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EPPO STANDARD ON DIAGNOSTICS

PM 7/114 (2) *Bactrocera zonata*

Specific scope: This Standard describes a diagnostic protocol for *Bactrocera zonata*.[1]
This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.
Specific approval and amendment: Approved in 2013–09. Revised in 2022–07.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Bactrocera zonata (the peach fruit fly) is one of the most harmful species of Tephritidae. It is a serious pest of *Prunus persica* (Rosaceae) and *Annona squamosa* (Annonaceae) in India, as well as *Psidium guajava* (Myrtaceae) and *Mangifera indica* (Anacardiaceae) in Pakistan. It is a polyphagous species attacking more than 50 cultivated and wild plant species (fruits and vegetables) mainly those with fleshy fruits (EPPO, 2010, 2022a; White & Elson-Harris, 1992).

Bactrocera zonata is native to India where it was first recorded in Bengal (Kapoor, 1993). It is present in Asia (Bangladesh, Bhutan, India, Iran, Iraq, Israel, Laos, Myanmar, Nepal, Oman, Pakistan, Saudi Arabia, Sri Lanka, Thailand, United Arab Emirates, Vietnam and Yemen (EPPO, 2022b)) and Africa since 1993 (Egypt) where the species has been causing fruit damage on *Mangifera indica*, *Psidium guajava*, *Prunus armeniaca*, *Prunus persica*, *Prunus domestica* and *Ficus carica* (Mosleh et al., 2011). It has also been recorded from Libya, Mauritius, Reunion Island and Sudan (EPPO, 2022b). In Europe, no established populations of this species have been reported so far, but incursions were recorded in Austria and France (Egartner et al., 2019; EPPO, 2022b). In North America, it has been trapped in California and Florida, but has then been eradicated (EPPO, 2022b; White & Elson-Harris, 1992).

Information on true fruit flies (Diptera, Tephritidae) in Africa is available in De Meyer and White (2004). For updated geographical distribution consult EPPO Global Database (EPPO, 2022b). A datasheet providing more information on the biology of *B. zonata* is also available in EPPO Global Database (EPPO, 2022a).

A flow diagram describing the diagnostic procedure for *B. zonata* is presented in Figure 1.

2 | IDENTITY

Name: *Bactrocera* (*Bactrocera*) *zonata* (Saunders, 1842).
Common name: peach fruit fly, guava fruit fly.
Other scientific names: *Dasyneura zonata* (Saunders), *Rivellia persicae* (Bigot), *Dacus ferrugineus* var. *mangiferae* Cotes (Norrbom et al., 1999), *Dacus zonatus* (Saunders), *Strumeta zonata* (Saunders; EPPO, 2022b).
Taxonomic position: Diptera, Brachycera, Tephritidae, Dacinae, Dacini (nomenclature and taxonomy suggested by Fauna Europaea are used as the reference).
EPPO code: DACUZO.
Phytosanitary categorization: EPPO A2 List No. 302, EU Annex II A.

3 | DETECTION

Fruit flies are mostly detected as larvae in fruits.[2] Holes (oviposition punctures) are visible on the fruits. Holes on fruits are usually accompanied by local discoloration (E. Recht (NPPO, IL), personal communication). Eggs might be found inside the fruit at the point where oviposition puncture marks are visible on the surface. Larvae will leave the fruits to pupate, and so consequently puparia may also be detected in packaging.

Larvae can be reared to the adult stage for species identification. Rearing of larvae is described in White and Elson-Harris (1992). A presumptive diagnosis may be feasible on the 3rd instar (Balmès & Mouttet, 2017), but 'confirmation' with molecular tests should also be performed on larvae (see Section 4.2).

For morphological identification, collected larvae should be killed by placing them in boiling water for a few seconds (until they become immobile) and transferred to 70% ethanol. For molecular identification, they can be killed by placing them in boiling water for a few seconds (until they become immobile) or by freezing them. They should then be transferred to

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them as others may also be suitable.

[2]'In the biological sense'.

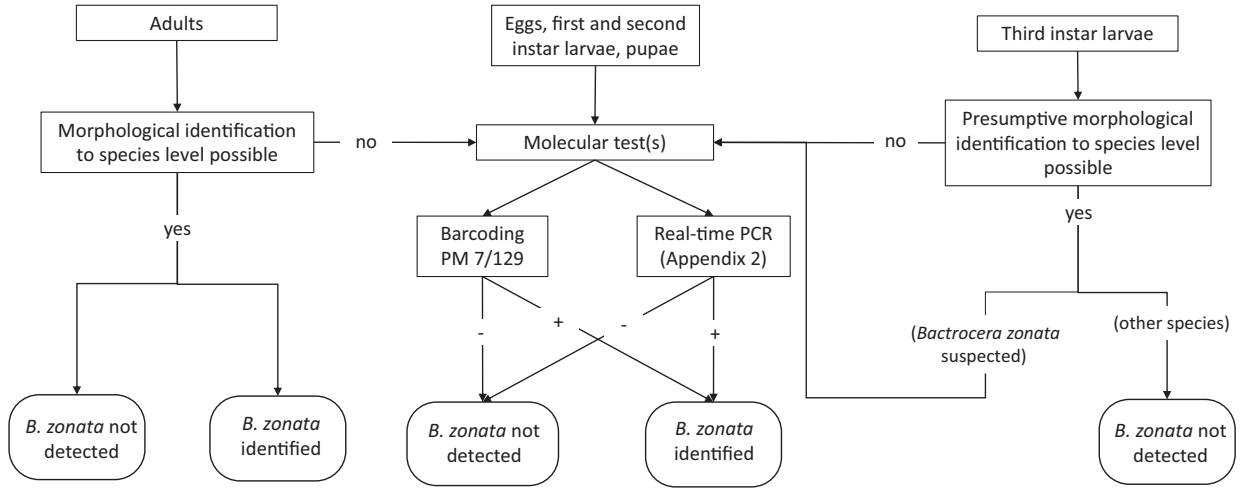

FIGURE 1 Diagnostic procedure for *Bactrocera zonata*. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

>90% ethanol until DNA extraction. Other procedures may be used.

Males of *B. zonata* are attracted to methyl eugenol (FAO/IAEA, 2018; White & Elson-Harris, 1992) and adults collected on traps can be used for identification.

4 | IDENTIFICATION

Identification is commonly based on the examination of adult specimens. Molecular tests (see Section 4.2) can be performed on all life stages, especially on those for which morphological identification to species level is not possible or on damaged adult specimens.

4.1 | Morphological identification

Morphological examination requires a stereo microscope for external examination of the adult and a compound microscope for examination of the larvae (for the preparation of the larvae see Appendix 1 part A) and of the adult female's aculeus (for the preparation of the aculeus see Appendix 1 part B). A reliable morphological identification to species level can only be made by examination of an adult specimen (either male or female) using the key presented in Table 1. A description of the larvae is also provided and may allow a presumptive diagnosis (see Section 4.1.1). Definitions and illustration of terms used and not specifically defined and illustrated in this protocol can be found in White and Elson-Harris (1992).

4.1.1 | Larva

A key for the identification of third-instar larvae at genus level is available in White and Elson-Harris (1992). However, it should be noted that the larvae of several *Ceratitis* species may also be assigned to the *Bactrocera* genus using this key (Steck & Ekesi, 2015). Therefore, its use is not recommended.

Examination of third instar larvae in combination with knowledge about the origin and the host, as well as the evidence provided by previously identified specimens from earlier and similar consignments, may allow a presumptive diagnosis (Balmès & Mouttet, 2017) but confirmation with molecular tests should also be performed on larvae suspected to be *B. zonata*.

Descriptions of larvae of several economically important fruit fly species are available in Carroll et al. (2004).

4.1.1.1 | *Description of a tephritid larva after Smith (1989) and Stehr (1991)*

Body cylindrical and rounded with a small tapering head, three thoracic and eight abdominal segments (Figure 2).

Head without sclerotization but with the cephalopharyngeal skeleton partially visible due to transparency (Figure 2, detail 2).

Anterior spiracle in a lateral position on each side of the first thoracic segment (Figure 2, detail 3).

Posterior spiracle on the surface of the last segment of the abdomen, unpigmented and without spine or lobe.

Two posterior spiracles with three spiracular openings or slits, arranged more or less parallel to each other (Figure 2; detail 4).

4.1.1.2 | *Partial description of 3rd instar larva of B. zonata after White and Elson-Harris (1992) and Carroll et al. (2004)*

Drawings of larvae are available at https://www.delta-intkey.com/ffl/www/bac_zona.htm.

Length: 10.0–11.0 mm (Figure 3). Carroll et al. (2004) extends this range to 9–12 mm.

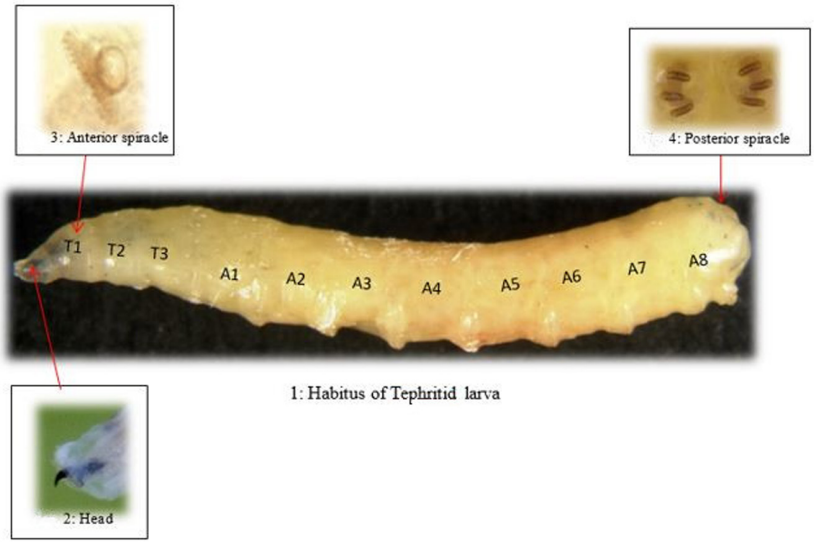

FIGURE 2 Habitus of tephritid larva. (detail 2 – Head, detail 3 – Anterior spiracles, detail 4 – Posterior spiracles) Courtesy: V. Balmès (ANSES, FR).

FIGURE 3 Larva. Courtesy: V. Balmès (ANSES, FR).

Head

Antenna 2-segmented. Stomal sensory organ small, rounded. Oral ridges with 10–11 deep, clearly defined rows. Accessory plates present.

Cephalopharyngeal skeleton

Mouthhook without preapical tooth; dental sclerite present; Parastomal bare elongate (Figure 4).

Anterior spiracles

Elevated, margin concave medially and with 13–15 short tubules (Figure 4).

Thoracic and abdominal segments

T1 with 6–9 rows of small spinules encircling anterior portion of segment; T2 with fewer rows encircling anterior portion of segment; T3 with a few spinules dorsally but forming rows laterally and ventrally; A1–A8 with rows of spinules ventrally forming creeping welts, with one anterior and 1–2 posterior rows of slightly larger spinules.

Anal area

Lobes well developed and surrounded by discontinuous rows of small spinules.

Posterior spiracles

Spiracular slits 3.0–3.5 times as long as they are broad, each with moderately sclerotised rima (=outer edge) (Figure 5); spiracular hairs slightly longer than half the length of a spiracular slit, frequently branched; dorsal and ventral bundles of 3–17 hairs, lateral bundles of 6–8 hairs.

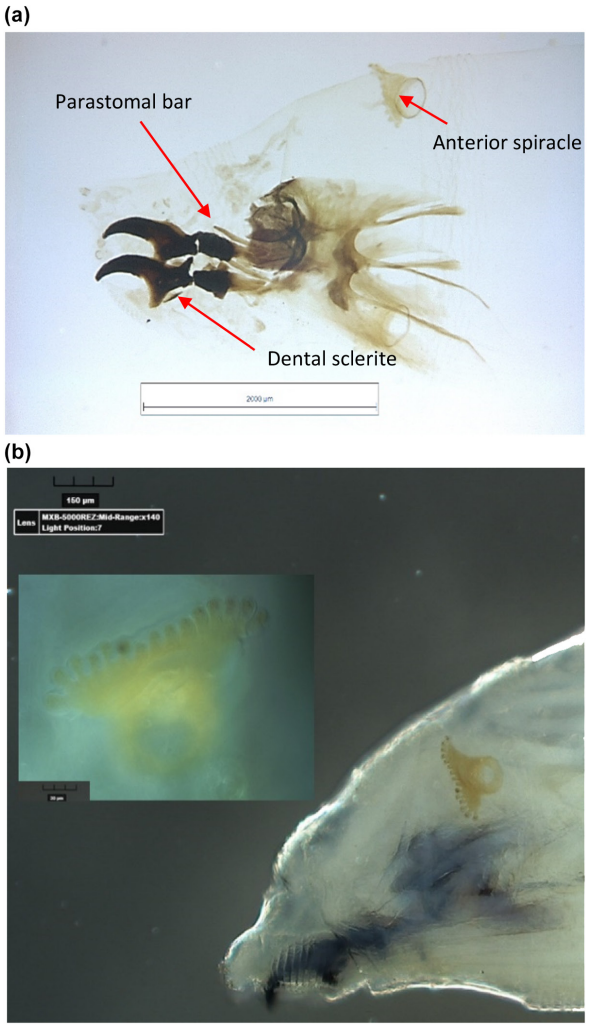

FIGURE 4 Head of larva, cephalopharyngeal skeleton . Courtesy: photo (a) V Balmès, ANSES (FR), photo (b) E Recht, NPPO (IL).

FIGURE 5 Posterior spiracles. Courtesy: V. Balmès (ANSES, FR).

4.1.2 | Adults

Characters to identify the subgenus *Bactrocera* (*Bactrocera*) are presented in section 4.2.1 of the IPPC Diagnostic Protocol (IPPC, 2019).

4.1.2.1 | *Description of the adult after White & Elson-Harris (1992) and Carroll et al. (2002)*

The specimen may be examined in ethanol or dry. Observation of colours is best achieved on specimens in ethanol as colours are more contrasted. However, they will fade after some time. Additional pictures of adults can be found in the virtual collections of the Royal Museum for Central Africa (BE).[3]

Predominantly pale orange-brown to red-brown (Figure 6).

Head

Head higher than long. Chaetotaxy reduced: ocellar seta absent or minute; postocellar seta absent; two pairs of frontal setae; one pair of reclinate orbital setae; inner and outer vertical setae present (one pair each). Dark round facial spots in each antennal furrow (Figure 7). First flagellomere elongate (at least three times as long as broad); rounded apically. Arista longer than flagellomere.

Thorax

Anterior supra-alar seta present. Presutural supra-alar seta absent. Intra-alar seta present and well developed, similar to posterior supra-alar seta. Prescutellar acrostichal seta present. Anterior notopleural seta present. Posterior notopleural seta acuminate (Figure 8). One anepisternal outstanding seta (Figure 9). Transverse suture with the lateral branches wide apart. Scutum orange-brown, or red-brown; without a large dark central stripe which broadens basally. Postpronotal lobe entirely pale whitish or yellowish; concolorous with lateral postsutural stripe. Posterior half of anepisternum pale whitish or yellowish. Two pale whitish to yellow/orange lateral postsutural stripes (vittae). Lateral postsutural stripes of scutum extending to intra-alar setae or beyond. Scutum without

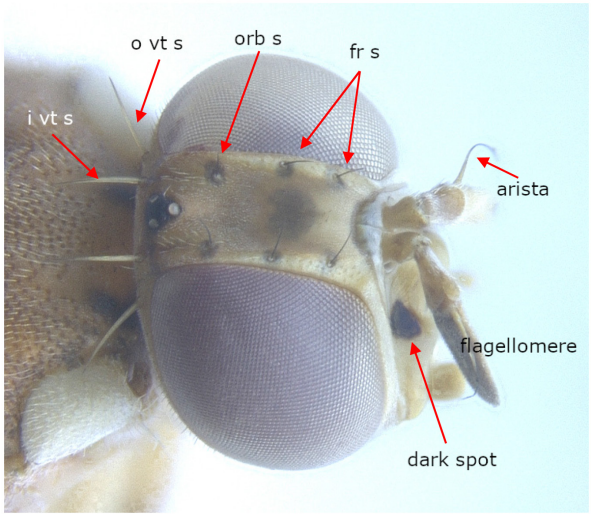

FIGURE 7 Head. fr s, frontal seta; i vt s, inner vertical seta; o vt s, outer vertical seta; orb s, orbital seta. Courtesy: V Balmès, ANSES (FR).

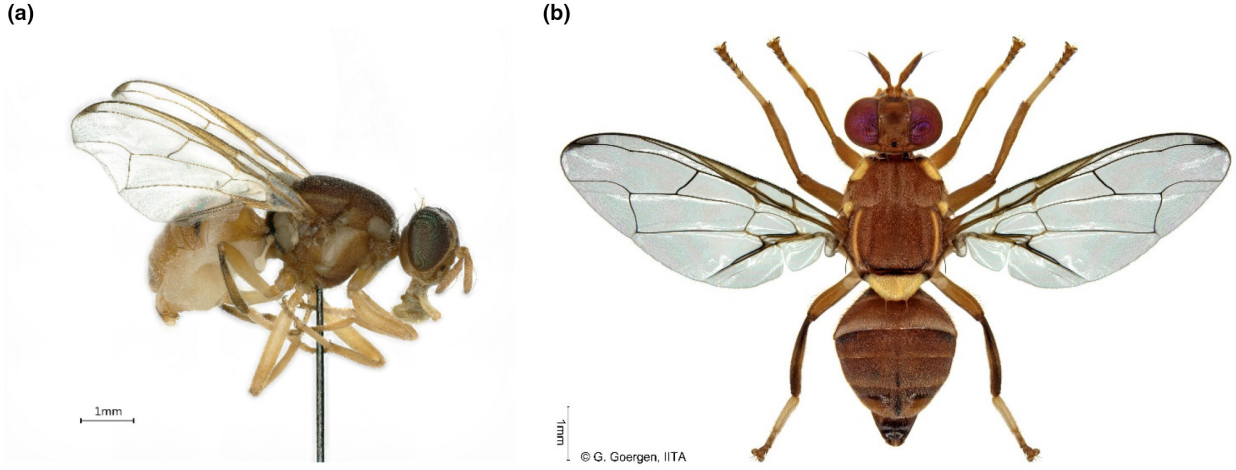

FIGURE 6 Habitus. Lateral view (a) and dorsal view (b) Courtesy: Photo (a) A Taddei, ANSES (FR) and Photo (b) G Goergen, IITA (NG).

[3]https://virtualcol.africamuseum.be/providence/pawtucket/index.php/Detail/objects/5563.

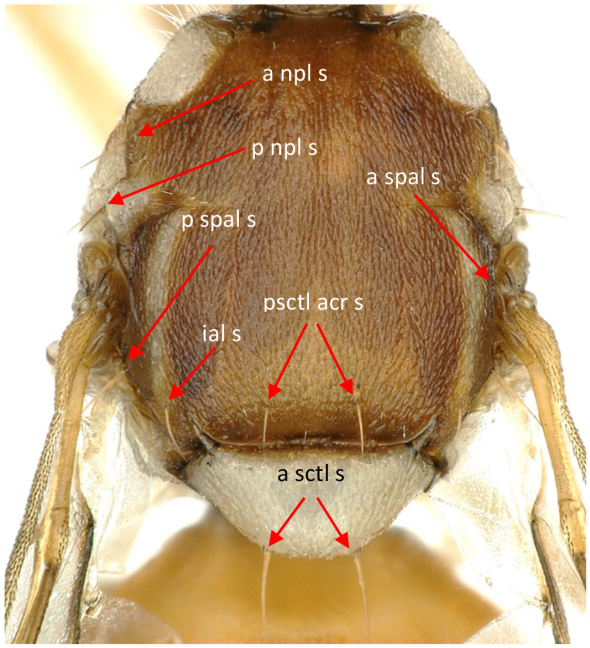

FIGURE 8 Thorax – dorsal view. a npl s, anterior notopleural seta; a sctl s, apical scutellar seta; a spal s, anterior supra-alar seta; ial s, intra-alar seta; p npl s, posterior notopleural seta; p spal s, posterior supra-alar seta; psctl acr s, prescutellar acrostichal seta. Courtesy: A Taddei, ANSES (FR).

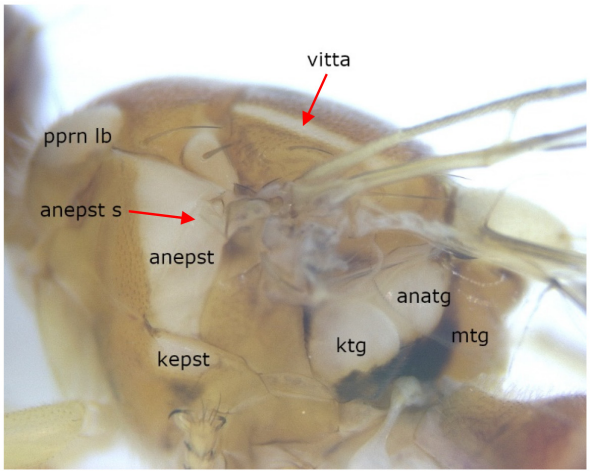

FIGURE 9 Thorax – lateral view. anatg, anatergite; anepst s, anepisternal seta; anepst, anepisternum; kepst, katepisternum; ktg, katatergite; mtg, mediotergite; pprn lb, postpronotal lobe. Courtesy: V Balmès, ANSES (FR).

blackish dorsoventral stripe. Katepisternum, katatergite and anatergite all with pale yellowish or whitish spot present and distinct. Mediotergite uniformly brown or yellowish to orange-brown medially, with distinct dark spot present (Figure 9). One pair of scutellar setae; pale and acuminate. Scutellum densely setulose; without a dark and pale pattern (at most a narrow dark basal line); without mark. Setulae on scutellum short, decumbent; unicolorous, acuminate (Figure 8).

Abdomen

Abdomen ovate or parallel sided. Abdominal tergites separate (Figure 10a). Abdomen in lateral view arched, dome-like, rather rigid. Abdominal tergite 1 broader at apex than at base; without a prominent hump laterally. Abdominal tergites 3–5 predominantly yellow to orange-brown. Abdominal tergites with medial dark stripe usually on T3–T5, or with medial dark stripe on T5 only; not brown with medial T-shaped yellow mark; with separate dark areas on anterolateral margins of T3–T5 (T3 only), or without isolated dark areas on lateral margins of T3–T5; without dark brown transverse bands. Male: pecten with dark setae on tergite 3 (Figure 11). Female: aculeus pointed; length 1.0–1.2 mm (Figure 12).

Legs

Femora slender. Fore femur with regular setae; without ventral spines; with 1–3 posterodorsal and one

FIGURE 10 Abdomen (ventro-lateral view) of *Bactrocera zonata* (a) with separate tergites and *Dacus vertebratus* (b) with fused tergites. Courtesy: A Taddei, ANSES (FR).

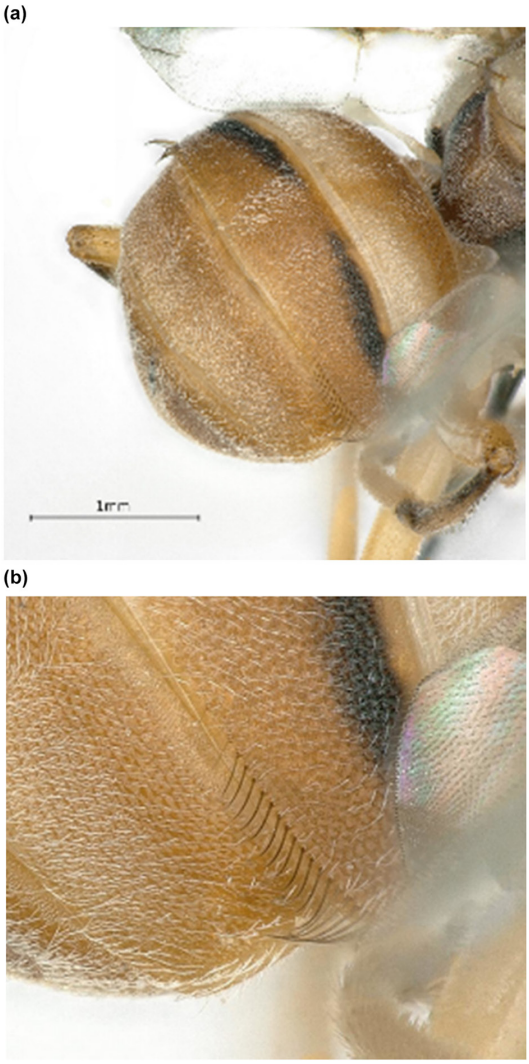

FIGURE 11 Part of abdomen of male (a). Close up on pecten on tergite 3 (b). Courtesy: A Taddei, ANSES (FR).

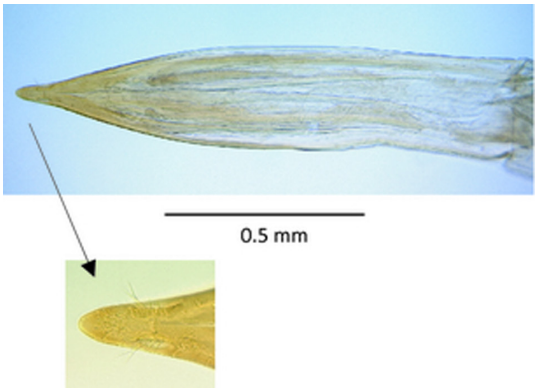

FIGURE 12 Aculeus (detail: tip of aculeus). Courtesy: V Balmès, ANSES (FR).

FIGURE 14 Wing detail, cell cu*p* extension. Courtesy: A Taddei, ANSES (FR).

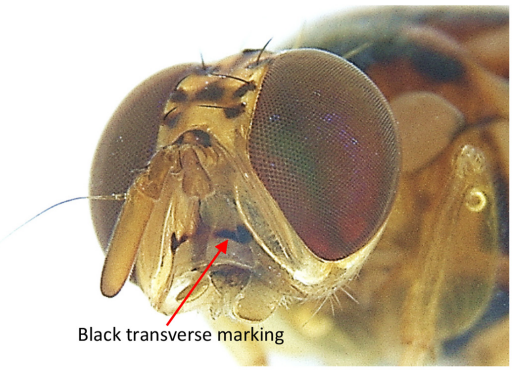

FIGURE 15 Head of *Bactrocera correcta*. Courtesy: V Balmès, ANSES (FR).

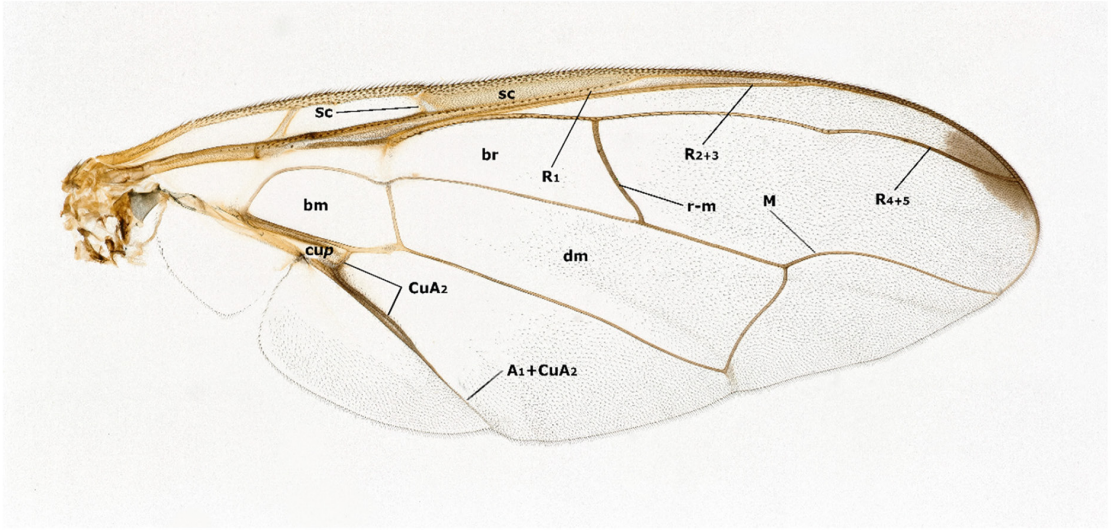

FIGURE 13 Wing. Courtesy: A Taddei, ANSES (FR).

TABLE 1 Identification of the adult of *Bactrocera zonata* (after Carroll et al., 2002; Kapoor, 1993; White & Elson-Harris, 1992).

1	Subcostal vein abruptly bent and dorsal side of vein R_1 with setulae (Figure 13)	Tephritidae 2
1*	Subcostal vein not abruptly bent or dorsal side of vein R_1 lacks setulae	Other families
2	Cell cu*p* very narrow and extension of cell cu*p* very long (Figures 13 and 14)	3
2*	Cell cu*p* broader and extension shorter	Other genera
3	Scutellum not bilobed and with one pair of scutellar setae (apical pair; Figure 8)	4
3*	Scutellum bilobed or with more than one pair of scutellar setae	Other genera/species
4	Scutum with prescutellar acrostichal and anterior supra-alar setae (Figure 8) and without medial yellow or orange vitta (Figure 8). Male with pecten on abdominal tergite 3 (Figure 11)	5
4*	Scutum different. Male with or without pecten on abdominal tergite 3	Other genera/species
5	Wing without any crossband (Figure 13)	6
5*	Wing with crossbands, at least covering crossvein r-m	Other species
6	Scutum with two light yellow to orange lateral vittae (Figure 9)	7
6*	Scutum without light yellow to orange lateral vittae	Other species
7	Face with a dark spot in each antennal furrow (Figure 7)	8
7*	Face without dark markings or with a transverse dark marking (Figure 15)	Other species
8	Wing without a distinct costal band, only cell sc and apex of vein R_{4+5} coloured (Figure 13)	9
8*	Wing different	Other species
9	Scutellum entirely pale coloured, except sometimes for a narrow black line across the base (Figure 8); apex of vein R_{4+5} covered by an elongate spot (Figure 13)	10
9*	Dorsal surface of scutellum with a large black triangular mark, lateral and apical areas yellow; apex of vein R_{4+5} not covered by an elongate spot	*B. psidii*
10	Thorax and abdomen pale orange-brown to red-brown (Figure 6). Aculeus length 1.0–1.2 mm (Figure 12)	*B. zonata*
10*	Thorax and abdomen black (if dark orange-brown then the wing without markin). Aculeus length 1.6 mm	*B. tuberculata*

Note: This simplified key will allow the distinction of *B. zonata* from most species of agronomic and/or economic importance.

posteroventral rows of setae only, or without major setae. Mid femur and hind femur without spine setae. Middle leg of male without feathering. Femora all entirely yellow without dark mark.

Wings

Length: 5.2–6.1 mm (Figure 13). Wing pattern reduced and mostly yellowish, or mostly brownish. Costal band with only cell sc and apex of vein R_{4+5} coloured. Apex of costal band distinctly expanded into a brown elongate spot. Cell bm broad, parallel-sided; ratio of length to width 2; ratio of width to cell cu*p* width 2. Cell dm widens apically gradually from base. Posterodistal corner of cell dm approximately a right angle. Cell cu*p* extension present, very long and coloured (Figures 13 and 14), vein CuA_2 abruptly bent; longer than vein A_1+CuA_2; with parallel margin. Anal band (= streak) absent, or not reaching nearly to wing margin (faint, short).

4.1.2.2 | *Key to adults*

For identification of the Family Tephritidae see Oosterbroek (2006).

Characters to identify the subgenus *Bactrocera* (*Bactrocera*) are presented in section 4.2.1 of the IPPC Diagnostic Protocol (IPPC, 2019).

The key for adult identification in Table 1 will allow the distinction of *B. zonata* from most species of agronomic and/or economic importance. Confirmation of the identification should be made using the description provided in Section 4.1.2.1.

4.2 | Molecular identification

Molecular tests are used for the identification of eggs, first and second instar larvae or pupae, or to support the morphological identification of third instar larvae or adults of *B. zonata*.

For the identification of *B. zonata*, a TaqMan real-time PCR test has been developed by Koohkanzade et al. (2018). This test can be applied on tissue from specimens. It was adapted and validated by the EURL for insects and mites (Appendix 2).

A protocol for DNA barcoding based on COI described in Appendix 1 of PM 7/129 (2) *DNA barcoding as an identification tool for a number of regulated pests*: DNA barcoding Arthropods (EPPO, 2021) allows the identification of *B. zonata*. The test was validated by the EURL for insects and mites and is suitable for the identification of *B. zonata*. Validation data are available in the EPPO database on diagnostic expertise, section on validation (https://dc.eppo.int/validation_data/validationlist). Sequences are available in EPPO-Q-bank https://qbank.eppo.int/arthropods/. Sequence analysis

should follow the guidelines described in appendices 7 and 8 of the EPPO Standard PM 7/129 (EPPO, 2021).

5 | REFERENCE MATERIAL

Not applicable.

6 | REPORTING AND DOCUMENTATION

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7 | PERFORMANCE CHARACTERISTICS

When performance characteristics are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

ANSES – EURL – Unite d'Entomologie et Plantes Invasives, 755 avenue du campus d'Agropolis CS30016, 34988 Montferrier sur Lez, France. E-mail: eurl-insects-mites@anses.fr

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share, please contact diagnostics@eppo.int.

10 | PROTOCOL REVISION

A regular review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

ACKNOWLEDGEMENTS

This protocol was originally drafted by V Balmès (ANSES, FR). Revision was prepared by A Egartner (AGES, AT), R Gottsberger (AGES, AT) and A Taddei (ANSES, FR). It was reviewed by the Panel on Diagnostics in Entomology.

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APPENDIX 1 - PREPARATION OF SAMPLES FOR MICROSCOPIC OBSERVATION

Part A: Preparation of larvae for observation using a stereo microscope and compound microscope with ×100 magnification

(1) Place the larva in a 10% potassium hydroxide solution for 1 h at room temperature or 15–20 min at between 60 and 80°C. Piercing the larva with a pin beforehand improves the digestion of internal tissues.
(2) Put the larva into distilled water, empty it from its digested content and flatten the body by applying gentle pressure with a fine spatula.
(3) Transfer the larva into clean distilled water for several minutes.
(4) The larva can then be mounted on a slide in a drop of glycerol with a cover slip for study and temporary storage or prepared in Canada balsam for permanent mounting.

Part b: preparation of aculeus for examination using a stereo microscope and compound microscope with ×400 magnification

(1) Break off the abdomen of the female and place it in a 10% potassium hydroxide solution for 1 h at room temperature or 20–30 min at between 60 and 80°C.
(2) When the abdomen is soft enough, use a pin to detach the aculeus from the rest taking care not to damage its tip.
(3) Transfer the aculeus into distilled water for several minutes and mount it on a slide in a drop of glycerol with a cover slip for study and temporary storage or prepare in Canada balsam for permanent mounting.

APPENDIX 2 - TAQMAN REAL-TIME PCR (KOOHKANZADE ET AL., 2018)

The test below differs from the one described in the original publication (see 1.2).

The test below is described as it was carried out by the European Union Reference Laboratory for Insects and Mites (EURL) to obtain the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification is carried out (see PM 7/98).

1. General information

1.1. This test is suitable for the identification of *B. zonata* eggs, larvae, pupae and adults. The test, as adapted by the EURL for Insects and Mites, was not evaluated on eggs, but similar results are expected as for other matrices. In the original paper (Koohkanzade et al., 2018), one egg sample of *B. zonata* was included and gave a positive result.
1.2. Real-time PCR primers were designed by Koohkanzade et al. (2018) and the test was further adapted by the EURL for Insects and Mites (AGES).
1.3. The target sequences are located in the *COI* gene.
1.4. Oligonucleotides:

	Primer	Sequence	Amplicon size (including primer sequences)
Forward primer	BzonF	5′-AGC CAC ATT ACA TGG TAC ACA ACT-3′	100 bp
Reverse primer	BzonR	5′-AGG ACA ACT CCT GTT AAT CCT CCT-3′	
Probe	BzonP	5′-FAM-CTC CAG CTA TAC TGT GGG CCC TAG GA- TQ2*-3′	

* Tide Quencher™ 2 phosphoramidite (TQ 2).

1.5. Enzyme: PerfeCTa qPCR ToughMix® Quanta Bio
1.6. Real-time PCR system: Eppendorf realplex Mastercycler, Bio Molecular Systems Magnetic Induction Cycler (MIC)

2. Methods

2.1. Nucleic acid extraction and purification:

2.1.1. Larvae:
Frozen specimens were ground directly in the first buffer of the DNeasy Blood & Tissue Kit (Qiagen) using the FastPrep-24TM homogenizer at a speed of 6.5 m/s for 30 s and DNA extraction was then performed according to manufacturer's instructions. For specimens stored in ethanol, the same procedure can be used but the specimens should be dried before grinding, e.g. on filter paper for 15 min.

For a non-destructive DNA extraction, the specimen can be incubated in the ATL buffer with proteinase K with very gentle shaking (<50 rpm) for more than 2 h and up to overnight. Pipette the buffer and use it for DNA extraction according to manufacturer's instructions.

2.1.2. Adults:

Destructive DNA extraction from single legs is performed as for larvae using the QIAamp DNA Micro Kit (Qiagen).

2.2. Real-time polymerase chain reaction

2.2.1. Master Mix

	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water		2	
PerfeCTa qPCR ToughMix	2×	5	1×
BzonF	10 µM	0.5	0.5 µM
BzonR	10 µM	0.5	0.5 µM
BzonP	1 µM	1.0	0.1 µM
Subtotal		9.0	
DNA extract		1.0	
Total		10.0	

2.2.2. PCR cycling conditions: Initial denaturation for 10 min at 95°C, followed by 45 cycles of (95°C for 15 s and 63°C for 60 s)

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification of clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of amplification: amplification of nucleic acid of the target organism.

As an alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately. The amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation to the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results

Verification of controls

- The PIC and PAC (and if relevant IPC) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative, if it does not produce an amplification curve or if it produces a curve which is not exponential.
- The test should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Validation data described below were produced by the EURL (unless stated otherwise). Additional validation data were produced by Koohkanzade et al. (2018) using a slightly different protocol.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity data

The analytical sensitivity of the test was 1.69 pg/µL of target DNA.

4.2. Analytical specificity data

Data from EURL.

Inclusivity evaluated on seven specimens (adults) from Egypt (2), India (1), Pakistan (3), Réunion Island (1): 100%.

Exclusivity evaluated on 23 specimens (adults) of 10 non-target species obtained from 12 different countries around the world (*B. albistrigata*, *B. correcta*, *B. dorsalis*, *B. latifrons*, *B. oleae*, *Dacus bivittatus*, *D. ciliatus*, *D. etiennellus*, *D. punctatifrons*, *Zeugodacus cucurbitae*): 100%.

Data from Koohkanzade et al. (2018).

Inclusivity evaluated on 15 specimens (nine adults, five larva, one pupa) collected from Iran (13) and India (2) and eggs from Guava: 100%.

Exclusivity evaluated on 40 specimens (34 adults, four larva, two pupae) of 19 non-target species obtained from 13 different countries around the world (*B. carambolae*, *B. tryoni*, *B. aquilonis*, *B. invadens*, *B. correcta*, *B. kandiensis*, *B. cucurbitae*, *B. dorsalis*,

B. jarvisi, *Ceratitis capitata*, *C. cosyra*, *C. rosa*, *D. pornia*, *B. melanotus*, *B. papayae*, *B. tau*, *B. latifrons*, *B. oleae*, *Carpomya vesuviana*): 100%.

4.3. Data on repeatability

100%, with three biological replicates of *B. zonata* (dilution near by the detection limit) analysed with three technical repetitions.

4.4. Data on reproducibility

100%, with two operators on two different real-time PCR cyclers, with three targets (adult, larva and pupa) and three non- targets (*B. correcta*, *B. latifrons* and *D. bivittatus*).

4.5. Other data

The performance of the test was evaluated in the following matrices: one adult specimen (female), one adult specimen (male), one larva, one pupa and one leg of *B. zonata* and gave 100% concordant results.