council, Reduit, Mauritius . 203-210.

White, I. M. and Elson-Harris, M. M. (1992). *Fruit flies of economic significance: their identification and bionomics*. CAB international.

Appendix 1 – Composition of the sample set and codification

Sample codification	New codification	Country of collection	Host plant	Identification
1500586_1	**1**	India	*Mangifera indica*	***Bactrocera zonata***
1901508	**2**	France	-	*Bactrocera oleae*
1800117_1	**3**	Laos	*Syzygium samarangense*	*Bactrocera correcta*
1401020_2	**4**	Sri Lanka	*Momordica charantia*	*Dacus ciliatus*
1702536_1	**5**	Egypt	*Mangifera indica*	***Bactrocera zonata***
1900081	**6**	Laos	*Solanum melongena*	*Bactrocera latifrons*
1901012_1	**7**	Ivory Coast	*Solanum sp.*	*Dacus bivittatus*
1600172	**8**	Congo	*Capsicum annuum*	*Dacus punctatifrons*
1500326_2	**9**	Réunion Island	-	*Dacus ciliatus*
2001513_3	**10**	Benin	*Mangifera indica*	*Bactrocera dorsalis*
1600249_1	**11**	Thailand	*Ziziphus*	*Bactrocera correcta*
1500858_2	**12**	Egypt	*Mangifera indica*	***Bactrocera zonata***
1700152_1	**13**	Réunion Island	*Mangifera indica*	***Bactrocera zonata***
1800117_2	**14**	Laos	*Syzygium samarangense*	*Bactrocera correcta*
1901524	**15**	France	-	*Bactrocera oleae*
1701066	**16**	India	*Capsicum annuum*	*Bactrocera latifrons*
2001405_1	**17**	Pakistan	*Mangifera indica*	***Bactrocera zonata***
1200101_1	**18**	Mayotte	-	*Dacus etiennellus*
2001513_1	**19**	Benin	*Mangifera indica*	*Bactrocera dorsalis*
1400557_1	**20**	Thailand	-	*Bactrocera albistrigata*
1600249_2	**21**	Thailand	*Ziziphus*	*Bactrocera correcta*
2001055	**22**	India	*Coccinia grandis*	*Zeugodacus cucurbitae*
1901012_2	**23**	Ivory Coast	*Solanum sp.*	*Dacus bivittatus*
1500326_3	**24**	Réunion Island	-	*Dacus ciliatus*
1901854	**25**	France	-	*Bactrocera oleae*
2001405_2	**26**	Pakistan	*Mangifera indica*	***Bactrocera zonata***
201057	**27**	Sri Lanka	*Psidium guajava*	*Bactrocera dorsalis*
1800889	**28**	Cambodia	*Capsicum frutescens*	*Bactrocera latifrons*
2001513_2	**29**	Benin	*Mangifera indica*	*Bactrocera dorsalis*
1901055_1	**30**	Pakistan	*Mangifera indica*	***Bactrocera zonata***

Appendix 2 - Check lists for the morphological analysis

Operator		Date	

Key for identification of adult *B. zonata* (modified from key on page 416, EPPO PM 7/114 (1) *Bactrocera zonata*)

Key for identification of adult *B. zonata*		go to (mark the decision with Y (yes) or N (no); note any comments)										
Morphological character		**Sample code**										
1	**Subcostal vein** abruptly bent and dorsal side of vein R1 with setulae (Fig. 10)	…	…	…	…	…	…	…	…	…	…	**2 (Tephritidae)**
	Subcostal vein not abruptly bent or dorsal side of vein R1 lacks setulae	…	…	…	…	…	…	…	…	…	…	**Other families**
2	**Abdominal segments** not fused	…	…	…	…	…	…	…	…	…	…	**3**
	Abdominal segments fused	…	…	…	…	…	…	…	…	…	…	***Dacus* sp.**
3	**Scutellum** not bilobed and with 2 marginal setae (Fig. 7)	…	…	…	…	…	…	…	…	…	…	**4**
	Scutellum bilobed	…	…	…	…	…	…	…	…	…	…	**Other species**
4	**Scutum** with prescutellar acrostichal and anterior supra-alar setae and without medial orange vitta (Fig. 7). Male with pecten on tergite 3 (Fig. 8). *Bactrocera* (*Bactrocera*) group of subgenera	…	…	…	…	…	…	…	…	…	…	**5**
	Scutum different	…	…	…	…	…	…	…	…	…	…	**Other subgenera**

5	**Mesonotum** with two postsutural yellow vittae. Head with black markings	…	…	…	…	…	…	…	…	…	…	**6**
	Mesonotum with three postsutural yellow vittae	…	…	…	…	…	…	…	…	…	…	**Other species**
6	**Face** with a black spot in each antennal furrow (Fig. 5)	…	…	…	…	…	…	…	…	…	…	**7**
	Face with transverse dark markings (Fig. 11)	…	…	…	…	…	…	…	…	…	…	***B. correcta***
7	**Wing** without any cross band. Area of cell br immediately above cell bm without microtrichia. Costal band with only cell sc and apex of vein R4 + 5 coloured. Apex of costal band expanded into an elongate (Fig. 10).	…	…	…	…	…	…	…	…	…	…	**8**
	Wing different	…	…	…	…	…	…	…	…	…	…	**Other species**
8	**Scutellum** entirely pale coloured, except sometimes for a narrow black line across the base (Fig. 7)	…	…	…	…	…	…	…	…	…	…	**9**
	Dorsal surface of scutellum with a large black triangular mark, lateral and apical areas yellow	…	…	…	…	…	…	…	…	…	…	***B. psidii***
9	**Thorax and abdomen** pale orange-brown to red-brown (Fig. 4). **Apex of costal band** distinctly expanded into a spot.	…	…	…	…	…	…	…	…	…	…	***Bactrocera zonata***
	Thorax and abdomen black (if dark orange-brown then the **wing** without marking).	…	…	…	…	…	…	…	…	…	…	***Bactrocera tuberculata***
	Comments / Results											

Summary Results sheet for the morphological test EPPO PM7/114 (1) *Bactrocera zonata*

Operator	
Stereomicroscope	

Sample code	Identification result	Date of analysis	Notes
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Appendix 3 – Specifications and parameters for the molecular tests

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: 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
∑	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of 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	∞	

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
∑	9	
DNA	1	

PCR conditions:

	°C	Duration (min., sec.)	Nr. of 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 (Real-time PCR)

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

BzonF: 5'- AGCCACATTACATGGTACACAACT-3'
BzonR: 5'- AGGACAACTCCTGTTAATCCTCCT-3'
BzonP: FAM-CTCCAGCTATACTGTGGGCCCTAGGA-TQ2*
* TQ2: Tide Quencher™ 2 phosphoramidite

Literature: Koohkanzade, M., Zakiaghl, M., Dhami, M. K., Fekrat, L., Sadeghi Namaghi, H. (2018) Rapid identification of *Bactrocera zonata* (Dip.: Tephritidae) using TaqMan real-time PCR assay. PLoS ONE 13(10): e0205136. https://doi.org/10.1371/journal.pone.0205136

Fragment length: 100bp

PCR - Parameters:

Eppendorf realplex Mastercycler with accompanying software, Bio Molecular Systems Magnetic Induction Cycler (MIC) with accompanying software.

Mastermix: PerfeCTa qPCR ToughMix® Quanta Bio. Contains AccuStart II Taq DNA polymerase, AccuVue plate loading dye, MgCl2, dNTPs

Composition:		**Final concentration:**
	Volume per reaction µl	
Water	2	
Mastermix	5	1x
Primer1:	0.5	0.5µM
Primer2:	0.5	0.5µM
Probe	1	0.1µM
∑	9	
DNA	1	

PCR conditions:

Step	**°C**	**Duration (min., sec.)**	**Nr. of Cycles**
Start	95	10 min	1
Denaturation	95	15 sec	45
Annealing/Extension and fluorescence reading	63	63 sec	

Appendix 4 – Summary Results sheets with the results from the three operators (morphological analysis)

Operator 1	
Instrument	ME BIN 08
Date of analysis/identification	26/11/20 – 27/11/20

Sample number	Analysis/Identification	Notes	Expected result	Assigned value
1	**Positive**	/	Positive	*Bactrocera zonata*
2	**Negative**	/	Negative	*Bactrocera oleae*
3	**Negative**	/	Negative	*Bactrocera correcta*
4	**Negative**	/	Negative	*Dacus ciliatus*
5	**Positive**	/	Positive	*Bactrocera zonata*
6	**Negative**	/	Negative	*Bactrocera latifrons*
7	**Negative**	/	Negative	*Dacus bivittatus*
8	**Negative**	/	Negative	*Dacus punctatifrons*
9	**Negative**	/	Negative	*Dacus ciliatus*
10	**Negative**	/	Negative	*Bactrocera dorsalis*
11	**Negative**	/	Negative	*Bactrocera correcta*
12	**Positive**	/	Positive	*Bactrocera zonata*
13	**Positive**	/	Positive	*Bactrocera zonata*
14	**Negative**	/	Negative	*Bactrocera correcta*
15	**Negative**	/	Negative	*Bactrocera oleae*
16	**Negative**	/	Negative	*Bactrocera latifrons*
17	**Positive**	/	Positive	*Bactrocera zonata*
18	**Negative**	/	Negative	*Dacus etiennellus*
19	**Negative**	/	Negative	*Bactrocera dorsalis*
20	**Negative**	/	Negative	*Bactrocera albistrigata*
21	**Negative**	/	Negative	*Bactrocera correcta*
22	**Negative**	/	Negative	*Zeugodacus cucurbitae*
23	**Negative**	/	Negative	*Dacus bivittatus*
24	**Negative**	/	Negative	*Dacus ciliatus*
25	**Negative**	/	Negative	*Bactrocera oleae*
26	**Positive**	/	Positive	*Bactrocera zonata*
27	**Negative**	/	Negative	*Bactrocera dorsalis*
28	**Negative**	/	Negative	*Bactrocera latifrons*
29	**Negative**	/	Negative	*Bactrocera dorsalis*
30	**Positive**	/	Positive	*Bactrocera zonata*

Operator 2	
Instrument	LEICA M205 c
Date of analysis/identification	20/11/20 – 01/12/20

Sample number	Analysis/ Identification_ 1	Analysis/ Identification_ 2	Analysis/ Identification_ 3	Notes	Expected result	Assigned value
1	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
2	**Negative**	**Negative**	**Negative**	*B. oleae*	Negative	*Bactrocera oleae*
3	**Negative**	**Negative**	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
4	**Negative**	**Negative**	**Negative**	?	Negative	*Dacus ciliatus*
5	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
6	**Negative**	**Negative**	**Negative**	*B. latifrons*	Negative	*Bactrocera latifrons*
7	**Negative**	**Negative**	**Negative**	*Dacus (bivittatus ?)?*	Negative	*Dacus bivittatus*
8	**Negative**	**Negative**	**Negative**	*Dacus punctatifrons*	Negative	*Dacus punctatifrons*
9	**Negative**	**Negative**	**Negative**	*Dacus ciliatus*	Negative	*Dacus ciliatus*
10	**Negative**	**Negative**	**Negative**	*Bactrocera sp.*	Negative	*Bactrocera dorsalis*
11	**Negative**	**Negative**	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
12	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
13	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
14	**Negative**	**Negative**	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
15	**Negative**	**Negative**	**Negative**	*B. oleae*	Negative	*Bactrocera oleae*
16	**Negative**	**Negative**	**Negative**	*B. latifrons*	Negative	*Bactrocera latifrons*
17	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
18	**Negative**	**Negative**	**Negative**	*Dacus (demmerezi?)*	Negative	*Dacus etiennellus*
19	**Negative**	**Negative**	**Negative**	*Bactrocera dorsalis complex*	Negative	*Bactrocera dorsalis*
20	**Negative**	**Negative**	**Negative**	*B. albistrigata* Doesn't key out at 6	Negative	*Bactrocera albistrigata*
21	**Negative**	**Negative**	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
22	**Negative**	**Negative**	**Negative**	*Z. cucurbitae*	Negative	*Zeugodacus cucurbitae*
23	**Negative**	**Negative**	**Negative**	*Dacus bivittatus*	Negative	*Dacus bivittatus*
24	**Negative**	**Negative**	**Negative**	*Dacus ciliatus*	Negative	*Dacus ciliatus*
25	**Negative**	**Negative**	**Negative**	*B. oleae*	Negative	*Bactrocera oleae*
26	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
27	**Negative**	**Negative**	**Negative**	*? acrostical setae?*	Negative	*Bactrocera dorsalis*
28	**Negative**	**Negative**	**Negative**	*B. latifrons*	Negative	*Bactrocera latifrons*
29	**Negative**	**Negative**	**Negative**	*B. dorsalis* complex	Negative	*Bactrocera dorsalis*
30	**Positive**	**Positive**	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*

Operator 3	
Instrument	ZEISS Stemi 2000-C
Date of analysis/identification	10/12/20 – 11/12/20

Sample number	Analysis/Identification	Notes	Expected result	Assigned value
1	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
2	**Negative**	/	Negative	*Bactrocera oleae*
3	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
4	**Negative**	*Dacus* sp.	Negative	*Dacus ciliatus*
5	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
6	**Negative**	/	Negative	*Bactrocera latifrons*
7	**Negative**	*Dacus sp.*	Negative	*Dacus bivittatus*
8	**Negative**	*Dacus sp.*	Negative	*Dacus punctatifrons*
9	**Negative**	*Dacus sp.*	Negative	*Dacus ciliatus*
10	**Negative**	/	Negative	*Bactrocera dorsalis*
11	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
12	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
13	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
14	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
15	**Negative**	/	Negative	*Bactrocera oleae*
16	**Negative**	/	Negative	*Bactrocera latifrons*
17	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
18	**Negative**	/	Negative	*Dacus etiennellus*
19	**Negative**	/	Negative	*Bactrocera dorsalis*
20	**Negative**	/	Negative	*Bactrocera albistrigata*
21	**Negative**	*B. correcta*	Negative	*Bactrocera correcta*
22	**Negative**	/	Negative	*Zeugodacus cucurbitae*
23	**Negative**	*Dacus sp.*	Negative	*Dacus bivittatus*
24	**Negative**	*Dacus sp.*	Negative	*Dacus ciliatus*
25	**Negative**	/	Negative	*Bactrocera oleae*
26	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*
27	**Negative**	/	Negative	*Bactrocera dorsalis*
28	**Negative**	/	Negative	*Bactrocera latifrons*
29	**Negative**	/	Negative	*Bactrocera dorsalis*
30	**Positive**	*B. zonata*	Positive	*Bactrocera zonata*

Sensitivity, specificity, accuracy :

Diagnostic sensitivity, specificity and accuracy is assessed on the basis of the analysis of the whole set carried out by operator 2 (Anses)

Operator_2_R1

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

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

		Expected result	
		positive	negative
Operator result	positive	7	0
	negative	0	23

Sensitivity	**100%**
Specificity	**100%**
Accuracy	**100%**

Repeatability : Operator_2_R1, Operator_2_R2, Operator_2_R3

Repeatability is assessed on the basis of the analysis of the whole set carried out by operator 2 (Anses) (three repetitions of analysis).

Operator_2_R1, Operator_2_R2, Operator_2_R3

Expressed as % level of agreement among repetitions by Operator 2

Sample code	Repetitions	Operator3_R 1	Operator3_R 2	Operator3_R 3	Agreement	Disagreement	Level of agreement %
1	3	Positive	Positive	Positive	3	0	100
2	3	Negative	Negative	Negative	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	Negative	Negative	Negative	3	0	100
10	3	Negative	Negative	Negative	3	0	100
11	3	Negative	Negative	Negative	3	0	100
12	3	Positive	Positive	Positive	3	0	100
13	3	Positive	Positive	Positive	3	0	100
14	3	Negative	Negative	Negative	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	Negative	Negative	Negative	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	Negative	Negative	Negative	3	0	100
25	3	Negative	Negative	Negative	3	0	100
26	3	Positive	Positive	Positive	3	0	100
27	3	Negative	Negative	Negative	3	0	100
28	3	Negative	Negative	Negative	3	0	100
29	3	Negative	Negative	Negative	3	0	100
30	3	Positive	Positive	Positive	3	0	100
	90				90	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 (Anses, first of the three repetitions of analysis in the case of operator 2) and 3 (AGES).

Operator_1, Operator_2_R1, Operator_3

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

Sample code	Operator1	Operator2_R1	Operator 3	Repetitions	Agreement	Disagreement	Level of agreement %
1	Positive	Positive	Positive	3	3	0	100
2	Negative	Negative	Negative	3	3	0	100
3	Negative	Negative	Negative	3	3	0	100
4	Negative	Negative	Negative	3	3	0	100
5	Positive	Positive	Positive	3	3	0	100
6	Negative	Negative	Negative	3	3	0	100
7	Negative	Negative	Negative	3	3	0	100
8	Negative	Negative	Negative	3	3	0	100
9	Negative	Negative	Negative	3	3	0	100
10	Negative	Negative	Negative	3	3	0	100
11	Negative	Negative	Negative	3	3	0	100
12	Positive	Positive	Positive	3	3	0	100
13	Positive	Positive	Positive	3	3	0	100
14	Negative	Negative	Negative	3	3	0	100
15	Negative	Negative	Negative	3	3	0	100
16	Negative	Negative	Negative	3	3	0	100
17	Positive	Positive	Positive	3	3	0	100
18	Negative	Negative	Negative	3	3	0	100
19	Negative	Negative	Negative	3	3	0	100
20	Negative	Negative	Negative	3	3	0	100
21	Negative	Negative	Negative	3	3	0	100
22	Negative	Negative	Negative	3	3	0	100
23	Negative	Negative	Negative	3	3	0	100
24	Negative	Negative	Negative	3	3	0	100
25	Negative	Negative	Negative	3	3	0	100
26	Positive	Positive	Positive	3	3	0	100
27	Negative	Negative	Negative	3	3	0	100
28	Negative	Negative	Negative	3	3	0	100
29	Negative	Negative	Negative	3	3	0	100
30	Positive	Positive	Positive	3	3	0	100
				90	90	0	**100**

Reproducibility **100%**

Appendix 6 – *In silico* testing of analytical specificity with DNA barcoding and real-time primer sets

In silico testing of analytical specificity by a database alignment (NCBI GenBank) was performed (11.08.2021) with the DNA barcoding primer sets (LCO1490/HCO2198 and LepF/LepR). The search set was limited to "*Bactrocera zonata* species complex (taxid:317241)". The results showed suitability of both primer sets (see Fig. A-D) for identification of several *Bactrocera* spp., although we have to state that barcoding is a generic test including targets and non-targets.

Distance trees of results from BLAST search were created with organism search set to *Bactrocera zonata* species complex with single primers (LepF, LepR, LCO1490, HCO2198).

Figure A: Phylogenetic tree for LepF constructed with the fast minimum evolution method by blast tree viewer.

Figure B: Phylogenetic tree for LepR constructed with the fast minimum evolution method by blast tree viewer.

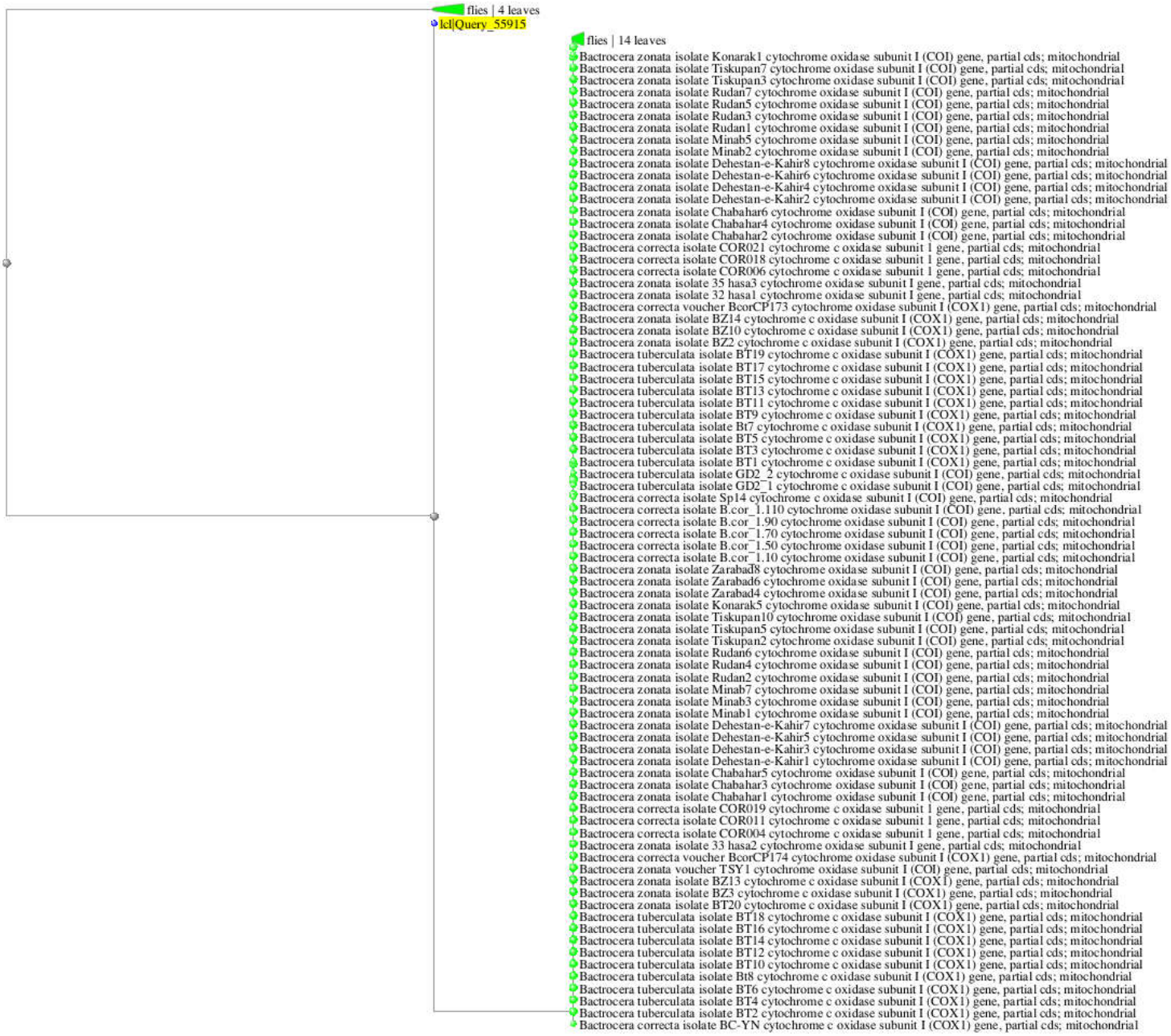
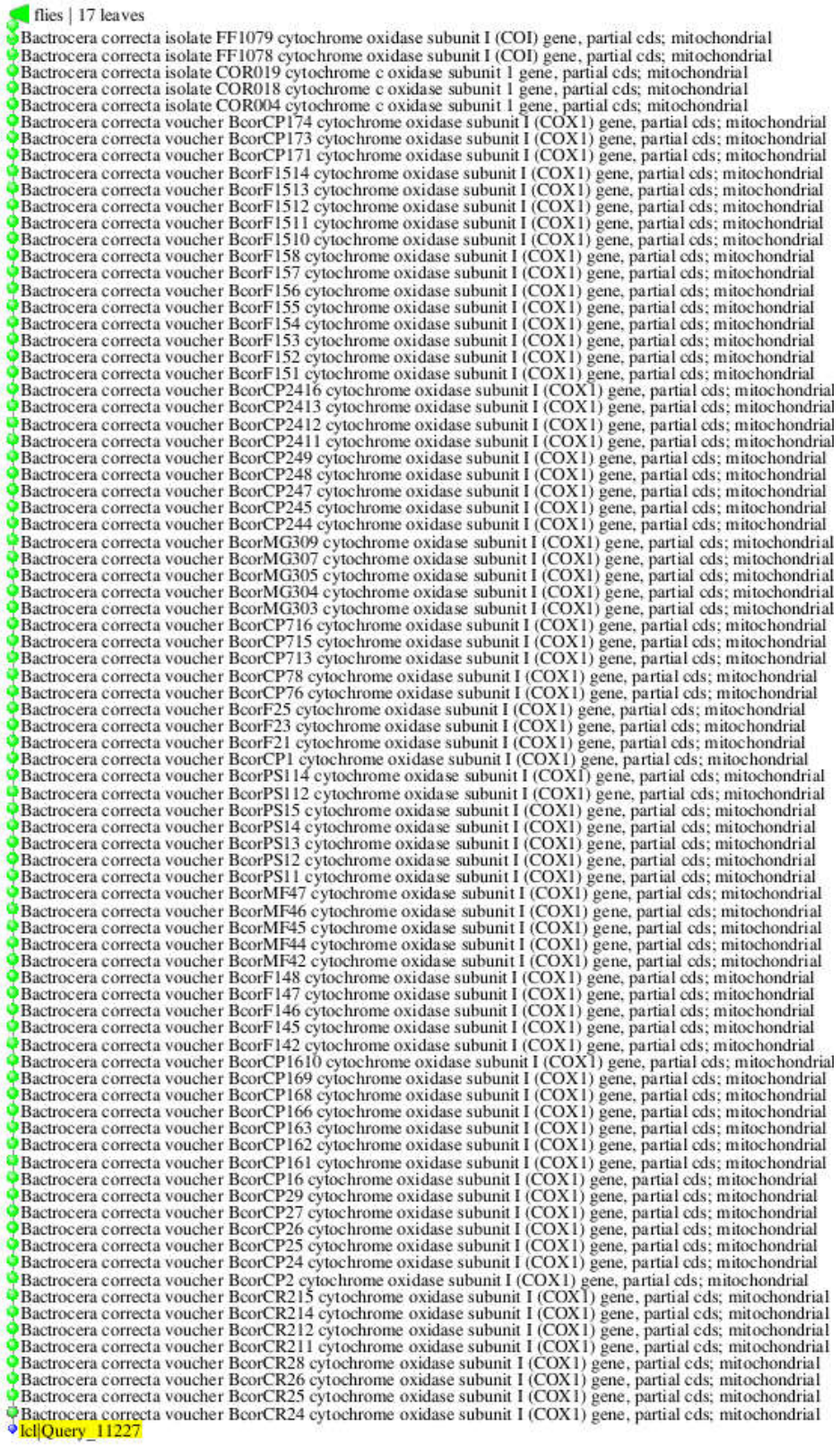


Figure C: Phylogenetic tree for LCO1490 constructed with the fast minimum evolution method by blast tree viewer.

Figure D: Phylogenetic tree for HCO2198 constructed with the fast minimum evolution method by blast tree viewer.



***In silico* testing of analytical specificity** by a database alignment (NCBI GenBank) was performed (11.08.2021) with the primer/probe set (BzonF/BzonR/BzonP). The primers and probe for the real-time PCR (BzonF/BzonR/BzonP) were aligned without restricted search set (Fig. E –G).

Figure E: Phylogenetic tree for BzonF constructed with the fast minimum evolution method by blast tree viewer.

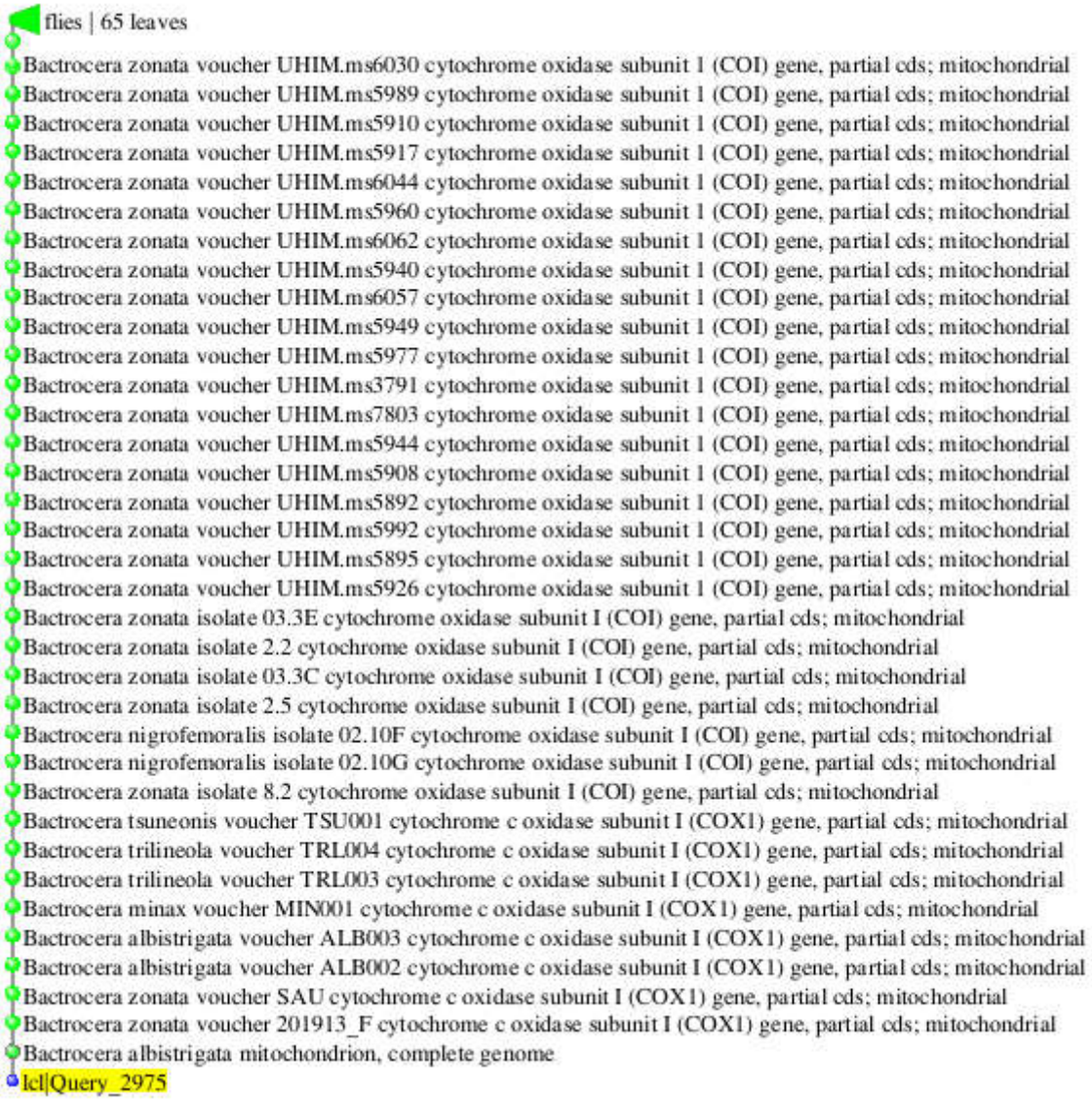


Figure F: Phylogenetic tree for BzonR constructed with the fast minimum evolution method by blast tree viewer.

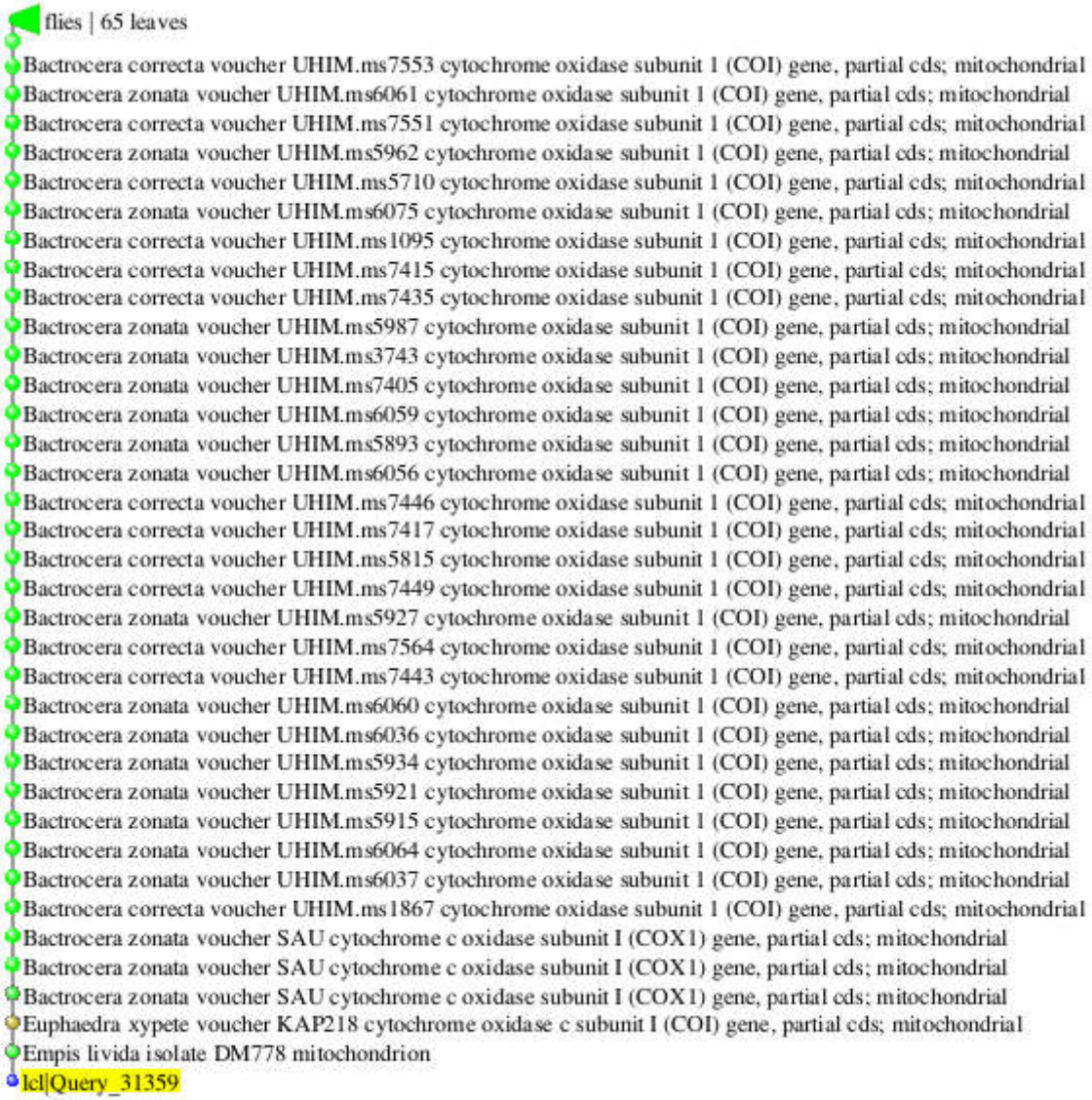
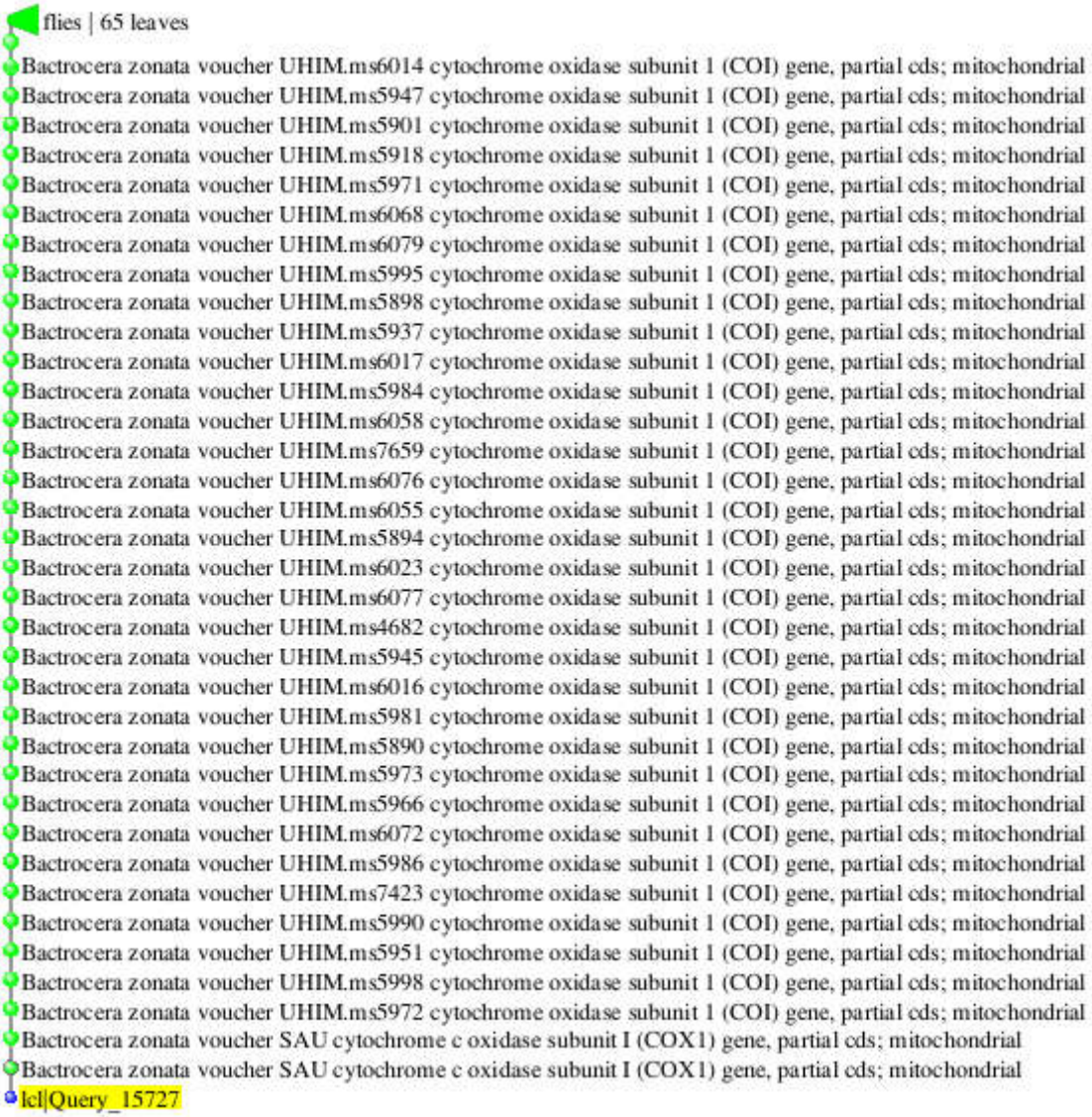


Figure G: Phylogenetic tree for BzonP constructed with the fast minimum evolution method by blast tree viewer.



Appendix 7 – Results of the analytical specificity with DNA barcoding and real-time PCR

Table A: Results of the analytical specificity with DNA barcoding (both primer sets) and real-time PCR

	EPPO PM7/129(2) (LCO1490/HCO2198)		**EPPO PM7/129(2) (LepF/LepR)**		**Real-time (Koohkanzade *et al.* 2018)**		
Sample Nb.	**Result**	**Note**	**Result**	**Note**	**Result**	**Expected result**	**Assigned value**
1	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
2	Negative	*Bactrocera oleae*	Negative	*Bactrocera oleae*	Negative	Negative	*Bactrocera oleae*
3	Negative	*Bactrocera correcta*	Negative	*Bactrocera correcta*	Negative	Negative	*Bactrocera correcta*
4		*excluded*		*excluded*			*excluded*
5	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
6	Negative	*Bactrocera latifrons*	Negative	*Bactrocera latifrons*	Negative	Negative	*Bactrocera latifrons*
7	Negative	*Dacus bivittatus*	Negative	*Dacus bivittatus*	Negative	Negative	*Dacus bivittatus*
8	Negative	*Dacus punctatifrons*	Negative	*Dacus punctatifrons*	Negative	Negative	*Dacus punctatifrons*
9	Negative	*Dacus ciliatus*	Negative	*Dacus ciliatus*	Negative	Negative	*Dacus ciliatus*
10	Negative	*Bactrocera dorsalis*	Negative	*Bactrocera dorsalis*	Negative	Negative	*Bactrocera dorsalis*
11	Negative	*Bactrocera correcta*	Negative	*Bactrocera correcta*	Negative	Negative	*Bactrocera correcta*
12	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
13	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
14	Negative	*Bactrocera correcta*	Negative	*Bactrocera correcta*	Negative	Negative	*Bactrocera correcta*
15	Negative	*Bactrocera oleae*	Negative	*Bactrocera oleae*	Negative	Negative	*Bactrocera oleae*
16	Negative	*Bactrocera latifrons*	Negative	*Bactrocera latifrons*	Negative	Negative	*Bactrocera latifrons*
17	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
18	Negative	*Dacus etiennellus*	Negative	*Dacus etiennellus*	Negative	Negative	*Dacus etiennellus*
19	Negative	*Bactrocera dorsalis*	Negative	*Bactrocera dorsalis*	Negative	Negative	*Bactrocera dorsalis*
20	Negative	*Bactrocera albistrigata*	Negative	*Bactrocera albistrigata*	Negative	Negative	*Bactrocera albistrigata*
21	Negative	*Bactrocera correcta*	Negative	*Bactrocera correcta*	Negative	Negative	*Bactrocera correcta*
22	Negative	*Zeugodacus cucurbitae*	Negative	*Zeugodacus cucurbitae*	Negative	Negative	*Zeugodacus cucurbitae*
23	Negative	*Dacus bivittatus*	Negative	*Dacus bivittatus*	Negative	Negative	*Dacus bivittatus*
24	Negative	*Dacus ciliatus*	Negative	*Dacus ciliatus*	Negative	Negative	*Dacus ciliatus*
25	Negative	*Bactrocera oleae*	Negative	*Bactrocera oleae*	Negative	Negative	*Bactrocera oleae*
26	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*
27	Negative	*Bactrocera dorsalis*	Negative	*Bactrocera dorsalis*	Negative	Negative	*Bactrocera dorsalis*
28	Negative	*Bactrocera latifrons*	Negative	*Bactrocera latifrons*	Negative	Negative	*Bactrocera latifrons*
29	Negative	*Bactrocera dorsalis*	Negative	*Bactrocera dorsalis*	Negative	Negative	*Bactrocera dorsalis*
30	Positive	*Bactrocera zonata*	Positive	*Bactrocera zonata*	Positive	Positive	*Bactrocera zonata*

Appendix 8 – Summary result sheets for analytical sensitivity, repeatability and reproducibility (molecular tests)

Sample panel analytical sensitivity and repeatability:
Sample 955/20: 1 adult specimen (female) of *B. zonata*
Sample 956/20: 1 adult specimen (male) of *B. zonata*
Sample 957/20: 1 larva of *B. zonata*
Sample 958/20: 1 pupa of *B. zonata*
Sample EURL_Pool: 1 leg of *B. zonata*

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 B).

Analytical sensitivity and repeatability:
5 samples were prepared in different dilutions (1:100, 1:1000; 1:10.000; 1:100.000, 1:1.000.000, 1:10.000.000) and PCRs with both barcoding primer sets, as well as the real-time PCR according to Koohkanzade *et al.* (2018) were performed in three technical repetitions per sample (Tables C, D, F and G).
Barcoding amplicons at the detection limit and the last dilution step before the detection limit were sent for SANGER sequencing. The quality of sequences was assessed by the length of the consensus sequences and % of high quality bases (%HQ), see Table E.

Sample panel reproducibility:

Targets

Bactrocera zonata (956/20, adult male)
Bactrocera zonata (957/20, larva)
Bactrocera zonata (958/20, pupa)

Non-targets

Bactrocera correcta (2539/20, leg)
Bactrocera latifrons (2542/20, leg)
Dacus bivittatus (2543/20, leg)

Results for analytical sensitivity (DNA barcoding)

Table B. – Extracted DNA concentration and PCR sensitivity for *B. zonata* sample panel used for sensitivity testing (DNA barcoding)

	DNA Concentration [ng/µl]					EPPO PM7/129	
Sample Nb. & Developmental Stage of *B. zonata*	Dilution	Repetition 1	Repetition 2	Repetition 3	Mean ± SD	Amplicons (LCO1490/HCO2198)	Amplicons (LepF/LepR)
955/20 (adult female)	Undiluted	212.5	212.4	212.7	212.53 ± 0.12	Strong	Strong
	1:10	20.0	20.4	20.2	20.20 ± 0.16	Strong	Strong
	1:100	1.7	1.0	2.1	1.60 ± 0.45	Strong	Strong
	1:1.000	N/A	N/A	N/A		Strong	Strong
	1:10.000	N/A	N/A	N/A		Weak	Strong
	1:100.000	N/A	N/A	N/A		Negative	Weak
	1:1.000.000	N/A	N/A	N/A		Negative	Negative
	1:10.000.000	N/A	N/A	N/A		Negative	Negative
956/20 (adult male)	Undiluted	59.3	59.8	59.5	59.53 ± 0.21	Strong	Strong
	1:10	5.2	4.7	5.5	5.13 ± 0.33	Strong	Strong
	1:100	N/A	N/A	N/A		Strong	Strong
	1:1.000	N/A	N/A	N/A		Strong	Strong
	1:10.000	N/A	N/A	N/A		Weak	Strong
	1:100.000	N/A	N/A	N/A		Weak	Weak
	1:1.000.000	N/A	N/A	N/A		Negative	Negative
	1:10.000.000	N/A	N/A	N/A		Negative	Negative
957/20 (larva)	Undiluted	304.8	309.7	311.0	308.50 ± 2.67	Strong	Strong
	1:10	30.6	30.7	30.8	30.70 ± 0.08	Strong	Strong
	1:100	2.9	2.2	1.6	2.23 ± 0.53	Strong	Strong
	1:1.000	N/A	N/A	N/A		Strong	Strong
	1:10.000	N/A	N/A	N/A		Weak	Strong
	1:100.000	N/A	N/A	N/A		Weak	Weak
	1:1.000.000	N/A	N/A	N/A		Negative	Negative
	1:10.000.000	N/A	N/A	N/A		Negative	Negative
958/20 (pupa)	Undiluted	288.6	285.7	289.7	288.00 ± 1.69	Strong	Strong
	1:10	28.0	27.7	28.5	28.07 ± 0.33	Strong	Strong
	1:100	2.0	2.1	1.8	1.97 ± 0.12	Strong	Strong
	1:1.000	N/A	N/A	N/A		Strong	Strong
	1:10.000	N/A	N/A	N/A		Weak	Weak
	1:100.000	N/A	N/A	N/A		Negative	Negative
	1:1.000.000	N/A	N/A	N/A		Negative	Negative
	1:10.000.000	N/A	N/A	N/A		Negative	Negative
EURL Pool (leg)	Undiluted	15.9	13.9	14.6	14.80 ± 0.83	Strong	Strong
	1:10	1.2	1.4	1.8	1.47 ± 0.22	Strong	Strong
	1:100	N/A	N/A	N/A		Strong	Strong
	1:1.000	N/A	N/A	N/A		Weak	Weak
	1:10.000	N/A	N/A	N/A		Negative	Negative
	1:100.000	N/A	N/A	N/A		Negative	Negative
	1:1.000.000	N/A	N/A	N/A		Negative	Negative
	1:10.000.000	N/A	N/A	N/A		Negative	Negative

N/A: not validly measurable

Results for repeatability (DNA barcoding)

Three replicates of *B. zonata* (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 Tables C and D.

Table C: Amplicon generation for DNA barcoding PCR repeatability test, primer set LCO1490/HCO2198

			Amplicon generation		
Test	Sample Nb. & Developmental Stage of *B. zonata*	Dilution	Repetition 1	Repetition 2	Repetition 3
EPPO PM7/129 (LCO1490/HCO2198)	955/20 (adult female)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Weak	Weak	Strong
		1:100.000	Negative	Negative	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	956/20 (adult male)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Weak	Weak	Weak
		1:100.000	Weak	Weak	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	957/20 (larva)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
		1:100.000	Weak	Weak	Weak
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	958/20 (pupa)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Negative	Weak	Weak
		1:100.000	Negative	Negative	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	EURL Pool (leg)	1:100	Strong	Strong	Strong
		1:1.000	Weak	Weak	Negative
		1:10.000	Negative	Negative	Negative
		1:100.000	Negative	Negative	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative

Table D: Amplicon generation for DNA barcoding PCR repeatability test, primer set LepF/LepR

			Amplicon generation		
Test	Sample Nb. & Developmental Stage of *B. zonata*	Dilution	Repetition 1	Repetition 2	Repetition 3
EPPO PM7/129 (LepF/LepR)	955/20 (adult female)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
		1:100.000	Weak	Weak	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	956/20 (adult male)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
		1:100.000	Weak	Strong	Weak
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	957/20 (larva)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Strong	Strong	Strong
		1:100.000	Weak	Strong	Weak
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	958/20 (pupa)	1:100	Strong	Strong	Strong
		1:1.000	Strong	Strong	Strong
		1:10.000	Weak	Weak	Weak
		1:100.000	Negative	Negative	Weak
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative
	EURL Pool (leg)	1:100	Strong	Strong	Strong
		1:1.000	Weak	Weak	Weak
		1:10.000	Weak	Negative	Negative
		1:100.000	Negative	Negative	Negative
		1:1.000.000	Negative	Negative	Negative
		1:10.000.000	Negative	Negative	Negative

Table E. Sequence quality criteria for *B. zonata* sample panel used for sensitivity testing (DNA barcoding)

Test	Sample Nb. & Developmental Stage of *B. zonata*	Dilution	Approx. Consensus Length (bp)	High Quality (HQ%) of Consensus	Calculated DNA Concentration [ng/µl]
EPPO PM7/129 (LCO1490/ HCO2198)	955/20 (adult female)	1:10.000	658bp	100	21.25 pg/µl
	956/20 (adult male)	1:10.000	658bp	100	5.95 pg/µl
	957/20 (larva)	1:10.000	658bp	100	30.85 pg/µl
	958/20 (pupa)	1:1.000	658bp	100	288 pg/µl
	EURL Pool (leg)	1:100	658bp	100	158 pg/µl
EPPO PM7/129 (LepF/LepR)	955/20 (adult female)	1:10.000	658bp	100	21.25 pg/µl
	956/20 (adult male)	1:10.000	658bp	100	5.95 pg/µl
	957/20 (larva)	1:10.000	658bp	100	30.85 pg/µl
	958/20 (pupa)	1:1.000	658bp	100	288 pg/µl
	EURL Pool (leg)	1:100	658bp	100	158 pg/µl

Results for analytical sensitivity and repeatability of the real-time PCR

Table F: Real-time PCR results for operator 1

			Ct value		
Test	Sample Nb. & Developmental Stage of *B. zonata*	Dilution	Repetition 1	Repetition 2	Repetition 3
Real-time PCR according Koohkanzade *et al.* 2018	955/20 (adult female)	1:100	-	22.50	22.62
		1:1.000	26.30	25.96	26.24
		1:10.000	31.11	30.87	30.61
		1:100.000	34.93	35.92	34.02
		1:1.000.000	38.05	38.84	36.27
		1:10.000.000	-	-	-
	956/20 (adult male)	1:100	23.43	-	23.74
		1:1.000	27.34	26.85	27.53
		1:10.000	31.15	31.82	31.81
		1:100.000	34.60	35.98	37.18
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-
	957/20 (larva)	1:100	21.92	22.62	22.08
		1:1.000	25.69	26.67	25.88
		1:10.000	29.87	29.81	29.58
		1:100.000	35.96	35.05	35.16
		1:1.000.000	38.69	41.27	-
		1:10.000.000	-	-	-
	958/20 (pupa)	1:100	24.31	23.89	23.51
		1:1.000	27.76	27.53	27.65
		1:10.000	31.97	31.76	31.39
		1:100.000	34.74	35.92	36.70
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-
	EURL Pool (leg)	1:100	30.03	29.50	29.42
		1:1.000	32.59	33.76	32.73
		1:10.000	-	-	-
		1:100.000	-	-	-
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-

Table G: Real-time PCR results for operator 2

			Ct value		
Test	Sample Nb. & Developmental Stage of B. zonata	Dilution	Repetition 1	Repetition 2	Repetition 3
Real-time PCR	955/20 (adult female)	1:100	21.57	21.73	21.91
		1:1.000	25.75	25.48	25.55
		1:10.000	30.31	30.36	30.49
		1:100.000	32.73	33.64	33.10
		1:1.000.000	-	37.43	36.27
		1:10.000.000	-	-	-
	956/20 (adult male)	1:100	22.98	22.93	22.86
		1:1.000	27.16	27.12	26.94
		1:10.000	30.59	30.93	30.59
		1:100.000	34.60	34.27	34.56
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-
	957/20 (larva)	1:100	21.33	21.49	21.59
		1:1.000	25.11	24.77	25.23
		1:10.000	28.96	29.30	29.68
		1:100.000	34.77	34.18	35.35
		1:1.000.000	35.04	36.20	37.44
		1:10.000.000	-	-	-
	958/20 (pupa)	1:100	23.20	23.08	23.30
		1:1.000	26.97	26.88	26.94
		1:10.000	31.28	30.94	30.75
		1:100.000	35.72	35.09	34.68
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-
	EURL Pool (leg)	1:100	28.03	28.13	28.28
		1:1.000	32.05	31.55	31.50
		1:10.000	35.59	34.21	35.94
		1:100.000	-	-	-
		1:1.000.000	-	-	-
		1:10.000.000	-	-	-

Results for PCR reproducibility of both barcoding 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 H and I.

Table H: Reproducibility of the PCR tests operator 1

Operator:	Pohn
Date of performance:	09.08.2021
Thermocycler machine:	BiometraT3000 (I)

	EPPO PM 7/129 (LCO1490/HCO2198)			**EPPO PM 7/129 (LepF/LepR)**		
Species & Sample Nb.	**Repetition 1**	**Repetition 2**	**Repetition 3**	**Repetition 1**	**Repetition 2**	**Repetition 3**
Bactrocera zonata (956/20, adult male)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Bactrocera zonata (957/20, larva)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Bactrocera zonata (958/20, pupa)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Bactrocera correcta (2539/20, leg)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Bactrocera latifrons (2542/20, leg)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon
Dacus bivittatus (2543/20, leg)	Amplicon	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon

*Sequenced

Table I: Reproducibility of the PCR tests operator 2

Operator:	Heiss
Date of performance:	10.08.2021
Thermocycler machine:	BiometraT3000 (II)

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Species & Sample Nb.	**Repetition 1**	**Repetition 2**	**Repetition 3**	**Repetition 1**	**Repetition 2**	**Repetition 3**
Bactrocera zonata (956/20, adult male)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera zonata (957/20, larva)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera zonata (958/20, pupa)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera correcta (2539/20, leg)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Bactrocera latifrons (2542/20, leg)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon
Dacus bivittatus (2543/20, leg)	Amplicon*	Amplicon	Amplicon	Amplicon*	Amplicon	Amplicon

*Sequenced

Results for PCR 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 was performed in three different data bases (NCBI GenBank, Bold, EPPO-Q-Bank). Tables J and K depict the results of reproducibility.

Table J: Reproducibility of the SANGER sequence analysis operator 1

Operator:	Pohn
Date of performance:	16.08.2021
Software:	Geneious prime® 10.1.3

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Species & Sample nb.	**NCBI GenBank**	**Bold**	**Q-Bank**	**NCBI GenBank**	**Bold**	**Q-Bank**
Bactrocera zonata (956/20, adult male)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera zonata (957/20, larva)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera zonata (958/20, pupa)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera correcta (2539/20, leg)	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*
Bactrocera latifrons (2542/20, leg)	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*
Dacus bivittatus (2543/20, leg)	*Dacus bivittatus*	*Dacus bivittatus*	*Dacus demmerezi*	*Dacus bivittatus*	*Dacus bivittatus*	*Dacus demmerezi*

Table K: Reproducibility of the SANGER sequence analysis operator 2

Operator:	Gottsberger
Date of performance:	16.08.2021
Software:	Geneious prime® 10.1.3

	EPPO PM7/129 (LCO1490/HCO2198)			**EPPO PM7/129 (LepF/LepR)**		
Species & Sample nb.	**NCBI GenBank**	**Bold**	**Q-Bank**	**NCBI GenBank**	**Bold**	**Q-Bank**
Bactrocera zonata (956/20, adult male)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera zonata (957/20, larva)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera zonata (958/20, pupa)	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*	*Bactrocera zonata*
Bactrocera correcta (2539/20, leg)	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*	*Bactrocera correcta*
Bactrocera latifrons (2542/20, leg)	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*	*Bactrocera latifrons*
Dacus bivittatus (2543/20, leg)	*Dacus bivittatus*	*Dacus bivittatus*	*Dacus demmerezi*	*Dacus bivittatus*	*Dacus bivittatus*	*Dacus demmerezi*

Appendix 9 – Calculations of the performance characteristics diagnostic sensitivity, diagnostic specificity and accuracy

Table L: Calculations of the applicable performance characteristics (diagnostic sensitivity, diagnostic specificity and accuracy) for the two EPPO PM7/129(2) barcoding primer sets (EPPO 2021) and the real-time PCR (Koohkanzade *et al.* 2018).

Target Species	**Criteria**	**EPPO PM7/129 (LCO1490/HCO2198)**	**EPPO PM7/129 (LepF/LepR)**	**Real-time PCR (Koohkanzade *et al.* 2018)**
Bactrocera zonata	Number of Positive Agreements	7	7	7
	Number of Negative Agreements	22*	22*	22*
	Number of Negative Deviations	0	0	0
	Number of Positive Deviations	0	0	0
	Diagnostic sensitivity	**100**	**100**	**100**
	Diagnostic specificity	**100**	**100**	**100**
	Accuracy	**100**	**100**	**100**

* Numbers are given without sample 4, which was excluded from the study.